Effects of *Limosilactobacillus reuteri* Strains PTA-126787 and PTA-126788 on Intestinal Barrier Integrity and Immune Homeostasis in an Alcohol-Induced Leaky Gut Model

Dharanesh Gangaiah[1*], Min Gu[2], Aline Zaparte[2], Olaf Will[3], Laurie C. Dolan[4], Anthony Goering[5], Jason Pillai[5], Shrinivasrao P. Mane[6], German Plata[1], Emily B. Helmes[1], David A. Welsh[2], and Arvind Kumar[1*]

Supplementary Data

Supplementary Materials and Methods

28-day rat toxicity study design and execution. The study was conducted according to the following guidelines: Good Laboratory Practice; OECD Guideline 407, Repeated Dose 28-Day Oral Toxicity Study in Rodents (2008) [1]; and US FDA Toxicological Principles for the Safety Assessment of Food Ingredients (Redbook 2000), IV.C. 3. a. Short-Term Toxicity Studies with Rodents (November 2003) [2].

Eighty rats (10/sex/group) were assigned to receive the vehicle control (sterile saline, Group 1) or the following nominal amounts of each strain calculated from CFUs/g, together in one dosing formulation: 1.7E+10 CFUs/kg body weight (BW)/day (Group 2), 3.3E+10 CFUs/kg BW/day (Group 3) or 1.0E+11 kg BW/day (Group 4). The results of the concentration verifications were below the targeted concentrations, presumably because of differences in sampling and enumeration between laboratories. The respective geometric doses of strains 3630 plus 3632 for Groups 2, 3 and 4 were as follows: 3.1E+9 CFU/kg BW/day plus 7.5E+9 CFUs/kg BW/day (Group 2), 4.5E+9 CFUs/kg BW/day plus 1.4E+10 CFUs/kg BW/day (Group 3), and 1.6E+10 CFUs/kg BW/day plus 5.7E+10 CFUs/kg BW/day (Group 4). All doses were administered by gavage at 5 mL/kg BW. The control group (Group 1) received the vehicle only, at the same dose volume as the test animals. Fresh formulations of the test article were prepared daily and continuously stirred until administration. Due to a technical oversight, one male in Group 2 was not dosed on Day 13. This animal was subsequently euthanized due to dosing injury on Day 19; this animal removal had no impact on the study outcome.

Body weights were recorded two times during the acclimation period, twice weekly thereafter, shortly before dose administration to adjust the dosing volume to current body weight, and immediately prior to termination. Body weight gain was calculated for selected intervals. Food consumption was measured and recorded twice weekly to coincide with body weight measurements. The animals were observed at least once daily for viability, behavioral changes, and signs of gross toxicity. Detailed observations of clinical condition were conducted weekly. The doses were administered for 29 or 30 days, based on scheduled termination date. On Day 30, animals were randomly selected from each group and designated for translocation analysis (5/sex/group). All animals selected for translocation analysis were treated with the test substance on Day 30 and terminated on Day 31. All remaining animals were terminated as scheduled on Study Day 30. Animals were fasted overnight prior to their scheduled termination. While fasting, the animals were placed in metabolism cages and urine was collected.

Urine samples were collected from each animal and stored on ice or under refrigeration until analyses for color, clarity, quality, volume, sediment (microscopic), specific gravity, pH, glucose, protein, ketones, blood, bilirubin, and urobilinogen. At study termination, blood samples for clinical chemistry [(aspartate aminotransferase (AST), alanine aminotransferase (ALT), sorbitol dehydrogenase (SDH), alkaline phosphatase (ALKP), gamma glutamyl transferase (GGT), total bilirubin (BILI), urea nitrogen (BUN), creatinine (CREA), glucose (GLUC), total protein (TP), albumin (ALB), globulin (GLOB), total cholesterol (CHOL), triglycerides (TRIG), calcium (Ca), chloride (Cl), inorganic phosphorus (IPHS),

potassium (K), and sodium (Na)], and hematology were collected from the inferior vena cava under isoflurane anesthesia. Blood samples collected for hematological analyses were placed in tubes containing K2EDTA and stored refrigerated until analysis, and samples for coagulation were collected in tubes containing 3.2% sodium citrate and then centrifuged to obtain plasma (which was stored at -80°C until analysis). Blood samples for clinical chemistry were collected into tubes that did not contain a preservative and were centrifuged under refrigeration to obtain serum (which was stored at -80°C until analysis). Hematological parameters included red blood cell count (RBC), hematocrit (HCT), hemoglobin concentration (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red blood cell distribution width (RDW), reticulocyte count (RET), platelet count (PLT), total white blood cell count (WBC), and differential leukocyte count [absolute lymphocytes (ALYM), monocytes (AMON), neutrophils (ANEU), basophils (ABAS), large unstained cells (ALUC), and eosinophils (AEOS)]. Mean corpuscular hemoglobin concentration (MCHC) was calculated. Additionally, separate blood smears were prepared from each animal undergoing hematological evaluation and if necessary, were stained with Wright-Giemsa stain and examined to clarify or substantiate the results of the hematology findings.

At scheduled termination, all animals were euthanized and subjected to a full necropsy. The following organs were collected and weighed: adrenals (combined), brain, epididymides (combined), heart, kidneys (combined), liver, spleen, thymus, ovaries with oviducts (combined), testes (combined) and uterus [3]. Following weighing, these organs were preserved in 10% buffered formalin [except for the eyes (with optic nerves), epididymides, and testes, which were preserved in modified Davidsons' fixative and stored in ethanol] for possible future histopathological examination. All tissues that contained gross lesions, accessory genital organs (prostate and seminal vesicles), aorta, bone (femur), bone marrow (femur and sternum), cecum, cervix, colon, duodenum, esophagus, Harderian gland, ileum, jejunum, larynx, lungs, mammary gland, mandibular and mesenteric lymph nodes, nose with nasal turbinates, pancreas, peripheral nerve (sciatic), pharynx, pituitary, rectum, salivary glands, skeletal muscle, skin, spinal cord (cervical, lumbar, and mid-thoracic), sternum, stomach, thyroid/parathyroid, trachea, urinary bladder and vagina were also collected from each animal at necropsy and preserved in 10% neutral buffered formalin. Portions of the blood (0.5 mL), liver and mesenteric lymph nodes (0.2-0.5 g each) were harvested from 5 randomly selected animals per sex per group for bacterial translocation evaluation (see below) and were not placed into fixative. The fixed organs and tissues were processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Histopathological examinations were performed on the preserved organs and tissues of all control and high dose animals.

Bacterial translocation analysis in rats. Blood collected for the analysis (approximately 500 µL/animal) was directly plated in duplicate on to MRS agar medium and brain heart infusion (BHI) agar. Cultures were incubated for 48 h at 37°C in an anaerobic box with AnaeroPaks (for MRS) or aerobically (for BHI). Portions of liver and mesenteric lymph nodes that were collected (0.5-2 g tissue/animal) were homogenized using a mechanical homogenizer in 2 ml of Dulbecco's phosphate buffered saline (1 g/10 mL) and 100 µL of the resulting homogenates were plated in triplicate on MRS agar and incubated at 37°C under anaerobic conditions (AnaeroPaks) for 24-48 h or until colony growth was adequate for counting. Plates were received by BioPrimate (Newark, Delaware) in anaerobic chambers immediately following 48 h of incubation at 37°C. Plates were held overnight at 4°C (maintained under anaerobic conditions) and processed the following morning. All plates were visually inspected for colony growth after incubation and individual colonies were counted. An automated plate counter was utilized to assist in enumerating highly populated plates. The mean CFUs/g of tissue was calculated using the amount of respective sample evaluated, plate colony growth and factoring for applicable dilutions. Colony morphology was assessed for six characteristics for all colonies present and colonies were picked and replated. If there were more than 5 colonies of a particular morphology on plates from a single sample, only 5 of that morphology were used for further analysis. Colonies were streaked on new MRS agar plates and incubated anaerobically at 37°C for 48 h.

Material from the regrown colonies was processed for DNA extraction and the DNA subjected to amplification of the 16S ribosomal RNA gene variable region 4 using the standard primers and methods described by the earth microbiome project [4]. Amplicon libraries were sequenced on an Illumina MiSeq instrument and a taxonomic designation was assigned for each isolate using the Mothur 16S MiSeq analysis pipeline [5]. By 16S V4 sequence analysis, *L. reuteri* can be differentiated from other species. The 16S sequences from the lyophilized test materials and all *L. reuteri* identified on the plated tissue samples generated identical 16S V4 sequences. For each tissue sample of origin, morphology was assessed, taxa assigned based on 16S V4, or for *L. reuteri* isolates further refined based on D-alanine-D-alanyl carrier protein ligase [*dltA*, a gene that clearly discriminates the monophyletic clades associated with host origin of the isolates [6]] gene sequence and random amplification of polymorphic DNA (RAPD) profiles.

Amplification and sequencing of a fragment of the *dltA* gene was carried out on test substance isolates as well as all tissue isolates identified as *L. reuteri* by 16S rRNA V4 sequence. The two test substances showed identical sequences over this region and were consistent with isolates of porcine origin, referred to going forward as genotype 1. By *dltA* gene sequence, over half of the *L. reuteri* tissue isolates from the study were different from the test strain, but identical to each other and consistent with isolates of rodent origin. These two *dltA* sequence variants have over a dozen single nucleotide differences over the region sequenced. Genotypes 1 and 2 were designated based on *dltA* gene sequences, where type 1 represented porcine origin and was consistent with all test substance isolates and genotype 2 was distinct and indicated rodent origin. A limitation of the study is that when more than 5 colonies of a given morphological type were present on plates from a single sample, only 5 representative colonies were picked and analyzed further. The total number of colonies were assigned proportionally to the taxa found among the five representatives.

Saponification, extraction, column chromatography, resin purification, HPLC purification and structural elucidation of PKS metabolite. Cells were removed from the culture by centrifugation and supernatant was discarded. Cell pellets were washed using a 10% saline solution and centrifuged once again. Supernatant was discarded and cell pellet was transferred into a clean 250 mL bottle. Cells were saponified using modified methods described previously [7]. Cells were saponified directly with 150 mL of 0.5M NaOH dissolved in 95% reagent ethanol. The mixture was then heated to 60°C with constant agitation for up to 2 h. The alcoholic solution was vacuum filtered using filter paper, cooled to room temperature, and then extracted using 100 mL of hexanes 3-5 times or until hexanes extractions were clear to remove unsaponifiable material. Hexanes were appropriately discarded. Alcoholic solution was then acidified to a pH of 1.0 using 37% hydrochloric acid and extracted using 100 mL of hexanes 3-5 times or until the extraction was clear. Extractions were pooled together, washed using a saturated saline solution, and concentrated to dryness under vacuum with a rotary evaporator.

The dried extract was resuspended in 20 mL of acetone. Five to ten grams of silica gel 60 was added to the suspension and then dried under vacuum in a rotary evaporator until a free-flowing powder was achieved. Extraction was dry loaded onto a prepared fritted glass column using 1:1 ethyl acetate: hexanes as the mobile phase. The first band to elute, “Band 1,” was deep yellow to orange in color. The second band was a light to pale yellow. Fractions for each band were pooled respectively and concentrated to dryness.

Ten grams of Diaion HP-20 was prepared according to protocol using methanol and RO water. Hydrated resin was then placed in a 35 mL fritted glass Buchner funnel. Dried “Band 1” extract was resuspended in 1 mL of 100% MeOH and then diluted to 10% MeOH using RO water. Extract was then applied to prepare Diaion HP-20 resin and washed with RO water three times. Resin was then washed using 30% MeOH three times, followed by 70% MeOH three times, then 100% MeOH three times, and finally with pure acetone three times with fractions being collected at each bump. The 100% MeOH fraction was then dried down completely and used as test samples for NMR analysis and AhR activity.

The 100% MeOH bump was resuspended in MeOH and further purified by HPLC using a Phenomenex Kinetex 2.6u C18 100A column (150 x 4.60mm, 00F-4462-E0, 609347-61). HPLC grade methanol was used as the mobile phase.

The structure of dodecapentaenoic acid produced by the PKS BGC was determined by high-resolution positive mode LC-MS using a Thermo Q-Exactive mass spectrometer and a Bruker DRX 500 MHz NMR in $CDCl_3$ utilizing 2D-^{1}H COSY, ^{1}H-^{13}C HMBC, and 1D-^{1}H experiments.

Supplementary Results

***L. reuteri* 3630 and 3632 administration had no adverse effects on clinical parameters, body weight and food consumption**. The results showed that there were no test substance-related deaths or clinical observations in rats. Clinical observations were observed only in a few Group 1 and Group 2 male rats and Group 1 female rats and were not attributed to test material administration due to lack of dose-dependence. One male in Group 2 was euthanized on Day 19. At necropsy, this animal had a visible tear of the esophagus, with fluid/edema noted in the thoracic cavity and around the lungs; these changes were attributed to inadvertent esophageal dosing trauma and served as the cause of morbidity. Other animals appeared healthy during the study. There were no effects of the test material on body weight (**Supplementary Figure 1 and Supplementary Figure 2**) or body weight gain (**Supplementary Table 1**) of either sex. There was no effect of the test articles on food consumption other than a significant increase in Group 4 males from Days 15-22 (**Supplementary Table 2**).

Administration of 1:1 mixture of *L. reuteri* 3630 and 3632 had no adverse effects on clinical chemistry in rats. The results showed that there were no test substance-related adverse effects on clinical chemistry. The only change in clinical chemistry was increased creatinine in Group 4 females (**Table 1**), which was not considered toxicologically relevant because the values were within ranges of historical controls. Further, there were no effects of the test material on renal pathology or urinalysis. A few changes in hematology were noted in Group 3 males (decreased HGB, HCT and increased ANEU) and Group 3 females (decreased HCT) (**Supplementary Table 3**), but these changes were not considered test-material related based on the slight degree of change, lack of dose-dependence and lack of corresponding changes in MCH, MCHC, or red blood cell count. Further, these values were within historical control ranges. There was no effect of the test material on either index of blood clotting (APTT or PT) (**Supplementary Table 3**) or urinalysis (**Supplementary Table 4**).

Administration of 1:1 mixture of *L. reuteri* 3630 and 3632 had no adverse effects on organ weights in rats. Organ weights were not affected by administration of the test substance, except for a significant increase in the spleen to body weight ratio in Group 2 males (**Supplementary Table 5**). At scheduled termination, no macroscopic or microscopic findings in any animal were attributed to the test material. Incidental macroscopic findings noted at necropsy were as follows (with microscopic correlates in parentheses): One female in Group 1 had a fluid filled uterus (dilatation); one male in Group 2 had small, bilateral testes (right – 11mm x 6mm, and left – 16mm x 4mm; atrophy), and small, bilateral epididymides (right – 32mm x 3mm, and left – 42mm x 3mm; hypospermia); one male in Group 3 had a small, right testis (1.8cm x 1.2cm; atrophy); and one female in Group 3 had a fluid filled uterus (dilatation). These findings are common incidental macroscopic findings in control rats of this age and strain. Minimal grade chronic progressive renal nephropathy (CPN) was found in 3 males in Group 1, 6 males in Group 4 and 1 female in Group 4. Due to the lack of elevated BUN or CREAT values in the Group 4 animals, no further correlated findings, the minimal severity level, the well-known etiology of this spontaneously occurring disease (especially in the Sprague-Dawley rat strain) and the absence of

renal counterpart in humans [8], the higher incidence of CPN in Group 4 males compared to Group 1 males is not considered to be of toxicological significance.

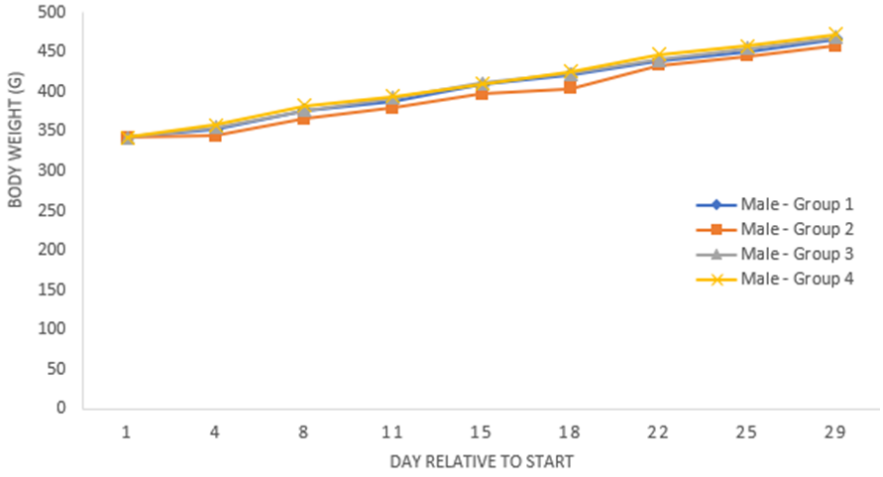
Administration of 1:1 mixture of *L. reuteri* 3630 and 3632 resulted in a small number of translocated bacteria in rats, which showed no dose dependence and correlation with any other clinical or histopathological findings. Translocated bacteria were found in the blood of three animals - one female in Group 1, one male in Group 2 and one male in Group 4 (**Supplementary Table 6**). In the Group 1 animal, 16 colonies were identified as *Staphylococcus*. The animal in Group 2 had four colonies, one *Lactobacillus*, two *L. reuteri* genotype 2 (of rodent origin, not test material related) and one of *L. reuteri* genotype 1, consistent with test substance origin. One animal in Group 4 had a single colony on the blood plates which types as *Limosilactobacillus* genotype 1, consistent with the yellow phenotype colonies from the test substance. The findings were rare (3 of 40 animals overall) and had no apparent association with dose of test material administered.

Translocated bacteria in the liver were found in one to four animals from each group, with no apparent relationship to treatment. Translocated bacteria were also identified in the mesenteric lymph nodes of both female and male rats from each treatment group, with three to five animals per group affected. Differences among the groups are not statistically significant with or without separating out animals by sex.

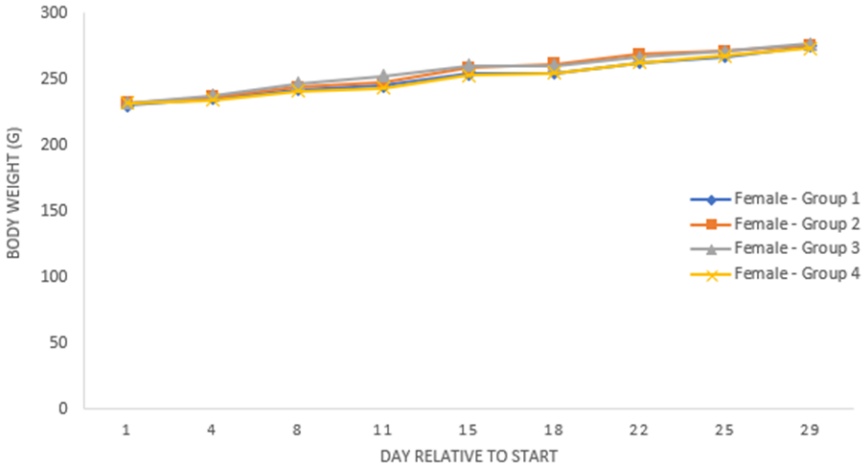
The number of translocated bacteria in liver and mesenteric lymph nodes also was assessed (**Supplementary Table 7**). Group 2 male and female groups showed similar levels of translocation to both mesentery and liver in comparison to vehicle control Group 1. Group 3 animals showed values not significantly different from vehicle control Group 1 (Student's t test, $P < 0.05$) with the exception of mean CFUs in mesentery from males. The high mean value in Group 3 males is driven by the mesentery sample from a single male that yielded 775 colonies on the three plates (representing 0.03 g of tissue) for a CFUs/g of 25,833 for mesentery from that animal. Taxonomic assignment of the colonies from this sample indicated that only 3 (0.38%) were consistent with the test strain (*L. reuteri* genotype 1) derived bacterium. The remainder were *Escherichia*/*Shigella* (733 colonies or 24,433 CFUs/g) and *Enterococcus* (41 colonies or 1,367 CFUs/g).

No statistically significant differences occurred between bacterial counts in the liver or mesentery of Group 4 and Group 1 females (Student's t test, $P < 0.05$) and no nominal findings were indicative of a need for enhanced scrutiny (**Supplementary Table 8**). Group 4 males exhibited nominally higher bacterial counts (means and medians) in both liver and mesentery when compared to vehicle control animals (**Supplementary Table 9**). The results of the analysis were skewed by the results of one high dose male (animal 3868) that exhibited 4,800 colonies in the mesentery and 147 colonies in the liver. Most translocated bacteria in the mesentery of this animal were not of test substance origin (**Supplementary Table 10**) and none of the translocated bacteria in the liver were of test substance origin. This animal did not exhibit translocation of the bacteria to blood. There was no significant difference in colony numbers in the liver or mesentery between Group 1 and Group 4 males (Student's t test, $P < 0.05$).

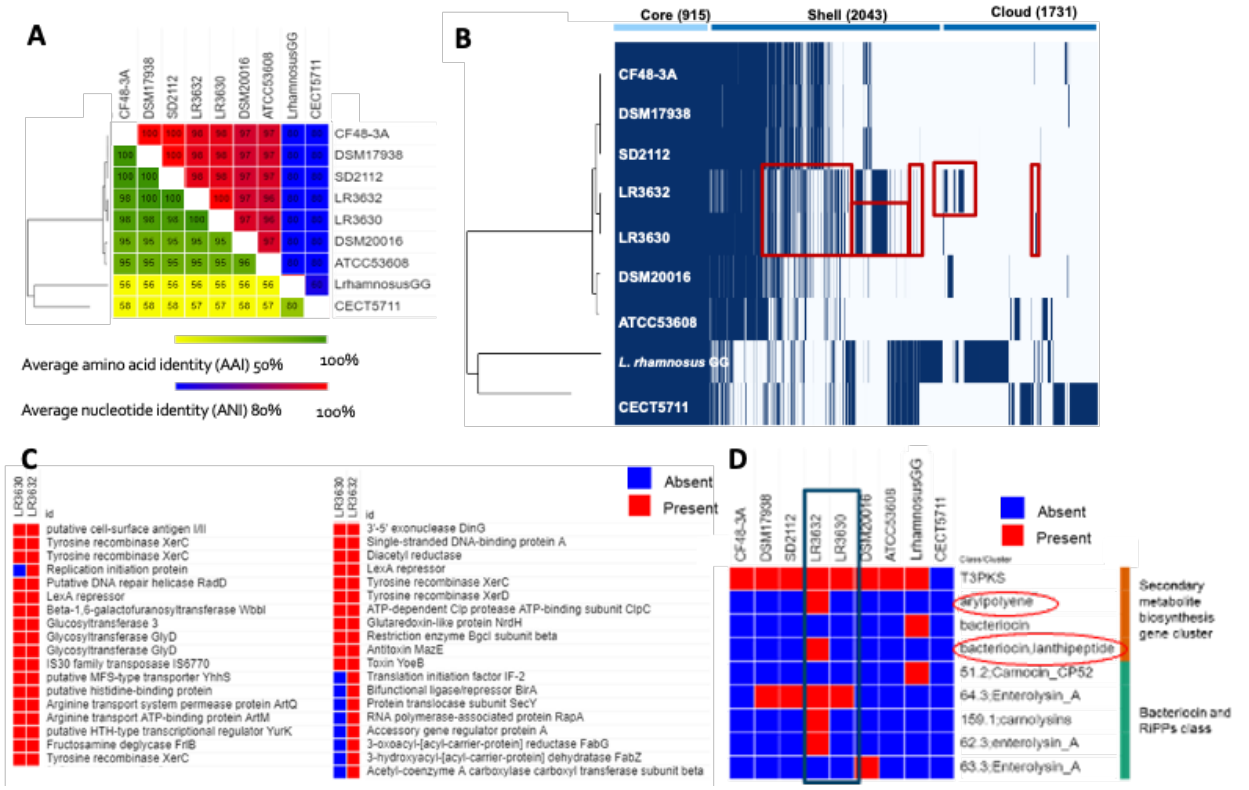
Supplementary Figures



Supplementary Figure 1. Mean body weight of male animals during the 28-day study.



Supplementary Figure 2. Mean body weight of female animals during the 28-day study.



Supplementary Figure 3. Average nucleotide identity (ANI) and ortholog analyses of *L. reuteri* 3630 and 3632 with other well-known human *L. reuteri* and non-*L. reuteri* strains. **A**. ANI of *L. reuteri* 3630 and 3632 with other *L. reuteri* and non-*L. reuteri* strains from humans. **B**. Ortholog relationship of *L. reuteri* 3630 and 3632 with other *L. reuteri* and non-*L. reuteri* strains from humans. Unique genes in *L. reuteri* 3630 and 3632 are highlighted in red boxes. **C**. Unique genes in *L. reuteri* 3630 and 3632 as identified using ortholog analysis. **D**. Unique genes present in *L. reuteri* 3632 as identified using ortholog analysis. Unique genes in *L. reuteri* 3632 that encode a potential secondary metabolite and a bacteriocin are highlighted in red boxes. CF48-3A, DSM17938, SD2112, LR3632, LR3630, DSM20016, ATCC53608 and CECT5711 refers to the respective *L. reuteri* strains. LrhamnosusGG refers to *L. rhamnosus* GG.

Supplementary Tables

Supplementary Table 1. Body weight gain of rats (g) administered *L. reuteri* strains 3630 and 3932 for 28 days.

Day of Study	Group 1 (Control)	Group 2	Group 3	Group 4
Males				
1 → 4	3.50 ± 3.59	0.57 ± 6.26	4.30 ± 7.40	4.97 ± 2.39
4 → 8	6.00 ± 1.05	5.53 ± 1.95	5.30 ± 6.57	6.15 ± 1.77
8 → 11	3.67 ± 1.24	4.50 ± 1.96	5.37 ± 1.05	4.10 ± 2.18
11 → 15	5.28 ± 1.39	4.48 ± 1.76	4.55 ± 1.98	3.93 ± 1.58
15 → 18	4.23 ± 1.83	2.27 ± 5.63	3.83 ± 1.63	5.13 ± 1.97
18 → 22	4.50 ± 1.13	4.78 ± 1.73	4.58 ± 1.72	5.23 ± 0.91
22 → 25	3.57 ± 1.23	3.89 ± 1.60	4.70 ± 1.49	3.60 ± 1.68
25 → 29	4.15 ± 1.14	3.14 ± 1.13	3.50 ± 1.20	3.73 ± 1.06
Females				
1 → 4	1.73 ± 2.37	1.60 ± 1.36	1.93 ± 1.76	0.70 ± 1.52
4 → 8	1.65 ± 0.84	1.78 ± 0.83	2.33 ± 1.31	1.80 ± 1.49
8 → 11	1.03 ± 2.25	1.10 ± 1.30	1.87 ± 3.14	0.70 ± 1.78
11 → 15	2.30 ± 1.06	2.95 ± 0.94	1.93 ± 1.54	2.38 ± 1.56
15 → 18	0.07 ± 2.39	0.80 ± 1.69	0.17 ± 1.55	0.60 ± 2.18
18 → 22	2.08 ± 0.70	1.85 ± 0.92	1.50 ± 0.86	2.05 ± 1.14
22 → 25	1.47 ± 2.15	0.83 ± 1.87	1.53 ± 2.45	1.60 ± 2.30
25 → 29	2.03 ± 1.03	1.15 ± 0.74	1.48 ± 1.17	1.48 ± 1.20

N = 10/group except for N=9 in Group 2 males from Day 18 onward (due to early termination of one animal in this group). Data are presented as mean ± standard deviation (SD).

Supplementary Table 2. Food consumption of rats administered *L. reuteri* strains 3630 and 3932 for 28 days (g/day).

Day of Study	Group 1 (Control)	Group 2	Group 3	Group 4
Males				
1 → 4	23.97 ± 2.89	20.70 ± 5.41	24.37 ± 1.84	24.80 ± 2.00
4 → 8	25.73 ± 1.27	22.98 ± 5.15	25.05 ± 1.05	27.25 ± 2.30
8 → 11	25.63 ± 1.63	25.60 ± 1.89	25.97 ± 1.32	28.70 ± 3.84
11 → 15	26.03 ± 1.75	25.90 ± 1.68	25.08 ± 0.93	27.40 ± 2.83
15 → 22	25.71 ± 1.34	26.41 ± 2.24	26.10 ± 1.68	31.11 ± 6.99*
22 → 25	27.33 ± 1.01	27.15 ± 1.33	27.97 ± 1.55	28.03 ± 1.93
25 → 29	28.55 ± 1.24	27.31 ± 1.76	28.53 ± 1.72	30.10 ± 2.68
Females				
1 → 4	18.00 ± 0.56	17.60 ± 1.05	17.73 ± 0.29	17.57 ± 1.15
4 → 8	18.15 ± 0.94	17.73 ± 1.10	18.38 ± 0.89	17.70 ± 1.58
8 → 11	17.60 ± 0.76	17.83 ± 1.56	17.93 ± 0.77	16.80 ± 2.34
11 → 15	18.73 ± 1.22	19.40 ± 1.09	18.88 ± 1.05	18.18 ± 1.47
15 → 22	17.59 ± 0.75	17.90 ± 1.50	17.66 ± 1.12	17.64 ± 1.39
22 → 25	19.13 ± 1.78	18.53 ± 1.92	19.33 ± 1.71	18.43 ± 1.69
25 → 29	19.28 ± 1.08	19.15 ± 1.23	19.25 ± 1.03	18.88 ± 1.60

N = 10/group except for N=9 in Group 2 males from Day 22 onward (due to early termination of one animal in this group) and in Group 4 males during the Day 18-22 interval, where n= 8 (inadvertent). Data are presented as mean ± standard deviation (SD). * Significantly different from control, ANOVA and Dunnett (Rank), $p< 0.05$.

Supplementary Table 3. Hematology and coagulation data for rats administered *L. reuteri* strains 3630 and 3632 for 28 days.

Parameter	Group 1 (Control)	Group 2	Group 3	Group 4
Males				
RBC (x106/µL)	8.48 ± 0.66	8.54 ± 0.30	8.09 ± 0.43	8.33 ± 0.32
HGB (g/dL)	15.6 ± 0.7	15.5 ± 0.5	14.8 ± 0.7*	15.4 ± 0.6
HCT (%)	49.3 ± 2.5	47.9 ± 1.8	45.7 ± 2.1*	47.6 ± 1.7
MCV (fL)	58.3 ± 3.9	56.1 ± 2.1	56.5 ± 1.9	57.2 ± 1.1
MCH (pg)	18.5 ± 0.8	18.2 ± 0.7	18.4 ± 0.7	18.5 ± 0.3
MCHC (g/dL)	31.8 ± 1.4	32.5 ± 0.8	32.5 ± 1.1	32.3 ± 0.6
RDW (%)	13.0 ± 0.9	12.9 ± 0.4	13.1 ± 0.6	13.0 ± 0.7
PLT (x103/µL)	952 ± 159	919 ± 148	852 ± 282	830 ± 304
WBC (x103/µL)	8.77 ± 2.03	10.75 ± 3.43	11.91 ± 3.75	10.58 ± 2.68
ANEU (x103/µL)	1.38 ± 0.47	1.81 ± 0.39	2.08 ± 0.77*	1.45 ± 0.31
ALYM (x103/µL)	6.98 ± 1.71	8.39 ± 2.95	9.22 ± 3.18	8.70 ± 2.41
AMON (x103/µL)	0.17 ± 0.09	0.23 ± 0.13	0.29 ± 0.18	0.21 ± 0.14
AEOS (x103/µL)	0.15 ± 0.05	0.18 ± 0.06	0.18 ± 0.07	0.14 ± 0.04
ABAS (x103/µL)	0.06 ± 0.03	0.11 ± 0.10	0.09 ± 0.07	0.04 ± 0.03
ALUC (x103/µL)	0.04 ± 0.02	0.05 ± 0.03	0.05 ± 0.02	0.04 ± 0.02
ARET (x103/µL)	225.4 ± 31.7	232.4 ± 30.5	224.3 ± 43.8	233.2 ± 18.9
APTT (seconds)	17.17 ± 2.20	18.76 ± 5.55	18.90 ± 2.23	18.33 ± 2.75
PT (seconds)	10.18 ± 0.36	10.14 ± 0.56	10.20 ± 0.43	10.17 ± 0.24
Females				
RBC (x106/µL)	8.60 ± 0.38	8.35 ± 0.58	8.11 ± 0.71	8.35 ± 0.27
HGB (g/dL)	15.6 ± 0.6	15.3 ± 0.9	15.0 ± 1.2	15.4 ± 0.5
HCT (%)	49.0 ± 3.2	46.0 ± 3.6	45.0 ± 3.8 *	46.4 ± 1.7
MCV (fL)	56.9 ± 2.6	55.1 ± 1.5	55.6 ± 2.1	55.7 ± 2.3
MCH (pg)	18.2 ± 0.5	18.3 ± 0.6	18.5 ± 0.5	18.5 ± 0.6
MCHC (g/dL)	32.0 ± 1.9	33.3 ± 1.1	33.4 ± 0.9	33.2 ± 1.0
RDW (%)	12.3 ± 0.7	12.1 ± 0.3	12.1 ± 0.4	11.8 ± 0.5
PLT (x103/µL)	1059 ± 233	1048 ± 183	1126 ± 194	939 ± 130
WBC (x103/µL)	7.28 ± 2.34	6.67 ± 1.35	7.24 ± 2.40	5.90 ± 1.65
ANEU (x103/µL)	0.72 ± 0.24	0.65 ± 0.22	0.69 ± 0.35	0.54 ± 0.16
ALYM (x103/µL)	6.22 ± 2.18	5.67 ± 1.26	6.25 ± 2.27	5.07 ± 1.50
AMON (x103/µL)	0.10 ± 0.05	0.13 ± 0.06	0.12 ± 0.07	0.13 ± 0.06
AEOS (x103/µL)	0.15 ± 0.10	0.16 ± 0.10	0.13 ± 0.03	0.11 ± 0.04
ABAS (x103/µL)	0.03 ± 0.02	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
ALUC (x103/µL)	0.04 ± 0.02	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.01
ARET (x103/µL)	188.8 ± 41.3	176.7 ± 34.3	194.5 ± 53.8	193.6 ± 45.5
APTT (seconds)	19.12 ± 3.54	17.48 ± 4.07	17.94 ± 2.17	17.96 ± 1.90
PT (seconds)	9.83 ± 0.26	9.76 ± 0.81	10.02 ± 0.33	9.91 ± 0.37

For hematology data, N = 10 animals per group except for Group 2 males and group 1, 2 and 4 females, where n = 9. For APTT, n = 9 for Group 2 and 4 males and females. For PT, n = 9 for Group 2 and 4 males and females and n = 8 for Group 1 females. Data are presented as mean ± standard deviation (SD). *Statistically different from the control group, Dunnett's test, $p < 0.05$. For males, historical control ranges for HGB, HCT and ANEU are 13.7-19.1 g/dL, 43.9-55.3% and 0.65-8.79 $x10^3$/µL, respectively. For females, the historical control range for HCT is 39.4-52.0%.

ABAS = basophils, AEOS = eosinophils, ALUC = large unstained cells, ALYM = lymphocytes, AMON = monocytes, ANEU = neutrophils, ARET = reticulocytes, APTT = activated partial thromboplastin time, HCT = hematocrit, HGB = hemoglobin, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, PLT = platelets, PT = prothrombin time, RBC = red blood cells, RDW = red blood cell distribution width, WBC = white blood cells (leukocytes). dL = deciliter, fL = femtoliters, g = grams, pg = picograms, µL = microliter.

Supplementary Table 4. Urinalysis data for rats administered *L. reuteri* strains 3630 and 3932 for 28 days.

Parameter	Group 1 (Control)	Group 2	Group 3	Group 4
Males				
Urine volume (mL)	10.55 ± 5.52	9.17 ± 8.02	7.45 ± 8.51	12.60 ± 8.67
pH	6.85 ± 0.58	6.67 ± 0.43	6.70 ± 0.35	6.70 ± 0.35
Glucose (mg/dL)	0.0 ± 0.0	0.0 ± 0.0	20.0 ± 42.2	0.0 ± 0.0
Ketones (mmol/L)	10.0 ± 5.3	10.6 ± 5.3	10.0 ± 5.3	7.0 ± 4.2
Protein (mg/dL)	51.0 ± 87.8	63.9 ± 43.1	83.5 ± 84.4	29.5 ± 25.9
Specific Gravity	1.023 ± 0.006	1.026 ± 0.005	1.027 ± 0.004	1.023 ± 0.005
Urobilinogen (EU/dL)	0.28 ± 0.25	0.20 ± 0.00	0.28 ± 0.25	0.20 ± 0.00
Females				
Urine volume (mL)	6.95 ± 6.13	7.15 ± 7.35	4.05 ± 4.15	3.40 ± 3.25
pH	6.55 ± 0.93	6.25 ± 0.54	6.65 ± 1.25	6.45 ± 0.44
Glucose (mg/dL)	10.0 ± 31.6	0.0 ± 0.0	10.0 ± 31.6	0.0 ± 0.0
Ketones (mmol/L)	0.5 ± 1.6	0.0 ± 0.0	0.5 ± 1.6	1.0 ± 2.1
Protein (mg/dL)	39.0 ± 43.5	29.0 ± 38.6	79.0 ± 88.1	64.5 ± 46.6
Specific Gravity	1.024 ± 0.008	1.024 ± 0.007	0.974 ± 0.166	1.026 ± 0.006
Urobilinogen (EU/dL)	0.28 ± 0.25	0.20 ± 0.00	0.52 ± 0.41	0.36 ± 0.34

N = 10 animals per group except for Group 2 males, where n = 9. Data are presented as mean ± standard deviation (SD).

Supplementary Table 5. Absolute organ weights (g) and relative organ to body weights (%) of rats administered *L. reuteri* strains 3630 and 3932 for 28 days (unless listed otherwise)†.

Parameter	Group 1 (Control)	Group 2	Group 3	Group 4
Males				
Adrenals (g)	0.077 ± 0.014	0.071 ± 0.013	0.066 ± 0.015	0.069 ± 0.011
Adrenals/TBW	0.172 ± 0.025	0.168 ± 0.029	0.151 ± 0.033	0.156 ± 0.029
Brain (g)	2.190 ± 0.079	2.210 ± 0.107	2.218 ± 0.089	2.185 ± 0.157
Brain/TBW	4.992 ± 0.359	5.164 ± 0.544	5.028 ± 0.312	4.908 ± 0.237
Epididymides (g)	1.320 ± 0.191	1.292 ± 0.294	1.312 ± 0.088	1.428 ± 0.104
Epididymides/TBW	3.005 ± 0.443	3.019 ± 0.714	2.977 ± 0.292	3.278 ± 0.452
Heart (g)	1.373 ± 0.148	1.417 ± 0.129	1.353 ± 0.110	1.422 ± 0.113
Heart/TBW	3.115 ± 0.237	3.291 ± 0.216	3.060 ± 0.193	3.194 ± 0.177
Kidneys (g)	3.189 ± 0.391	3.250 ± 0.286	3.212 ± 0.403	3.287 ± 0.283
Kidneys/TBW	7.222 ± 0.452	7.549 ± 0.418	7.251 ± 0.710	7.393 ± 0.614
Liver (g)	12.381 ± 1.847	13.009 ± 2.010	12.807 ± 1.327	13.406 ± 3.129
Liver/TBW	27.998 ± 2.398	30.152 ± 3.582	28.897 ± 1.581	29.908 ± 5.559
Spleen (g)	0.768 ± 0.113	0.896 ± 0.135	0.890 ± 0.148	0.853 ± 0.144
Spleen/TBW	1.748 ± 0.271	2.084 ± 0.333*	2.004 ± 0.252	1.902 ± 0.178
Spleen/TBrW	0.350 ± 0.048	0.406 ± 0.063	0.401 ± 0.061	0.389 ± 0.046
Testes (g)	3.474 ± 0.356	3.177 ± 0.911	3.384 ± 0.390	3.467 ± 0.276
Testes/TBW	7.921 ± 0.983	7.439 ± 2.227	7.681 ± 1.007	7.792 ± 0.522
Thymus (g)	0.364 ± 0.080	0.441 ± 0.123	0.360 ± 0.097	0.374 ± 0.097
Thymus/TBW	0.830 ± 0.187	1.018 ± 0.252	0.812 ± 0.195	0.839 ± 0.214
Females				
Adrenals (g)	0.073 ± 0.012	0.073 ± 0.018	0.076 ± 0.011	0.068 ± 0.015
Adrenals/TBW	0.288 ± 0.059	0.287 ± 0.079	0.293 ± 0.038	0.269 ± 0.066
Brain (g)	1.942 ± 0.076	1.948 ± 0.083	1.963 ± 0.088	2.000 ± 0.077
Brain/TBW	7.650 ± 0.526	7.581 ± 0.391	7.592 ± 0.387	7.912 ± 0.575
Heart (g)	0.879 ± 0.060	0.933 ± 0.078	0.876 ± 0.063	0.841 ± 0.063
Heart/TBW	3.458 ± 0.242	3.627 ± 0.279	3.385 ± 0.202	3.317 ± 0.170
Kidneys (g)	1.845 ± 0.153	1.836 ± 0.155	1.790 ± 0.109	1.823 ± 0.153
Kidneys/TBW	7.252 ± 0.515	7.134 ± 0.488	6.919 ± 0.377	7.208 ± 0.691
Liver (g)	7.424 ± 0.943	7.479 ± 0.931	7.450 ± 0.495	7.617 ± 1.154
Liver/TBW	29.148 ± 3.227	29.014 ± 2.740	28.782 ± 1.494	30.065 ± 4.360
Ovaries (g)	0.125 ± 0.014	0.120 ± 0.010	0.128 ± 0.023	0.137 ± 0.021
Ovaries/TBW	0.493 ± 0.064	0.469 ± 0.049	0.492 ± 0.074	0.540 ± 0.087
Spleen (g)	0.563 ± 0.141	0.486 ± 0.039	0.573 ± 0.066	0.512 ± 0.047
Spleen/TBW	2.212 ± 0.545	1.891 ± 0.153	2.212 ± 0.225	2.022 ± 0.189
Thymus (g)	0.359 ± 0.079	0.338 ± 0.083	0.361 ± 0.078	0.299 ± 0.092
Thymus/TBW	1.411 ± 0.301	1.310 ± 0.294	1.400 ± 0.314	1.168 ± 0.291
Uterus (g)	0.647 ± 0.252	0.495 ± 0.055	0.535 ± 0.165	0.567 ± 0.345
Uterus/TBW	2.539 ± 0.991	1.934 ± 0.285	2.064 ± 0.635	2.241 ± 1.338

N = 10 animals per group except for Group 2 males, where n = 9 for all organ weights except adrenal, where n= 9, 8, 10 and 10. Data are presented as mean ± standard deviation (SD). Relative organ weights (ratios) presented in the table are times 1000. †Data for organ weight as a % of brain weight is included in the table if absolute organ weight or organ weight as a % of body weight in main study animals was affected. TBW = terminal body weight; TBrW = terminal brain weight.*Significantly different from control ANOVA and Dunnet, $p<0.05$.

Supplementary Table 6. Incidence of translocation of any bacteria in rats administered *L. reuteri* strains 3630 and 3632 for 28 days.

Group (treatment and sex)	Number of animals with translocated bacteria in blood	Number of animals with translocated bacteria in liver	Number of animals with translocated bacteria in mesentery	Number of animals showing any translocation
1 Male	0	2	4	5
1 Female	1	3	4	5
2 Male	1	1	3	4
2 Female	0	2	3	4
3 Male	0	3	3	4
3 Female	0	2	3	3
4 Male	1	3	5	5
4 Female	0	4	3 (of 4[#])	5
Total All Groups	3	20	28	35

n = 5/sex/group [#]No evaluable plates received for this tissue from one animal in this group.

Supplementary Table 7. Mean and median detected CFU/g of tissue of any bacteria in rats administered *L. reuteri* strains 3630 and 3632 for 28 days.

Group (treatment and sex)	Mean CFU/g in liver	Median CFU/g in liver	Mean CFU/g in Mesentery	Median CFU/g in Mesentery
1 Male	12	None detected (< 33)	240	200
1 Female	40	33	886	300
2 Male	13	None detected (<33)	160	33
2 Female	27	None detected (<33)	100	33
3 Male	53	33	5233	67
3 Female	53	None detected (<33)	460	267
4 Male	1067	133	33947[&]	3667
4 Female	67	33	3467[&&]	2150[&&]

n = 5/sex/group unless listed otherwise. In instances where no bacteria are detected the limit of detection based on the amount of tissue interrogated is listed in parentheses as < the 95% confidence limit of detection.

[&]Should be considered a minimum. One animal generated plates with colonies too numerous to accurately count. The count generated was based on identifiable single colonies and represents an estimated minimum.

[&&]Interpretable plates from only 4 animals were received.

Supplementary Table 8. Colonies counted by animal in liver and mesentery of control and high dose female rats administered *L. reuteri* strains 3630 and 3932 for 28 days.

Vehicle Control Female Mesentery	High Dose Female Mesentery	Vehicle Control Female Liver	High Dose Female Liver
0	92	0	5
4	37	3	3
90	No Plates	0	0
30	0	3	1
9	387	1	1

Supplementary Table 9. Colonies counted by animal in liver and mesentery of control and high dose male rats administered *L. reuteri* strains 3630 and 3932 for 28 days.

Vehicle Control Male Mesentery	High Dose Male Mesentery	Vehicle Control Male Liver	High Dose Male Liver
11	110	0	4
6	10	1	0
4	4800	0	147
0	47	1	0
15	125	0	5

Supplementary Table 10. Enumeration by taxa of translocated bacteria from animal 3868 mesentery and liver after administration of *L. reuteri* strains 3630 and 3932 for 28 days.

Taxa	Number of colonies	Extrapolated CFU/gram
Mesentery		
L. reuteri genotype 2*	2580	86000
Lactobacillus	1720	57333
L. reuteri genotype 1	375	12500
Ligilactobacillus	120	4000
Liver		
Lactobacillus	109	3633
L. reuteri genotype 2*	28	933
Escherichia/Shigella	2	67
Ligilactobacillus	1	33
Enterococcus	1	33

*not test substance derived.

Supplementary Table 11. Comparative genomics analyses of *L. reuteri* strains 3630 and 3632 along with 206 publicly available genomes to identify putative biosynthetic gene (BGC) clusters using antiSMASH.

BGC_name	Predicted product (antiSMASH)	Gene cluster family (GCF) label	Incidence	Percent of Strains	Remarks	BGC description
GCA_000722535_JOKX02000004.1.region001	T3PKS.NRPS	NRPS_35 PKS-NRP_Hybrids_19	3	1.40%	Occurs adjacent to PKSother_1	Reutericyclin
GCA_009389475_QKQO01000053.1.region001	NRPS	NRPS_65	2	0.93%		
GCA_003703885_PUXG01000030.1.region001	Aryl polyene	Others_13	5	2.34%		pks
GCA_009649605_WJND01000018.1.region001	Aryl polyene	Others_16	3	1.40%	Present in *L. reuteri* 3632	pks
GCA_001703865_MBLR01000164.1.region001	T3PKS	PKSother_1	208	97.20%	Most strains have this GCF but product is not known	
GCA_002128585_MIMK01000029.1.region001	T3PKS	PKSother_88	1	0.47%		
GCA_004556015_SFJC01000184.1.region001	T3PKS	PKSother_232	1	0.47%		
GCA_002128495_MIMI01000115.1.region001	T3PKS	PKSother_342	1	0.47%		
GCA_003703875_CM011639.1.region001	hglE-KS	PKSother_391	1	0.47%		fun
GCA_002254085_NGPY01000033.1.region001	bacteriocin	RiPPs_1	66	30.84%	Likely gassericin	Gassericin
GCA_002253945_NGPO01000011.1.region001	bacteriocin	RiPPs_2	24	11.21%		
GCA_002254175_NGQA01000005.1.region001	bacteriocin	RiPPs_3	31	14.49%		
GCA_001703875_MBLT01000045.1.region001	bacteriocin	RiPPs_4	11	5.14%		
GCA_009389465_QKQN01000034.1.region001	lanthipeptide.bacteriocin	RiPPs_11	2	0.93%		
GCA_002253925_NGPR01000119.1.region001	bacteriocin	RiPPs_22	2	0.93%		

PTA-126788_CP065850.region001	bacteriocin	RiPPs_31	2	0.93%	Present in *L. reuteri* 3632	Mersacidin
GCA_000179435_AEAW01000001.1.region001	bacteriocin	RiPPs_95	1	0.47%		
GCA_011028005_JAAJBL010000010.region001	bacteriocin	RiPPs_158	1	0.47%		
GCA_000168255_AAPZ02000001.1.region001	bacteriocin	RiPPs_165	1	0.47%		
GCA_003790365_RJWE01000103.1.region001	lanthipeptide	RiPPs_184	1	0.47%	This is the other half of the mersacidin BGC	

References

1 OECD. *Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents*. (2008).

2 FDA. Redbook 2000 standards. Toxicological principles for the safety assessment of food ingredients. IV.C.4.a. Subchronic toxicity studies with rodents. *FDA*, 121-131 (November 2003).

3 Dolan, L., Matulka, R., Worn, J. & Nizio, J. Safety studies conducted on pecan shell fiber, a food ingredient produced from ground pecan shells. *Toxicol Rep* **3**, 87-97 (2016). https://doi.org:10.1016/j.toxrep.2015.11.011

4 Earth Microbiome Project. <http://earthmicrobiome.org/protocols-and-standards/16s/> (2021).

5 Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and environmental microbiology* **79**, 5112-5120 (2013).

6 Oh, P. L. *et al.* Diversification of the gut symbiont Lactobacillus reuteri as a result of host-driven evolution. *The ISME journal* **4**, 377-387 (2010).

7 Ibáñez González, M. J., Robles Medina, A., Grima, E.M. et al. . Optimization of fatty acid extraction from Phaeodactylum tricornutum UTEX 640 biomass. *J Amer Oil Chem Soc* **75**, 1735–1740 (1998). https://doi.org:https://doi.org/10.1007/s11746-998-0325-z

8 Hard, G. C., Johnson, K. J. & Cohen, S. M. A comparison of rat chronic progressive nephropathy with human renal disease-implications for human risk assessment. *Crit Rev Toxicol* **39**, 332-346 (2009). https://doi.org:10.1080/10408440802368642