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PERSISTENCE OF *LACTOBACILLUS REUTERI* DSM17938 IN THE HUMAN INTESTINAL TRACT: RESPONSE TO CONSECUTIVE AND ALTERNATE-DAY CONSUMPTION WITH VARYING STORAGE CONDITIONS

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14. ABSTRACT

Probiotic-containing foods are consumed to enhance gastrointestinal (GI) health and immune function. How storage of probiotic-containing foods affects colonization and persistence of probiotics in the GI tract after the stored foods are consumed is undefined. Similarly, the amount or "dose" of probiotics that must be consumed to achieve colonization and persistence of probiotics in the GI tract is not known. Volunteers ate "fresh" pudding with *Lactobacillus reuteri* DSM17938 (*L. reuteri*; BioGaia AB, Stockholm, Sweden; 10^8 CFU/serving; room temperature ≤ 1 week followed by freezer) daily (n = 9) or on alternate days (n = 9) over 7 days; or, "stored" pudding with *L. reuteri* (10^9 CFU/serving; 37ºC for ~7 days followed by freezer) daily over 7 days (n = 10). Fecal samples were collected daily during probiotic consumption (D1-7) and after dosing ended (D13-15 and D20-22), and analyzed for the presence of *L. reuteri*. All volunteers who consumed "fresh" probiotic-containing pudding, and one volunteer who consumed "stored" probiotic-containing pudding, had detectable *L. reuteri* during consumption (D1-7). *L. reuteri* count significantly rose in response to daily consumption and alternate-day consumption of "fresh" probiotic-containing pudding, and significantly fell in both groups one week after dosing ended. In contrast, *L. reuteri* count did not rise in response to "stored" probiotic-containing pudding. The total number of volunteers with detectable *L. reuteri* one and two weeks after dosing ended was similar in response to daily feeding and alternate-day feeding of "fresh" probiotic-containing pudding; however, *L. reuteri* was not detected after dosing ended in response to "stored" probiotic-containing pudding. Alternate-day probiotic intake achieves equivalent colonization to daily intake, but colonization declines rapidly once dosing stops. Colonization of *L. reuteri* is achievable with daily or alternate day intake of "fresh", but not "stored" *L. reuteri*-containing pudding.

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BACKGROUND

Diarrhea is a persistent problem in deployed forces. Probiotic organisms are believed to be a solution for sustaining gut health, and could thereby mitigate the effects of exogenous diarrhea-causing pathogens that Warfighters are likely to encounter in operational environments. Commercial food products containing probiotic organisms are available, but cannot meet the shelf-stability requirements established for combat rations. Combat Feeding Directorate Natick Soldier Research Development and Engineering Center, Natick, MA developed a food product containing a commercially-available strain of the probiotic L. Reuteri (DSM17938, BioGaia, Stockholm, Sweden) that withstands environmental stressors (i.e. heat, oxygen, moisture and acidity), and appears to meet the rigorous shelf-life specifications for combat rations. However, the ability of L. Reuteri to persist in the human intestinal tract when consumed after a typical storage life and in response to different dosing strategies (i.e. practical for implementation in a military field ration) was not yet demonstrated.

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EXECUTIVE SUMMARY

Probiotic-containing foods are consumed to enhance gastrointestinal (GI) health and immune function. How storage of probiotic-containing foods affects colonization and persistence of probiotics in the GI tract after the stored foods are consumed is undefined. Similarly, the amount or "dose" of probiotics that must be consumed to achieve colonization and persistence of probiotics in the GI tract is not known. Volunteers ate "fresh" pudding with *Lactobacillus reuteri* DSM17938 (*L. reuteri*; BioGaia AB, Stockholm, Sweden; 10^8 CFU/serving; room temperature ≤ 1 week followed by freezer) daily (n = 9) or on alternate days (n = 9) over 7 days; or, "stored" pudding with *L. reuteri* (10^9 CFU/serving; 37ºC for ~7 days followed by freezer) daily over 7 days (n = 10). Fecal samples were collected daily during probiotic consumption (D1-7) and after dosing ended (D13-15 and D20-22), and analyzed for the presence of *L. reuteri*. All volunteers who consumed "fresh" probiotic-containing pudding, and one volunteer who consumed "stored" probiotic-containing pudding, had detectable *L. reuteri* during consumption (D1-7). *L. reuteri* count significantly rose in response to daily consumption and alternate-day consumption of "fresh" probiotic-containing pudding, and significantly fell in both groups one week after dosing ended. In contrast, *L. reuteri* count did not rise in response to "stored" probiotic-containing pudding. The total number of volunteers with detectable *L. reuteri* one and two weeks after dosing ended was similar in response to daily feeding and alternate-day feeding of "fresh" probiotic-containing pudding; however, *L. reuteri* was not detected after dosing ended in response to "stored" probiotic-containing pudding. Alternate-day probiotic intake achieves equivalent colonization to daily intake, but colonization declines rapidly once dosing stops.

Colonization of *L. reuteri* is achievable with daily or alternate day intake of “fresh”, but not “stored” *L. reuteri*-containing pudding.

INTRODUCTION

The human gut is a complex and diverse microbial environment. Probiotics, referred to as “healthy bacteria”, are living organisms that exert positive health effects on the host (21). The efficacy of some probiotic strains on the prevention and/or treatment of gastrointestinal illnesses has been demonstrated (7; 14), as has the ability of probiotics to enhance immune function (3; 4; 8-10; 17; 19).

Lactobacillus reuteri ATCC 55730 (*L. reuteri*) is one probiotic strain that has shown positive clinical benefits, such as reducing the incidence and/or duration of infectious diarrhea (2; 6; 13). At the species level, *L. reuteri* has been isolated from all parts of the human digestive tract (e.g. oral cavity, stomach, small intestine, colon and feces) and is a part of the normal indigenous intestinal flora in ~10% of individuals (12; 17). Studies involving consumption of *L. reuteri* ATCC 55730 have demonstrated that *L. reuteri* survives in the gastrointestinal (GI) tract and can be isolated alive from mucosal samples from the small intestine and from feces (1; 17; 20).

Reports indicate that recovery of *L. reuteri* in the feces is achievable after 7 consecutive days of ingestion (1; 20); however, it is unclear if *L. reuteri* can colonize after only a few days of feeding, or if alternate-day dosing strategies are similarly effective. Further, studies have demonstrated that *L. reuteri* is detectable in the feces up to 4 weeks following long-term consumption (21-28 days) of *L. reuteri* (17; 20), but it is unclear how long colonization persists following the discontinuation of short-term consumption (7 days).

Even if those questions are addressed, there are other barriers to providing probiotic-containing food to deployed military personnel. Commercially available food

products containing probiotic organisms cannot survive the shelf-stability requirements established for combat rations. However, Combat Feeding Directorate Natick Soldier Research Development and Engineering Center, Natick, MA developed a prototype food product containing a commercially-available strain of the probiotic *L. Reuteri* (DSM17938, BioGaia, Stockholm, Sweden) that withstands environmental stressors (i.e. heat, oxygen, moisture and acidity) and meets the rigorous shelf-life specifications for combat rations. The ability of this particular strain to persist in the human intestinal tract when consumed after being subjected to high storage temperatures typical of combat rations remains to be tested.

The purpose of this investigation was to assess the persistence of *L. reuteri* DSM17938 (BioGaia AB, Stockholm, Sweden) in the human intestinal tract during and after discontinuation of consumption of: 1) "fresh" (room temperature ≤ 1 week followed by freezer) *L. reuteri*-containing pudding for 7 consecutive days; 2) "fresh" *L. reuteri*-containing pudding on alternate-days (4 doses, every-other-day, for 7 days); and, 3) "stored" (37ºC for ~7 days followed by freezer) *L. reuteri*-containing pudding for 7 consecutive days. We hypothesized that *L. reuteri* levels would be higher during the consumption period in response to consecutive-day dosing of "fresh" *L. reuteri*-containing pudding compared to alternate-day dosing of "fresh" and consecutive day dosing of "stored" *L. reuteri*-containing pudding; and, that *L. reuteri* would persist for a longer period of time after the discontinuation of consumption in response to consecutive-day dosing of "fresh" *L. reuteri*-containing pudding compared to alternate-day dosing of "fresh" and consecutive-day dosing of "stored" *L. reuteri*-containing pudding.

METHODS

SUBJECTS

Volunteers were active duty Soldiers residing at Natick Soldier Research, Development and Engineering Center in Natick, MA. Fifty-one healthy volunteers (45 men, 6 women) gave their free, informed, voluntary, written consent to participate in this investigation following an oral and written explanation of the study procedures and risks. The protocol for this research was reviewed and approved by institutional scientific and human subjects committees. All subjects completed an initial screening form and were medically cleared for participation in accordance with the United States Army Research Institute of Environmental Medicine guidelines for human use. Volunteers were excluded from participation if they did not meet age requirements (18-50), were pregnant, had an allergy to any ingredients in the probiotic-containing pudding, were taking medications that affected energy metabolism, appetite, or gastrointestinal health (e.g. antibiotics), or if they had medical conditions that affected energy metabolism, appetite, or gastrointestinal health (e.g. irritable bowel syndrome, gastroenteritis, Crohn's Disease, etc…). The investigators have adhered to the policies for protection of human subjects as prescribed in Army Regulation 70-25 and USAMRMC Regulation 70-25, and the research was conducted in adherence with the provisions of 32 CFR Part 219.

EXPERIMENTAL DESIGN

This was a non-randomized trial. "Fresh" or "stored" portions of a probiotic-containing food item (vanilla pudding) were administered either daily or every-other-day for 7 days (D1-7). Thus, there were four groups of test subjects classified as "fresh, daily"; "fresh, alterate-day"; "stored, daily"; and, "stored, alternate-day". Fecal samples

were collected on study days 1-7, 13-15 and 20-22 (Figure 1) and assessed for presence of *L. reuteri*.

PREPARATION OF THE PROBIOTIC-CONTAINING PUDDING

The preparation of "fresh" probiotic-containing pudding was achieved as by adding approximately 10^8 of *L. reuteri* DSM17938 to each serving of powdered pudding mix, after which time the powder was sealed in a tri-laminate pouch, and stored in a freezer (0°F) until consumption. Pudding was tested immediately after production (before freezer storage), and after the last volunteer in each group completed the study, to ensure quantity of live *L. reuteri* (Table 1).

The preparation of "stored" probiotic-containing pudding was achieved by adding approximately 10^{10} CFU of *L. reuteri* DSM17938 to each serving of powdered pudding mix, after which the powder was sealed in a tri-laminate pouch and stored in a 37ºC incubator for approximately 7 days (i.e., until *L. reuteri* count was matched to the "fresh" probiotic-containing pudding). Upon removal from the incubator, microbial plate counts were obtained (Table 1) and samples were placed in frozen storage until consumption by the group receiving the "stored" product (consecutive- day "stored").

The following procedure was used to determine *L. reuteri* viability in pudding. Bottles of sterile molten MRS agar and ½ strength MRS broth were tempered to 50°C. A 20g sample of pudding powder was added to 180mL of tempered ½ strength MRS broth in a filtered Whirl Pak stomacher bag and agitated at 230 rpm for 2 minutes. Next, 1.0 mL was transferred to the first serial dilution tube containing 9.0 ml Butterfield's buffer (pH 7.2). Serial dilutions were carried out in this manner until appropriate dilution was reached. Pour plates were prepared with 1.0 mL of desired dilution and approximately

20 ml molten MRS agar added to each sterile Petri plate, in duplicate. Plates were swirled, allowed to solidify at room temperature, inverted, and incubated at 37^{o}C under anaerobic conditions for 48 hours**.**

Immediately prior to consumption, the pudding powder was removed from the freezer and reconstituted with water. Volunteers consumed the pudding in the presence of study staff, thus ensuring 100% compliance.

DIET INSTRUCTION

Prior to beginning the study, volunteers completed a diet history questionnaire (Diet History Questionnaire, Version 1.0. National Institutes of Health, Applied Research Program, National Cancer Institute, 2007), which was analyzed using software provided by the National Cancer Institute (Diet*Calc Analysis Program, Version 1.4.3. National Cancer Institute, Applied Research Program. November 2005). The main purpose of the diet history questionnaire was to assess typical fiber intake, since some forms of soluble fiber may stimulate growth of probiotics (18).

Approximately two weeks prior to the intervention, volunteers were instructed to maintain their typical diet and refrain from consuming any dietary supplements or food products containing probiotics (e.g. yogurt, kefir, etc…) until study completion. Volunteers were given a reference list of food products containing probiotics, and a questionnaire assessing their intake of probiotic-containing food items was administered weekly throughout the study.

FECAL SAMPLING & ANALYSIS

All volunteers were given pre-labeled fecal collection containers with covers. Volunteers refrigerated their fecal samples within 30 minutes of defecation in specially

designated refrigerators. Time of defecation was self-recorded by volunteers. Trained laboratory technicians transferred ~5g of feces into a sterile container within 24 hours of defecation and samples were frozen at -70 C prior to enumeration of bacteria.

Thawed fecal samples were initially weighed and placed in sterile stomacher bags (Nasco, Modesto, CA), diluted in 0.85% NaCl (1:5, w/v) and stored at -70^{o}C until bacterial enumerations were performed. When ready to process, thawed and weighed samples were further diluted (1:2, w/v) with 0.85% NaCl, and stomached for 2 minutes at 230 rpm (Lab-Blender 400 stomacher, Tekmar Company, Cincinnati, Ohio). After stomaching, standard serial dilutions were prepared by adding 0.5 ml of fecal sample to 4.5 ml of dilutent (0.85% NaCl) in sterile dilution tubes. Subsequently, 0.1 ml of an appropriate dilution was spread plated on modified Rogosa Sharpe plus 2% sodium acetate (MRS-3) agar with vancomycin (50 mg/liter; (17)) and plates were incubated anaerobically (glove box: 5% CO_2, H_2, 85% N_2) at 37ºC for 48 h after which time colonies were confirmed as *L. reuteri* using an overlay technique developed by BioGaia AB (Stockholm, Sweden)

The overlay technique relies on the fact that *Lactobacillus reuteri* produces reuterin in the presence of glycerol. Plates with suspected *L. reuteri* colonies (≤ 150 colonies per plate) were selected, and the colonies were counted and recorded. Five mL of soft Bacto agar (1%, w/v) containing 20 ml of glycerol/liter soft agar (46- 47^{o}C) was then added over the plate. The plate was allowed to solidify for 5 minutes and incubated at 37^{o}C for 1 hour. Plates were removed from the incubator and placed, open, on a paper towel, then flooded with 5 ml of 2-4 Dinitrophenylhydrazine (DNPH) solution (1 g DNPH, 170 ml HCl and 830 ml distilled water). After 5 minutes, DNPH was

discarded from plates, and 5M potassium hydroxide was added, after which time a reddish, brown color appeared around the reuterin-producing colonies. Reuterin positive colonies were then counted and reported as *L. reuteri*.

The fecal sample taken at baseline, prior to the probiotic intervention, represents the volunteer's natural microbiota and served as the control. The D1 fecal sample, taken on the first day of probiotic consumption, was used to confirm baseline *L. reuteri* levels, and omitted from further analysis. Fecal counts were averaged in 3-day increments (D2-4, D5-7, D13-15, and D20-22) since some volunteers may not defecate daily.

SAMPLE SIZE CALCULATION

Sample size estimates were made using SamplePower (release 2.0, SPSS Inc, Chicago, IL) paired t-test (mean=0) procedure. Wolf et al. (20) administered 10^{11} CFU/day of *L. reuteri* (n=30) for 21 days, and reported subsequent fecal counts on days 7, 14 and 21. Since the current investigation administered 10^8 CFU/day of the probiotic for only 7 days, the mean difference between baseline and day 7 *L. reuteri* fecal counts was taken into account: 3.0 $\log_{10}$, $SD_{difference}$= 2.1 (20). Nine volunteers were required to achieve a power of 90% (alpha 0.05; two-tailed) with regard to detecting a significant mean difference between baseline and day 7 fecal counts. Since 10% of adults are natural carriers of *L. reuteri* and 80% of volunteers exhibit colonization after 7 days of consumption (20), we recruited 15 volunteers in each group to ensure that adequate power was achieved.

STATISTICAL ANALYSES

Data were analyzed using SPSS statistical software. Descriptive statistics included mean and standard deviations for continuous variables (i.e. age, height,

weight, *L. reuteri* count, etc…) and frequencies for categorical variables (detection of *L. reuteri*). Significance was established at $p \leq 0.05$. The Shapiro-Wilk test was used to examine the normality of each variable. Statistical analysis employing one-way repeated measures ANOVA was used to examine differences in *L. reuteri* counts over time. Dosing scheme was used as a between-subjects factor to measure differences between the two groups. Epsilon, using the Greenhouse-Geiser correction (when epsilon is <0.75) or Huynh-Feldt correction (if Greenhouse-Geiser epsilon is >0.75), was used to adjust the degrees of freedom if Mauchly's Test of Sphericity resulted in significant deviation from the assumption of sphericity. If a significant F-ratio was observed for a main effect, post-hoc comparisons were made using Tukey's HSD. Results are expressed as mean ± standard deviation (SD) unless otherwise noted.

RESULTS

FINAL SAMPLE SIZE AND REASONS FOR WITHDRAWALS

The consecutive-day trial of "fresh" *L. reuteri* was conducted first, wherein 15 volunteers were initially recruited. One additional volunteer was recruited due to volunteer withdrawal (i.e., inability to produce a baseline fecal sample within 3 days). Fifteen volunteers completed the consecutive-day trial; however, the final analysis included data for 9 of the 15 volunteers since 1 volunteer was colonized with *L. reuteri* at baseline and 5 volunteers were unable to provide at least one fecal sample within each of the analysis groupings (D2-4, D5-7, D13-15, and D20-22).

For the alternate-day trial of "fresh" *L. reuteri*, 15 volunteers were initially recruited, with an additional 4 volunteers recruited due to volunteer withdrawal. Reasons for volunteer withdrawal included: inability to provide a baseline fecal sample (n = 1), moved to another geographic location (n = 1), antibiotic usage (n = 2), diarrhea

(n = 1), and medical event not associated with the study (n = 1). Thirteen volunteers completed the alternate-day trial; however, the final analysis included data for 9 of the 13 volunteers, since 1 volunteer was colonized with *L. reuteri* at baseline, and 4 volunteers were unable to provide at least one fecal sample within each of the analysis groupings (D2-4, D5-7, D13-15, and D20-22). Of note, one volunteer reported diarrhea of undetermined etiology during the probiotic intervention period. No other adverse events were reported.

The consecutive-day trial of "stored" *L. reuteri* was conducted last, wherein 12 volunteers were initially recruited. Four additional volunteers were recruited due to volunteer withdrawal. Reasons for volunteer withdrawal included the inability to produce a baseline fecal sample within 3 days (n = 1) and moved to another geographic location (n = 3). Twelve volunteers completed this trial; however, the final analysis included data for 10 of the 12 volunteers since 2 volunteers were unable to provide at least one fecal sample within each of the analysis groupings (D2-4, D5-7, D13-15, and D20-22).

BASELINE CHARACTERISTICS

Baseline characteristics of the volunteers included in the final analysis, as well as typical fiber consumption, are shown in Table 2. There were no significant differences between groups for any of these baseline characteristics. None of the volunteers included in the final analysis reported consumption of any probiotic-containing food items during the study (aside from the study-related pudding).

***L. REUTERI* ISOLATION FROM FECES**

L. reuteri counts in response to consecutive-day and alternate-day intake of *L. reuteri*-containing pudding are shown in Figure 2. All volunteers consuming "fresh" *L. reuteri* had detectable levels of *L. reuteri* in their feces D2-4 and D5-7. In contrast, only one volunteer consuming "stored" *L. reuteri* had detectable levels of *L. reuteri* in their feces D2-4 and D5-7. In response to consecutive-day intake of "fresh" *L. reuteri*, average fecal count of *L. reuteri* significantly increased from baseline to D2-4 and baseline to D5-7 ($p < 0.05$); and, significantly declined after discontinuation of feedings compared to D2-4 and D5-7 ($p < 0.05$). In response to alternate-day intake of "fresh" *L. reuteri*, average fecal count of *L. reuteri* significantly increased from baseline to D2-4 ($p < 0.05$) and significantly declined from D2-4 after the consumption period ended ($p < 0.05$). In response to consecutive-day intake of "stored" *L. reuteri*, there were no significant differences in fecal count of *L. reuteri* over time.

In response to daily consumption of "fresh" *L. reuteri*, fecal shedding of the live *L. reuteri* bacteria was detected in 44% of volunteers (n = 4) on D13-15 and 22% of volunteers (n = 2) on D20-22. In response to alternate-day consumption of "fresh" *L. reuteri*, 33% of volunteers (n = 3) demonstrated fecal shedding of the live bacteria on D13-15, while 22% (n = 2) had detectable levels on D20-22. In response to consecutive-day intake of "stored" *L. reuteri*, none of the volunteers demonstrated fecal shedding of the live bacteria after the consumption period ended.

L. reuteri counts were higher on D2-4 in response to alternate-day consumption compared to consecutive day consumption ($p < 0.05$). No other significant between group differences were observed.

DISCUSSION

This study sought to compare the persistence of *L. reuteri* from feces in response to consuming "fresh" *L. reuteri*-containing pudding for 7 consecutive days, "fresh" *L. reuteri*-containing pudding for alternate-days (4 doses, every-other-day, for 7 days), and "stored" *L. reuteri*-containing pudding for 7 consecutive days. We also sought to determine how long *L. reuteri* persists in the gastrointestinal tract after discontinuation of consumption. We hypothesized that *L. reuteri* levels would be higher during the consumption period in response to consecutive-day dosing of "fresh" *L. reuteri*-containing pudding versus alternate-day dosing of "fresh" and consecutive day dosing of "stored" *L. reuteri*-containing pudding; and, that *L. reuteri* would persist for a longer period of time after completion of "fresh" consecutive-day doses compared to "fresh" alternate-day doses and "stored" consecutive-day doses. We found levels of *L. reuteri* isolation from feces was equivalent with alternate-day compared to daily intake of "fresh" *L. reuteri*-containing pudding, and that colonization declines rapidly once dosing stops regardless of the dosing strategy. Additionally, we found that persistence of *L. reuteri* was unachievable during or after consumption of "stored" *L. reuteri*-containing pudding.

During the consumption period, both groups consuming "fresh" *L. reuteri* experienced an increase in *L. reuteri* levels from baseline, which is consistent with the literature. Colonization by *L. reuteri* in the adult human intestinal tract has been demonstrated with administration of 4×10^8 CFU/d of the probiotic for 28 days (17). Further, Bjorkman et al (1) and Wolf et al (20) reported colonization in 8 of 10 volunteers given 10^9 CFU/day of *L. reuteri* for 12 days and in 15 of 15 volunteers given 10^{11}

CFU/day of L. reuteri for 21 days, respectively. In the current study, fecal recovery of *L. reuteri* was achieved within 4 days of consuming "fresh" *L. reuteri*-containing pudding everyday or every-other-day.

In contrast to our findings that *L. reuteri* persisted in the human intestinal tract in response to consecutive and alternate-day intake of "fresh" *L. reuteri*-containing pudding, fecal recovery of *L. reuteri* was unachievable during consumption of "stored" *L. reuteri*-containing pudding. Indeed, *L. reuteri* levels did not increase from baseline and *L. reuteri* was recovered in the feces from only one volunteer. Plate counts indicated that the pudding contained 10^9 CFU of *L. reuteri* per serving after storage in a 37ºC incubator for approximately 7 days; however, the high temperature treatment seems to have injured the cells and compromised their ability to remain alive through GI transit to the feces (16). It is unclear how far *L. reuteri* persisted in the GI tract before dying, e.g., it is possible that the *L. reuteri* persisted through the small intestine, but not the large intestine. Biopsies of the intestinal wall are necessary to confirm the persistence of *L. reuteri* in different portions of the GI tract (20). Nevertheless, our findings do not provide support for inclusion of *L. reuteri* in combat rations at this time.

Surprisingly, we observed that *L. reuteri* counts were significantly higher on D2-4 in response to alternate-day feeding compared to consecutive-day feeding of both "fresh" and "stored" *L. reuteri*-containing pudding. It is conceivable that discrepancies in fecal collection and processing methodology might have contributed to that observation. *L. reuteri* could have multiplied if fecal samples were not refrigerated within the allotted time period (30 minutes) after defecation, if fecal samples were not frozen within 24-hours of defecating, or if refrigeration time prior to processing differed between the

groups. However, data collection records indicated that all fecal samples included in the final analysis were, indeed, properly refrigerated, frozen and processed within this 24-hour time-frame, and refrigeration time did not differ between groups. Therefore, we believe the differences between groups are real and of biological origin, not a methodological artifact.

It is possible that some volunteers in the alternate-dosing group were more responsive to *L. reuteri* consumption, thus explaining why we observed higher *L. reuteri* counts during alternate-day feeding compared to consecutive-day feeding. Individual differences in dietary prebiotic intake (i.e. non-digestible carbohydrates, found in common foods, that act as nutritional substrates for probiotics) may be one factor that could alter the response to probiotics, since intake of prebiotics has been shown to promote colonization of probiotics (11; 15). We did not observe a significant difference in total dietary fiber intake (i.e. a crude measure of assessing prebiotic intake) between groups; however, it is possible that prebiotic intake may have differed between groups which was not quantifiable via the dietary software employed in this study. Further, we observed large inter-subject variations in total fiber intake, thus potentially masking differences between groups. In addition to dietary intake, there may be other physiological factors affecting responsiveness to *L. reuteri* consumption, for example, gut microbiota of the individual (5). If volunteers in the alternate-day feeding group were more responsive to probiotics, then we might have expected higher *L. reuteri* values D5-7 in response to alternate-day compared to consecutive-day consumption; however, this did not occur. It is possible that *L. reuteri* values were not higher D5-7 for the alternate-day dosing group, because D5-7 reflects only 3 days of consumption whereas the group

receiving probiotics everyday received 6 doses. Future studies should survey prebiotic intake, in order to determine if "day-to-day" prebiotic intake affects probiotic colonization. It would also be valuable for future studies to employ a cross-over design, with an adequate wash-out period, in order to eliminate individual responsiveness as a potential confounder.

In the current study, *L. reuteri* was detected in the feces of some volunteers, but not others, one and two weeks after consumption of "fresh" *L. reuteri*-containing pudding was discontinued regardless of the dosing strategy employed. This outcome may, again, be attributable to differences in prebiotic intake or other unknown individual variations. Although fecal shedding of *L. reuteri* after the consumption period was apparent in some volunteers, *L. reuteri* levels were low and likely insufficient to confer a clinical benefit. Similar to our findings, Valeur et al. detected low levels of *L. reuteri* in the feces 2-4 weeks after the discontinuation of long-term *L. reuteri* intake (4×10^8 CFU/day for 28-days) (17). In contrast, Wolf et al. (20) reported that *L. reuteri* levels in the feces one week after 21-days of consumption (10^{11} CFU/day) were similar to the levels achieved during the consumption period (i.e. levels peaked after 21 days of consumption but levels on day 14 were similar to levels one week after consumption was discontinued). The discrepancy between Wolf et al.'s findings and the current study may be due to the fact that Wolf et al. supplemented for 21 days compared to the 7-day consumption period described herein. It unclear if Valeur et al. would have achieved similar results to Wolf et al., since Valeur et al. did not measure *L. reuteri* levels one week after the conclusion of consumption. Taken together, these findings suggest short-term consumption produces a transient colonization. Additional studies

are required to determine the time course of colonization following discontinuation of long-term consumption.

Consumption of *L. reuteri* appeared to be safe and was largely free of side effects. One volunteer reported a one-day bout of diarrhea on day 5 of the alternate-day dosing trial, which spontaneously resolved on day 6. It is unclear if this isolated case of diarrhea was attributable to the probiotic-containing food item or if it was of food-borne or viral etiology. Our findings of no serious adverse effects are consistent with the literature. Connolly (2) reviewed 12 clinical studies with approximately 600 volunteers ranging in age from premature infants to adults, and reported no serious adverse effects associated with administration in doses of 10^7 - 10^{11} CFU of *L. reuteri* per day. In one study, 3 of 30 volunteers who received 10^{11} CFU/day for 21 days noted a slight, although transient, increase in gas formation (20). Further, when *L. reuteri* was administered in multiple daily doses (i.e. up to 4 x 10^8 CFU), there were no significant side effects reported, aside from a mild increase in flatus in some volunteers (17). Only one volunteer in the current study reported flatus. Therefore, *L. reuteri* appears to be safe and well tolerated in healthy, young volunteers.

A weakness of this study is that persistence of *L. reuteri* was measured via fecal shedding as opposed to direct biopsy of GI tract tissues. Valeur et al. (17) reported that *L. reuteri* in the feces was lower than *L. reuteri* isolated for the GI tract; however, there was a correlation between methods (17). Therefore, we contend that the detection of live bacteria in the feces is an acceptable method for providing insight into the persistence of probiotics in the GI tract. Further, these findings may not be applicable to other species and strains of probiotics. Lastly, although these findings provide insight

into alternative dosing strategies, in terms of persistence of *L. reuteri* in the GI tract, they do not provide evidence related to clinical outcome measures.

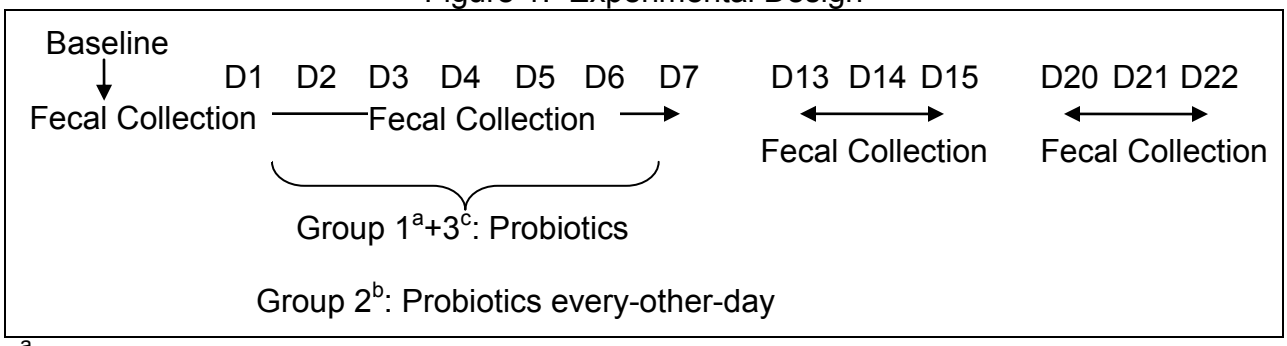
CONCLUSIONS

Probiotics are gaining popularity and are heavily marketed to consumers for their purported health benefits. The findings presented herein indicate that it may not be necessary to consume probiotics every day, and that colonization may be achieved via intermittent dosing. Further, once consumption of "fresh" probiotics is discontinued, there is a significant decrease in *L. reuteri* counts, regardless of dosing regimens. Outcomes from this study also indicate that colonization of *L. reuteri* is unachievable in response to daily consumption of heat-treated *L. reuteri*-containing pudding.

RECOMMENDATIONS

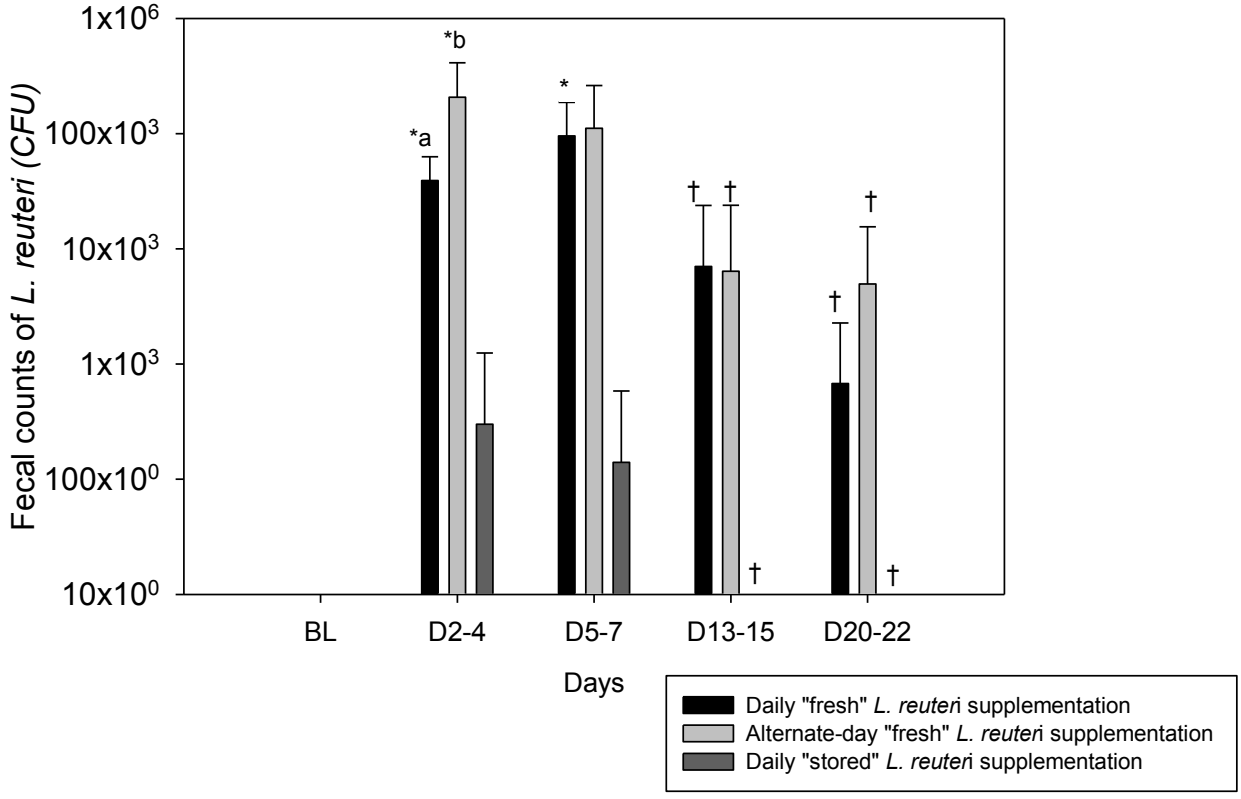
Future studies investigating clinical outcomes should investigate persistence of colonization in alternative dosing strategies, which may be more practical (in terms of convenience and cost savings) for consumers. The addition of *L. reuteri* to combat rations is not advisable at this time. However, it would be worthwhile to determine if a ration component containing both prebiotics and probiotics positively affects persistence of probiotics in the GI tract.

Figure 1. Experimental Design



[a]daily administration of "fresh" probiotics
[b]alternate-day administration of "fresh" probiotics
[c]daily administration of "stored" probiotics

Figure 2. Fecal Counts of *L. reuteri* During and After Consumption



*significant within group difference from baseline, $p < 0.05$.
†significant within group difference from consumption period
Unlike superscripts indicate significant between group differences, $p < 0.05$

Table 1. Quantity of *L. reuteri* in pudding

	Quantity of *L. reuteri* after production of pudding (CFU/serving)	Quantity of *L. reuteri* at completion of study (CFU/serving)
Consecutive Day "Fresh" Group	5.0×10^8	6.9×10^8
Alternate-day "Fresh" Group	5.7×10^8	2.0×10^8
Consecutive Day "Stored" Group	$1.4 \times 10^{9*}$	9.7×10^8

L. = *Lactobacillus*; CFU = colony forming units; *count was obtained after storage at 37ºC for 7 days.

Table 2. Baseline Characteristics

	Group 1[a] (n = 9)	Group 2[b] (n = 9)	Group 3[c] (n = 10)
Sex (male, female)	9, 0	7, 2	10, 0
Age (years)	22 ± 4	22 ± 4	23 ± 5
Height (cm)	176.6 ± 5.8	171.9 ± 8.3	181.1 ± 8.1
Weight (kg)	83.9 ± 12.2	82.6 ± 19.5	77.7 ± 11.7
Fiber (g)	29.9 ± 11.3	24.8 ± 13.7	26.9 ± 12.3

[a]daily administration of "fresh" probiotics

[b]alternate-day administration of "fresh" probiotics

[c]daily administration of "stored" probiotics

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