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Lack of genotoxicity and subchronic toxicity in safety assessment studies of *Akkermansia muciniphila* formulation

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ABSTRACT

A powder formulation of viable *Akkermansia muciniphila* bacteria (AMUC) was evaluated in a 90-day repeated-dose toxicity study in rats and a battery of genotoxicity studies to evaluate AMUC as a food ingredient. All studies followed Organisation for Economic Co-operation and Development protocols (OECD TG 408, 471 473, 474). AMUC was administered to rats *via* gavage at 0, 500, 1000, and 2000 mg/kg body weight/day (equivalent to 0, 4.1×10^{10}, 9.2×10^{10}, and 1.64×10^{11} CFU/kg body weight/day). No mortality or treatment-related adverse effects were reported in any endpoints that were attributed to AMUC consumption. No bacterial translocation of viable *A. muciniphila* from the intestinal tract was found to the liver, mesenteric lymph nodes, or blood. The no-observed-adverse-effect level was concluded to be the highest dose tested (2000 mg/kg body weight/day), approximately 1.64×10^{11} CFU/kg body weight/day. AMUC (nonviable) was not mutagenic when examined in an *in vitro* bacterial reverse mutation assay and not clastogenic in an *in vitro* mammalian chromosomal aberration test. Viable AMUC was not genotoxic when evaluated in an *in vivo* mammalian cell micronucleus assay when administered at up to 1.64×10^{11} CFU/kg body weight/day. These results confirm that AMUC is not toxic under the conditions of these studies.

1. Introduction

Akkermansia muciniphila is a Gram-negative, anaerobic, non-motile, non-spore-forming, oval-shaped, commensal, mucin-degrading bacterium abundantly present in the gastrointestinal tract of adults and children where it represents 1–4 % of the microbial community [11,12,20,24,71]. *A. muciniphila* preferentially colonizes the mucin-rich intestinal mucus layer, as found in the colon [62]. *A. muciniphila* enzymatically degrades the mucin, which is used as a source of carbon, nitrogen, and energy to synthesize oligosaccharides and short-chain fatty acids (*e.g.*, acetate and propionate) required for bacterial growth [12,23,39]. The mucin degradation results in positive modulation of mucosal thickness and gut epithelial function [79].

A. muciniphila was initially isolated and characterized from the fecal sample of a healthy adult [12], but subsequent studies have shown that *A. muciniphila* is present in different parts of the intestinal mucosa as well as feces [19,27,7,73]. *A. muciniphila* has also been detected in human breast milk, and may be transferred from mothers to newborns through human milk as this bacterium colonizes the intestinal tract early in life achieving levels of abundance similar to those observed in healthy adults within a year [11,20,43,7]. Collado et al. [7] assessed the levels of *A. muciniphila* at various life stages (*i.e.*, 1-, 6-, and 12-month-old infants; 25- to 35-year-old adults; and 80- to 82-year-old elderly) and reported that *A. muciniphila* was detected in 16–90 %, 100 %, and 96 % of fecal samples, respectively. Median values were approximately 1×10^4 to 1×10^5, 1×10^8, and 1×10^6 *A. muciniphila* cells/gram in infants, adults, and elderly, respectively.

Observational studies have shown that *A. muciniphila* is less

Abbreviations: AFU, active fluorescent units; AMUC, *Akkermansia muciniphila* bacteria lyophilized into powder form; ANOVA, analysis of variance; BHI, Brain Heart Infusion; CFU, colony-forming units; CPA, cyclophosphamide; EFSA, European Food Safety Authority; EMS, ethyl methanesulfonate; GLP, Good Laboratory Practice; HDL, high-density lipoprotein; IL, interleukin; MF, mutation factor; MIE, micronucleated immature erythrocytes; MN-NCE, micronucleated normochromatic erythrocytes; MN-RET, micronucleated reticulocytes; OECD, Organisation for Economic Co-operation and Development; PBS, phosphate-buffered saline; PSL, Product Safety Labs; PT, prothrombin time; RDW, red cell distribution width; RET, reticulocytes; V4, variable region 4.

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abundant in fecal samples from adults with several disease states, including diabetes, obesity, and metabolic syndrome [27,34,5,57,6,66,77,9]. Results of animal model studies suggest that supplementation with *A. muciniphila* may have positive effects on glucose maintenance and metabolic parameters [22,64,75,78]. However, other studies suggest that *A. muciniphila* is not beneficial in all circumstances. Luo et al. [41] indicates that (i) not all *A. muciniphila* act in the same manner, and (ii) in mouse models with specific gut microbiota or in certain disease states, *A. muciniphila* may not act in a beneficial manner. However, this may be species-specific, as the temporal increase in *A. muciniphila* over a human's lifetime is an inverse of what has been reported in mice [41].

Nonclinical [2,29,56] and clinical [10,56] studies have shown that oral administration of *A. muciniphila* is well tolerated and no test item–related mortalities, adverse effects, or safety concerns have been reported. However, most of these studies were proof-of-concept type investigations rather than traditional safety assessments. A comprehensive safety assessment of pasteurized *A. muciniphila* was previously conducted by Druart et al. [13] who performed a 90-day toxicity study and genotoxicity assays comprised of bacterial reverse mutation and *in vitro* mammalian cell micronucleus tests. Findings of these studies support that pasteurized *A. muciniphila* is safe as a food ingredient, as the results of both *in vitro* genotoxicity studies were negative and no adverse effects were observed in the 90-day study. The authors concluded a no-observed-adverse-effect-level (NOAEL) to be the highest dose tested of 1500 mg/kg body weight/day, equivalent to 9.6×10^{10} *A. muciniphila* cells/kg body weight/day [13].

Thermal pasteurization is a mild form of heat treatment that, although limiting the denaturation of cellular components, results in inactivation of heat-sensitive microorganisms and some degradation of the cellular components. Therefore, in the present work, a series of nonclinical safety studies were conducted to determine the safety of a viable *A. muciniphila* formulation lyophilized into powder form (AMUC) for use as a food ingredient. A 90-day oral toxicity study in rats and an *in vivo* mammalian cell micronucleus assay were completed to assess the potential for subchronic toxicity and genotoxic effects, respectively. A nonviable AMUC formulation was utilized to assess potential mutation activity in a bacterial reverse mutation assay and potential chromatid-type or chromosomal-type aberrations in an *in vitro* mammalian chromosomal aberration test.

The 90-day oral toxicity study evaluated clinical observations, body weight, food consumption, clinical chemistry, hematological parameters, and gross and histopathological alterations following repeated daily gavage administration of viable *A. muciniphila* to male and female Sprague-Dawley rats. A component of the study included a determination of the level of bacterial translocation from the intestinal track to the mesenteric lymph nodes, liver, and blood following repeated administration of viable AMUC to ascertain whether the translocated bacteria (if any) were derived from the test substance.

2. Materials and methods

2.1. Test materials

The viable *A. muciniphila* ingredient (AMUC) is an off-white powder manufactured using traditional fermentation methods. Briefly, a production strain derived from *A. muciniphila* strain DSM 22959 (ATCC BAA-835) is fermented using a proprietary process and its growth curve is monitored *via* optical density at a wavelength of 600 nanometers (OD600). After optimal growth is achieved, the *A. muciniphila* cell culture is concentrated to a biomass that is then mixed with a 10 % sucrose cryoprotectant solution at a 1:1 ratio (w:v), then dried by lyophilization. The resulting viable *A. muciniphila* cake is milled to a fine powder to obtain the final ingredient comprised of 90 % viable *A. muciniphila* and 10 % sucrose.

Stability analysis and concentration verification were conducted at the beginning (Day 1), middle (Day 45), and end (Day 92) of the study in samples from all dose preparations. Furthermore, homogeneity was assessed in samples collected from the top, middle, and bottom of Day 1 dose preparations. Levels of heavy metals and microbiological contaminants were confirmed to be within company specifications for viable *A. muciniphila* powder (data not shown).

2.2. Experimental design

2.2.1. Bacterial reverse mutation assay

Viable AMUC was evaluated *in vitro* for its potential to induce gene mutations in bacteria (*i.e.*, *Salmonella* (ser.) Typhimurium and *Escherichia coli*) using the bacterial reverse mutation assay (Ames test). This assay was conducted at Product Safety Labs (PSL; Dayton, NJ) and was in compliance with GLP guidelines [50], Organisation for Economic Co-operation and Development (OECD) Test Guideline No. 471, *Bacterial Reverse Mutation Test* [54], and general guidance from the U.S. FDA Redbook 2000, IV.C.1.a., *Bacterial Reverse Mutation Test* [68] and ICH S2 (R1) *Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use* [32].

Based on results from the preliminary sterility check, the viable AMUC was sterilized with *gamma* irradiation (25–40 kGy). Testing was conducted on the post-sterilized test substance. Using the plate incorporation method and subsequent confirmatory pre-incubation test, *S.* Typhimurium strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* strain WP2 uvrA (obtained from Molecular Toxicology Inc.) were incubated with the test substance in sterile water at levels of 1.58, 5.0, 15.8, 50, 158, 500, 1580, and 5000 μg/plate in both the presence and absence of metabolic activation (rat liver S9 microsomal fraction, obtained from Molecular Toxicology Inc., Boone, NC). The plates were evaluated in triplicate at each dose level. Sodium azide (15 μg/mL for TA100 and TA1535), 9-aminoacridine hydrochloride monohydrate (500 μg/mL for TA1537), 2-nitrofluorene (10 μg/mL for TA98), and 4-nitroquinoline N-oxide (5 μg/mL for WP2 uvrA) were used as positive controls in the absence of metabolic activation. In the presence of metabolic activation, 2-aminoanthracene (20 μg/mL for TA1535 and TA1537; 100 μg/mL for WP2 uvrA) and benzo[*a*]pyrene (50 μg/mL for TA100, TA98) served as positive control substances. Sterile water served as the negative (vehicle) control under both conditions of metabolic activation.

An evaluation of toxicity was based on the partial or complete absence of a background lawn of non-revertant bacteria or a substantial dose-related reduction in revertant colony counts compared with lower dose levels and concurrent vehicle control, considering the historical control range. Where precipitation obscured observations of the background lawn, the lawn was considered normal and intact if the revertant colony counts were within the expected range based on the results of the lower dose levels and historical control counts for that strain. An evaluation of mutagenicity was based on the mutation factor (MF) for the revertant colony. The MF was calculated by dividing the mean revertant colony count by the mean revertant colony count for the corresponding vehicle control group. A significant increase in mutagenicity was determined by resultant MFs of $\geq$2 for TA98, TA100, and WP2 uvrA and $\geq$3 for TA1535 and TA1537. The increase was considered significant if occurring in a dose-related or reproducible manner and with mean values out of historical controls levels.

2.2.2. In vitro mammalian chromosomal aberration test

The *in vitro* mammalian chromosomal aberration test was conducted in accordance with OECD Test Guideline No. 473 [51] and standard operating procedures of the Eurofins BioPharma Product Testing laboratory under Good Laboratory Practice (GLP) standards (Munich, Germany). The potential for the test substance to produce clastogenic effects was evaluated in human peripheral blood lymphocytes obtained by venipuncture from a healthy non-smoking human donor with no known recent exposure to genotoxic chemicals and radiation. The test article was treated with *gamma*-irradiation (VPTRad; Chelmsford, MA) prior to

culture. The lymphocytes were cultured in Chromosome Medium 1 A (Gibco®) after treatment with an anti-coagulant (heparin) and pre-cultured in the presence of the mitogen phytohematogglutinin. Preliminary solubility tests determined that the best-suited test article vehicle was Minimum Essential Media cell culture medium. A pre-experiment cytotoxicity test was conducted with concentrations of the test substance at 125, 250, 750, 1250, 2500, and 5000 μg/mL. Based on the results of the cytotoxicity study, the exposure concentrations for Experiment I with a short-term (4-hour) treatment with and without S9 metabolic activation and for Experiment II with a long-term (24-hour) treatment without S9 metabolic activation were 125, 250, 750, 1250, 2500, and 5000 μg/mL. After treatment, the cells were washed and resuspended in complete cell culture medium, incubated for 20 hours (recovery time) for Experiment I and for 24 hours (preparation interval) for both Experiments I and II, then treated with colcemid and harvested, according to the OECD protocol [51]. Precipitation was recorded at 1250 μg/mL in Experiment I, both with and without metabolic activation, and therefore the evaluated experimental points were 250, 750, and 1250 μg/mL in Experiment I, both with and without S9, and 125, 250, and 1250 μg/mL, in Experiment II without S9 metabolic activation. Ethyl methanesulfonate (EMS; Sigma-Aldrich) was utilized as the positive control for Experiments I and II without metabolic activation; cyclophosphamide (CPA; Sigma-Aldrich) was utilized as the positive control for Experiment I with metabolic activation. The cultures for Experiments I and II were prepared in duplicate. Metaphases (n=150) were scored for structural chromosomal aberrations, except in the positive controls in which the number of metaphases scored was reduced to 170 (CPA) in Experiment I and 25 (EMS) in Experiment II.

All slides were independently coded and evaluated for structural chromosomal aberrations breaks, fragments, deletions, exchanges, and chromosomal disintegrations. Gaps were recorded but not included in aberration rate calculations. The mitotic index was determined to assess potential cytotoxicity and the number of polyploid cells was scored.

2.2.3. *In vivo mammalian erythrocyte cell micronucleus test*

An *in vivo* mammalian cell micronucleus test was conducted by PSL (Dayton, NJ) in accordance with GLP guidelines [50], OECD Test Guideline No. 474, *Mouse Micronucleus Test* [52] and general guidance from the U.S. FDA *Toxicological Principles for the Safety Assessment of Food Ingredients*, Redbook 2000, IV.C.1.d., *Mammalian Erythrocyte Micronucleus Test* [69]. Fifty CD-1 (Swiss-derived) mice (n=25/sex) approximately seven weeks of age were acclimated for six days before being randomized into five groups, each containing five mice/sex. The mice were grouped as follows: Group 1 – 0.05 % L-cysteine–phosphate-buffered saline (PBS) solution (control); Groups 2, 3, and 4 – targeted doses of *A. muciniphila* at 4.1 $\times 10^{10}$, 8.2 $\times 10^{10}$, and 1.64 $\times 10^{11}$ AFU [active fluorescent units] of *A. muciniphila*/kg body weight/day, respectively. These doses corresponded to 500, 1000, and 2000 mg test item/kg bw. Group 5 was administered CPA monohydrate (40 mg/kg body weight) on Day 2 only, as a positive control group. All substances were dosed *via* gavage. At study termination, mice were anesthetized with carbon dioxide and then euthanized by exsanguination followed by cervical dislocation. IACUC Approval for the study was obtained prior to study commencement.

The following parameters were analyzed: body weights/body weight changes, clinical observations, average of reticulocyte percentage (% RET), percentage micronucleated normochromatic erythrocytes (% MN-NCE), and frequency of micronucleated reticulocytes (% MN-RET), also stated as micronucleated immature erythrocytes (MIE). Blood was obtained from each animal (cardiac puncture), processed according to the Litron In Vivo Micronucleus Kit (MicroFLowBASIC; Rochester, NY), fixed, permeabilized, and stored frozen in methanol until evaluation. The cells were immunostained with dyes to identify immature *vs.* mature erythrocytes *via* flow cytometry (fluorescent labeled anti-CD71 antibody), platelets (fluorescent labeled anti-CD61 antibody), and DNA content (propidium iodide, following RNAse treatment). A minimum of 4000 immature erythrocytes/animal was the analysis target for MIE enumeration. DNA content was measured in mature (CD71-/CD61-) and immature (CD71+/CD61-) erythrocytes.

Test validity was accepted if (i) negative control group animals had MIE values within the expected ranges of published method values and laboratory control data, and (ii) the positive control caused a statistically significant increase in MIE with mean values outside the historical negative control range. Cytotoxicity occurred if the reticulocyte fraction was less than 5 % of the respective vehicle control. A result was positive if the treatment group exhibited a statistically significant increase in MIE frequency when compared to the concurrent negative control, and if the treatment group mean was outside the historical negative control data 95 % control limits.

2.2.4. *90-Day oral toxicity study*

The 90-day oral toxicity study was conducted at PSL in compliance with OECD GLP Guidelines [50], OECD Test Guideline No. 408, *Repeated Dose 90-Day Oral Toxicity Study in Rodents* [53] and U.S. FDA Redbook 2000, IV.C.4.a., *Subchronic Toxicity Studies with Rodents* [70]. Male and female CRL Sprague-Dawley CD IGS rats were obtained from Charles River Laboratory, Inc. PSL is AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) accredited and certified in the appropriate care of all live experimental animals and maintained current staff training, ensuring animals were handled humanely during the experimental phase of this study, and met all guideline standards.

After a six-day acclimation period, seven- to eight-week-old rats free of clinical signs of disease or injury, and with body weight variation within ±20 % of the mean body weight for each sex, were randomly distributed and stratified by body weight for males (142–195 g) and females (186–244 g) on the day of study initiation. Animals were group-housed in polycarbonate cages with two rats of the same sex per cage under a 12-hour light/dark cycle at a room temperature of 17–24°C and relative humidity of 31–62 %. Food and water were available *ad libitum*.

CRL Sprague-Dawley rats (10/sex/group) were administered 0 (sterile PBS as vehicle control) (Group 1), 500 (Group 2), 1000 (Group 3), or 2000 (Group 4) mg/kg body weight/day of the test substance *via* oral gavage for 90 consecutive days, yielding targeted dose-equivalents of viable *A. muciniphila* of 0, 4.1×10^{10}, 8.2×10^{10}, and 1.64×10^{11} CFU [colony-forming units]/kg body weight/day, respectively. Doses for the 90-d study were established on the basis of potential human exposure and noting that 2000 mg/kg bw/day is conventionally used as the highest doses gavage studies of food ingredients not expected to cause signs of toxicity.

Ophthalmological examinations (*i.e.*, focal illumination, indirect ophthalmoscopy, and slit-lamp microscopy) were performed during the acclimation period and at the end of the study (Day 89). Cage-side observations of all experimental animals were performed daily, and mortality was determined at least twice daily. Food consumption, body weight and detailed clinical observations were recorded twice during the acclimation period, prior to study start on Day 1, and at weekly intervals (7±1 days) thereafter. To calculate organ-weight-to-body-weight ratios, the experimental animals were also weighed prior to euthanasia. Parameters evaluated in the clinical evaluations included changes in gait, posture, behavior, skin, fur, eyes, and mucous membrane. Presence of secretions and excretions, autonomic activity (*e.g.*, lacrimation, piloerection, pupil size, unusual respiratory pattern), stereotypies (*e.g.*, repetitive circling, excessive grooming), clonic or tonic movements, and abnormal behavior (*e.g.*, walking backwards, self-mutilation) were also assessed.

Following overnight fasting, blood samples were collected *via* the inferior vena cava, under isoflurane anesthesia, prior to terminal sacrifice at the end of the study for clinical pathology, including hematology,[1] clinical chemistry,[2] and coagulation.[3] IThyroid hormone measurements were not carried out as part of the protocol for this study given that data supports that there is either no target involvement or only suggested health benefits of probiotics on gut-thyroid or hypothalamic-pituitary axis thyroid function [25,36,63,72].

At the end of the study, all animals (including decedents) were euthanized by exsanguination from the abdominal aorta under isoflurane anesthesia and subjected to full necropsy. Necropsy included evaluation of the external surface of the body, all orifices, musculoskeletal system, and the thoracic, abdominal, pelvic, and cranial cavities and their contents. Wet organ weights were evaluated for the following organs: brain, liver, thymus, heart, kidneys, adrenals, spleen, pituitary gland, thyroid/parathyroid, uterus, ovaries with oviducts, testes, epididymides, prostate, and seminal vesicles. The day before schedule sacrifice, animals were placed in metabolism cages and urine was collected for urinalysis.[4]

Organs and tissues of all animals were preserved in 10 % neutral buffered formalin except for eyes, optic nerve, epididymides, and testes, which were preserved in modified Davidson's fixative and stored in ethanol for possible histopathological analysis. Histopathological analysis was conducted on the organs and tissues of all animals from the control (Group 1) and the high-dose group (Group 4). The histopathological examination included all gross lesions; liver; kidneys; spleen; urinary bladder; organs of the gastrointestinal tract (esophagus, colon, stomach, cecum, duodenum, jejunum, ileum with Peyer's patches, and rectum); endocrine system (pancreas, pituitary gland, thymus, thyroid, parathyroid, and adrenals); respiratory tract (nose, nasal turbinates, larynx, pharynx, trachea, and lungs); female (ovaries, oviducts, uterus, vagina, and cervix) and male (prostate, testes, epididymides, and seminal vesicles) reproductive tract; brain (sections including medulla/pons, cerebellar, and cerebral cortex); spinal cord (cervical, mid-thoracic, and lumbar); lymph nodes (mandibular and mesenteric); salivary glands (sublingual, submandibular, and parotid); mammary gland; heart; aorta; skin; skeletal muscle, eyes; optic nerve; sciatic nerve; Harderian gland; femur; bone marrow from femur; and sternum. Slide preparation and histological assessment was conducted by a board-certified veterinary pathologist at StageBio, Mount Jackson, VA.

2.2.5. Bacterial translocation analysis

As part of the 90-d study translocation analysis was conducted on samples of whole blood (0.5 mL), liver, and mesenteric lymph nodes (0.2–0.5 g) collected from four to five randomly selected rats/sex/group. To determine bacterial growth and achieve adequate colony growth for counting, homogenized and/or diluted samples were plated on pre-reduced Brain Heart Infusion (BHI) Agar plates (n=3/rat/tissue) and incubated anaerobically for nine days at approximately 37°C. After incubation, all plates were visually inspected for CFU growth and individual colonies were counted and their morphology was assessed for seven characteristics (size, form, elevation, margin, surface, opacity, and pigmentation). The average CFU/mL of blood or gram of tissue was calculated based on the amount of sample evaluated, the plate colony growth, and the respective dilution factor.

The plates were provided to BioPrimate (Chester, PA) and the colonies were further characterized using phylogenetic and random amplification of polymorphic DNA (RAPD) analysis. First, colonies were replated on new BHI Agar plates and incubated at 37°C for nine days for DNA isolation. If more than five colonies of a single morphological type were present on plates from a single tissue from an experimental animal, only five representative colonies of that particular morphology were selected for further analysis. DNA was extracted from the regrown colonies and the 16S ribosomal RNA gene variable region 4 (V4) was amplified using the standard primers and methods [18]. The amplified fragments of the V4 region of the 16S rRNA gene were subject to massively parallel sequencing by Illumina for taxonomic identification. Briefly, amplicon libraries were sequenced on an Illumina MiSeq instrument and the consensus sequence for each isolate was generated, and a taxonomic designation was assigned by comparison to the GenBank database [1,46]. The 16S rRNA V4 region of *Akkermansia* differentiates it from other genera. Importantly, for *Haloferula*, the closest non-*Akkermansia* genetic match, this region differs by over 10 %. Further, this region can differentiate among isolates of the species *A. muciniphila*.

2.3. Statistical analysis

2.3.1. Bacterial reverse mutation assay

PSL calculated the mean values and standard deviations for all quantitative data.

2.3.2. In vitro mammalian chromosomal aberration test

The data from the main Experiments I and II were evaluated for concentration-related increases in chromosomal aberrations by the Fischer's exact test with statistical significance at the 5 % level ($p<0.05$), when compared to the corresponding negative control. Aberrant cells without gaps were only used for calculations. Statistical trend analysis was also evaluated (chi-square test for trend; $p<0.05$).

2.3.3. In vivo mammalian erythrocyte cell micronucleus test

The proportions of immature *vs.* total erythrocytes and micronucleated erythrocytes were analyzed by the paired t-test (GraphPad Prism software; San Diego, CA). For all comparisons, a p-value of ≤ 0.05 indicated a significant effect.

2.3.4. 90-day oral toxicity study

PSL performed statistical analysis of all data collected during the in-life phase of the 90-day oral toxicity study as well as organ weight data. Statistical significance was judged at a probability value of $p<0.05$.

2.3.4.1. In-life data. For in-life endpoints (*e.g.*, food intake and body weight), statistical analysis was performed between each treatment group and the control group using a repeated-measures analysis of variance (ANOVA), testing the effects of both time and treatment, with methods accounting for repeated measures in one independent variable (time) [45]. The p-value for each individual factor was further analyzed by a *post hoc* multiple comparisons test (*e.g.*, Dunnett's test; [16,17]).

2.3.4.2. Organ weight and bacterial translocation data. Homogeneity of variances [4] and normality were evaluated for parameters with single measurements of continuous data within groups (*e.g.*, absolute and relative organ weight, bacterial translocation data). Treatment and control groups were compared using a one-way ANOVA where normal

[1] Hematological analysis included assessments of hematocrit, hemoglobin concentration, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, red blood cell (erythrocyte) count, platelet count, red cell distribution width, reticulocyte count, white blood cell count, and differential leukocyte count.

[2] Clinical chemistry parameters included alkaline phosphatase, serum alanine aminotransferase, serum aspartate aminotransferase, *gamma*-glutamyl trans-peptidase, sorbitol dehydrogenase, total bilirubin, urea nitrogen, low-density lipoprotein, high-density lipoprotein, blood creatinine, albumin, globulin, triglycerides, total cholesterol, fasting glucose, total serum protein, calcium, inorganic phosphorus, sodium, potassium, and chloride.

[3] Coagulation analysis included activated partial thromboplastin time and prothrombin time.

[4] Urinalysis included microscopic examination of urine sediment and assessment of pH, volume, color, clarity, quality, specific gravity, blood, bilirubin, total protein, urobilinogen, and ketones.

distribution and homogeneous variances were observed. A comparison of the treated groups to control was performed with a multiple-comparisons test (*e.g.*, Dunnett's test; [16,17]). Treatment groups were compared using a non-parametric method such as Kruskal-Wallis non-parametric ANOVA [37] where variances were considered significantly different. A comparison of treated groups to control (*e.g.*, Dunn's test; [15]) was performed where non-parametric ANOVA was significant.

2.3.4.3. Clinical pathology. Clinical pathology data were preliminarily analyzed for homogeneity (Bartlett's test; [4]) and normality (Shapiro-Wilk test; [61]). Where homogeneity and normality were not significant, data were compared using a one-way ANOVA followed by a Dunnett's test [16,17]. Where homogeneity and normality were significant, a log transformation was applied to the data. If the log transformation failed to achieve normality and variance homogeneity, the data were analyzed *via* a non-parametric method (*e.g.*, Kruskal-Wallis non-parametric ANOVA; [37]). When the non-parametric ANOVA was significant, a Dunn's test was utilized to compare treatment groups to control group (*e.g.*, Dunn's test; [15]).

3. Results

3.1. Bacterial reverse mutation assay

No treatment- or dose-related increases in the number of revertant colonies in the main test (Table 1) or in the confirmatory test (Table 2) were observed in *S.* Typhimurium strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* strain WP2 uvrA in either the presence or absence of metabolic activation upon incubation with nonviable AMUC up to 5000 μg/plate. These results were consistent when analyzing the MF results for these tests (Supplementary Tables S1 and S2). For all strains, at least eight non-toxic dose levels without precipitate or contamination were evaluated. No signs of precipitation, contamination, or toxicity were observed in any of the strains at all dose levels. Further, the mean revertant colony counts of all strains treated with sterile water (vehicle) were within range of historical controls [26,44] and the positive controls induced a substantial increase in revertant colonies, in both the absence and presence of metabolic activation, confirming the validity of the tests.

3.2. In vitro chromosomal aberration assay

Exposure of human peripheral blood lymphocytes to nonviable AMUC (short-term both with and without metabolic activation, or long-term without metabolic activation) did not result in any biologically or statistically significant changes, nor did it demonstrate dose-dependent increases in the frequency of cells with chromosomal aberrations, when compared to the respective control groups under the conditions of this study (Table 3). No biologically relevant increases in polyploid cell frequencies were found after nonviable AMUC treatment. A biologically relevant decrease of the relative mitotic index (decrease below 70 %), an indication of toxicity, was noted in Experiment I at 1250 μg/mL without metabolic activation and at 750 μg/mL with metabolic activation. In Experiment II, a biologically relevant relative mitotic index decrease was reported at 1250 μg/mL (Table 3) without metabolic activation. The positive control treatments resulted in significant increases in chromosomal aberrations in the test system, confirming the ability of the test to indicate clastogenic effects.

3.3. In vivo mammalian erythrocyte cell micronucleus test

All animals appeared healthy during the study and no clinical signs or mortality were reported for any animal during the observation period until exsanguination. Flow cytometry analysis was performed on samples from all animals of the study. No biologically or statistically significant change in the % RET, % MN-NCE, and % MN-RET (MIE) were observed in the highest viable AMUC treatment group, when compared to the negative (vehicle) control (Table 4). Treatment with CPA resulted in a reduction in the %RET and increases in the % MN-RET, confirming the validity of the test and test system.

3.4. 90-day oral toxicity study

3.4.1. Test substance and dose preparation analyses

To determine whether the experimental animals received the target concentrations at the dose levels tested in the 90-day oral toxicity study, test substance stability and dose preparation homogeneity was assessed and concentration verification was conducted (Supplementary Tables S3 to S5). Viable AMUC was determined to be stable under the conditions of storage over the course of the study as the difference in the viable CFU content assessed at Day 1, Day 45, and Day 92 was 0.11 %. The overall stability was determined to be 100.11 % (Supplementary Table S3). Concentration of *A. muciniphila* was verified in the dose preparations of Day 1, Day 45, and Day 92. The analysis of Day 1 mixtures resulted in 79.7, 118.0, and 129.4 % of target concentrations; the analysis of Day 45 mixtures resulted in 45.3, 93.3, and 80.0 % of target concentrations; and the analysis of Day 92 mixtures resulted in 66.7, 116.7, and 108.3 % of target concentrations for Groups 2, 3, and 4, respectively (Supplementary Table S4). Homogeneity analysis indicated that the dose mixtures of Day 1 for Groups 2, 3, and 4 had a relative standard deviation of 1.5, 0.3, and 1.1 %, respectively (Supplementary Table S5). AMUC was therefore homogeneously distributed in the dose preparations at all tested concentrations. These results indicate that the dose preparations met the target concentrations in the mid- and high-dose preparations to provide viable *A. muciniphila* at up to 1.64 $\times 10^{11}$

Table 1
Summary of bacterial reverse mutation assay results (plate incorporation method – main test; up to 5000 μg/plate) – mean revertant colonies per plate.

μg/plate	TA98		TA100		TA1535		TA1537		WP2 uvrA	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
1.58	28±2.1	27±3.5	109±7.0	111±16.1	13±6.1	13±2.6	14±3.6	15±1.5	33±0.6	43±5.6
5	29±4.7	29±3.6	111±4.2	107±10.2	16±3.1	9±3.2	12±3.2	13±2.3	33±2.0	42±5.9
15.8	25±3.8	30±4.0	106±5.0	115±9.5	14±2.5	9±1.2	13±0.0	16±3.2	41±6.7	43±5.5
50	29±3.1	26±0.6	111±7.8	113±4.4	12±2.1	12±2.5	13±2.1	12±0.0	36±3.6	42±2.5
158	25±4.2	27±7.6	109±4.7	113±6.7	21±4.9	14±1.7	10±2.1	11±2.0	34±2.5	42±2.6
500	26±5.3	31±5.3	98±2.6	122±4.6	14±3.2	13±2.3	12±2.9	12±3.2	41±4.0	44±0.6
1580	25±2.6	26±2.1	110±8.3	115±10.4	14±3.1	11±2.1	12±1.0	14±2.5	43±3.1	47±1.2
5000	30±1.5	34±2.1	112±10.6	108±2.9	13±0.6	15±4.0	10±1.0	18±0.6	45±4.5	43±5.5
Sterile water	24±2.9	24±4.2	108±5.3	118±3.5	13±5.0	11±1.7	13±2.0	14±2.3	34±1.2	38±3.1
Positive control	175±23.6[a]	165±20.5[b]	450±43.5[c]	638±61.2[b]	542±44.5[c]	370±27.0[d]	218±10.7[e]	256±52.4[d]	710±19.9 [f]	229±18.7[d]

SD = standard deviation.
[a] 2-Nitrofluorene; [b] benzo[*a*]pyrene; [c] sodium azide; [d] 2-aminoanthracene; [e] 9-aminoacridine HCl H_2O; [f] 4-nitroquinoline N-oxide.
Data are shown as mean ± SD revertant colonies per plate for three replicates for each concentration in each experiment.

Table 2
Summary of bacterial reverse mutation assay results (pre-incubation method – confirmatory test; up to 5000 μg/plate) – mean revertant colonies per plate.

μg/plate	TA98		TA100		TA1535		TA1537		WP2 uvrA	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
1.58	25±4.0	30±2.3	100±8.4	101±5.6	14±3.8	10±0.6	8±1.7	13±4.6	43±2.9	43±4.2
5	23±4.2	26±3.2	110±7.8	106±10.5	15±5.2	10±4.2	9±3.5	10±1.7	41±6.4	43±5.6
15.8	25±3.1	29±5.0	97±18.1	110±9.5	15±2.1	11±3.2	11±1.2	12±3.8	38±3.5	43±5.0
50	29±5.5	28±1.0	98±16.5	102±9.0	16±3.6	12±3.5	11±1.5	13±3.6	40±2.0	45±7.8
158	22±5.1	31±1.5	106±5.8	114±8.1	20±4.0	15±5.5	10±3.1	13±3.5	33±2.0	44±0.6
500	25±3.6	27±6.1	88±7.6	110±3.8	19±3.5	12±4.4	12±3.1	13±4.0	34±4.2	44±5.1
1580	28±3.8	33±2.6	87±11.1	115±3.2	12±1.0	13±4.0	10±2.1	14±3.5	32±1.5	44±5.2
5000	34±2.1	33±3.5	82±5.5	115±7.6	20±2.9	13±2.6	10±3.8	13±1.5	46±1.0	45±3.5
Sterile water	23±4.0	23±1.5	116±5.6	99±8.0	17±1.2	11±2.0	15±1.7	11±1.0	33±0.6	46±2.5
Positive control	179±8.7[a]	160±14.5[b]	496±85.9[c]	603±25.5[b]	553±43.0[c]	322±36.6[d]	272±29.1[e]	243±37.5[d]	1020±5.9 [f]	189±14.1[d]

SD = standard deviation.
[a] 2-Nitrofluorene; [b] benzo[*a*]pyrene; [c] sodium azide; [d] 2-aminoanthracene; [e] 9-aminoacridine HCl H_2O; [f] 4-nitroquinoline N-oxide.
Data are shown as mean ± SD revertant colonies per plate for three replicates for each concentration in each experiment.

Table 3
Chromosome analysis of AMUC in human peripheral blood lymphocytes with and without exogenous metabolic activation (S-9).

Treatment time (hours)	S9 (+/-)	Dose group	Concentration (μg/mL)	Numerical aberration (%)		Cytotoxicity	
				Including gaps	Excluding gaps	Relative mitotic index (%)	Precipitation (+/-)
Experiment I							
4	-	Control[a]	0	3.0	1.7	100	-
		AMUC Group 2	250	1.7	1.3	94	-
		AMUC Group 3	750	1.0	0.3	74	-
		AMUC Group 4	1250	1.0	1.0	55	+
		Positive control[b]	900	6.3	5.0	63	-
4	+	Control[a]	0	3.0	1.3	100	-
		AMUC Group 2	250	3.0	2.0	97	-
		AMUC Group 3	750	3.0	0.7	60	-
		AMUC Group 4	1250	2.7	2.0	71	+
		Positive control[c]	900	12.9	11.8	131	-
Experiment II							
24	-	Control[a]	0	2.7	1.3	100	-
		AMUC Group 2	125	2.0	0.7	95	-
		AMUC Group 3	250	1.0	1.7	73	-
		AMUC Group 4	1250	4.0	1.0	45	-
		Positive control[b]	400	80.0	76.0	17	-

AMUC = *Akkermansia muciniphila* bacteria lyophilized into a powder form.
[a] Culture medium; [b] ethyl methanesulfonate; [c] cyclophosphamide.

CFU/kg body weight/day. Slightly lower than target concentrations of viable *A. muciniphila* were noted in the low-dose group. The source of the variation is unknown, but the result is of no concern as the target concentrations were achieved in the higher dose groups.

3.4.2. Mortality and clinical observations

No mortalities or clinical observations related to the administration of AMUC were observed. Clinical signs in the study were considered to be incidental and of no toxicological significance. Incidental clinical observations in male animals included slight to moderate hair loss on the head and superficial eschar of the head or back with corresponding detailed clinical observations of hair loss and eschar, in the control group (Group 1) and in groups receiving the mid and high doses of AMUC (Groups 3 and 4, respectively). Incidental clinical observations in female animals included slight to moderate hair loss on the left/right forepaw, back, or head with corresponding detailed clinical observations of hair loss among the control and all treatment groups, and superficial eschar on the head, nose/snout, or back with corresponding detailed clinical observations of eschar in groups receiving the low and high dose of AMUC (Groups 2 and 4, respectively).

3.4.3. Body weight, food consumption, and ophthalmological observations

No significant changes in terminal body weight (Tables 5 and 6), mean weekly body weights (Fig. 1), or food consumption (data not shown) were reported in male or female rats following administration of the test substance. Although mean daily body weight gain was significantly increased on Days 1–8 in male rats receiving AMUC at all doses (p<0.05–0.01) and on Days 8–15 in male rats receiving 1000 mg/kg body weight/day of AMUC (Group 3; p<0.05) (data not shown), mean weekly body weights for male rats were comparable across all groups (Fig. 1). Similarly, mean daily and weekly body weight gain for female rats receiving all doses of AMUC were comparable to the control group (data not shown).

No ophthalmological changes associated with the administration of AMUC were observed. On Day 89, a single male in the high-dose group (Group 4) presented an incipient cataract in its right eye, a common sporadic finding in young Sprague-Dawley rats. Thus, AMUC is not considered an ocular toxicant.

3.4.4. Organ weights, necropsy, and histopathological observations

Examination of absolute and relative organ weights revealed no changes related to AMUC administration (Tables 5 and 6). Thyroid-parathyroid absolute weight was significantly increased in female rats of Groups 2 (p<0.01) and 4 (p<0.01). Consistently, thyroid-parathyroid-to-body-weight ratio was increased in females of Group 2 (p<0.001) and 4 (p<0.5), while thyroid-parathyroid-to-brain-weight ratio was significantly increased in females of Group 2 (p<0.001). However, these increases were not seen in female rats of Group 3 nor in any of the male rats receiving all doses of AMUC. These organ weight changes were therefore considered incidental, unrelated to the administration of

Table 4
Results of *in vivo* mouse micronucleus test with viable AMUC.

Group	AMUC dose (AFU/kg BW/day)	Mean ± SEM		p-value	
		Male	Female	Male	Female
% RET					
Negative control	0	1.73 ±0.07	1.37 ±0.12	-	-
AMUC	3.58×10^{10}	1.83 ±0.19	1.19 ±0.12	na	na
	7.35×10^{10}	1.64 ±0.15	1.42 ±0.21	na	na
	1.32×10^{11}	1.70 ±0.18	1.84 ±0.22	ns	ns
Positive control	-	0.34 ±0.04	0.45 ±0.11	$p<0.0001$	$p=0.0010$
% MN-NCE					
Negative control	0	0.14 ±0.01	0.12 ±0.01	-	-
AMUC	3.58×10^{10}	0.13 ±0.01	0.12 ±0.01	na	na
	7.35×10^{10}	0.14 ±0.01	0.10 ±0.01	na	na
	1.32×10^{11}	0.12 ±0.01	0.12 ±0.03	ns	ns
Positive control	-	0.13 ±0.01	0.15 ±0.01	ns	ns
% MN-RET					
Negative Control	0	0.16 ±0.01	0.18 ±0.04	-	-
AMUC	3.58×10^{10}	0.17 ±0.02	0.16 ±0.02	na	na
	7.35×10^{10}	0.16 ±0.02	0.15 ±0.01	na	na
	1.32×10^{11}	0.14 ±0.01	0.15 ±0.05	ns	ns
Positive control	-	2.51 ±0.18	2.02 ±0.40	$p=0.0002$	$p=0.0114$

- = not applicable; AFU = active fluorescent units; AMUC = *Akkermansia muciniphila* bacteria lyophilized into powder form; BW = body weight; MN-NCE = micronucleated normochromatic erythrocytes; MN-RET = micronucleated reticulocytes (*i.e.*, micronucleated immature erythrocytes [MIE]); na = not analyzed; ns = not significant; RET = reticulocytes; SEM = standard error of the mean.

AMUC, and of no toxicological significance, as no patterns, dose-response relationship, or correlations were identified.

No macroscopic and microscopic alterations were related to the administration of AMUC to rats *via* oral gavage. No abnormalities were observed at necropsy; histopathological evaluation of tissues from rats in the control group (Group 1) and from rats administered 2000 mg/kg body weight/day of AMUC (Group 4) revealed thyroid follicular cell hypertrophy in male and female rats of both groups, as well as minimal liver mixed cell infiltration, mild pancreatic fibrosis, and minimal chronic progressive nephropathy. Mild to severe thyroid follicular cell hypertrophy was observed in all in male rats of the control group (1) (n=10) and in nine out of ten rats of the high-dose group (Group 4) (Table 7). Eight out of ten female rats of Control Group 1 presented mild to marked thyroid follicular cell hypertrophy while the change ranged from mild to severe in nine out of ten female rats of Group 4. The study director concluded that the thyroid follicular cell hypertrophy may represent an exacerbation of a background change and the severity of change indicated that the hypertrophy may have been present prior to study initiation. All microscopic findings were determined to be incidental, of the nature commonly observed in this strain and age of rats, and/or of similar incidence and severity in control rats and in animals administered 2000 mg/kg body weight/day of AMUC. As such, they were considered unrelated to the oral administration of AMUC.

3.4.5. Hematology, clinical chemistry, and urinalysis

Hematological analysis found red cell distribution width (RDW)

Table 5
Absolute and relative organ weights of male rats administered AMUC for 90 days.

Parameter	Group 1, 0 mg/kg BW/day AMUC	Group 2, 500 mg/kg BW/day AMUC	Group 3, 1000 mg/kg BW/day AMUC	Group 4, 2000 mg/kg BW/day AMUC
Male terminal body weight (g)	570.2±41.5	570.0±61.6	596.9±89.2	555.8±68.8
Absolute organ weights				
Adrenals (g)	0.0675 ±0.0152	0.0644 ±0.0075	0.0627 ±0.0132	0.0669 ±0.0083
Brain (g)	2.254 ±0.125	2.280 ±0.102	2.299 ±0.117	2.286 ±0.087
Epididymides	1.7036 ±0.3239	1.7119 ±0.2915	1.7434 ±0.1569	1.7190 ±0.1430
Heart (g)	1.669 ±0.191	1.603 ±0.122	1.722 ±0.236	1.627 ±0.190
Kidneys (g)	3.601 ±0.394	3.675 ±0.369	3.866 ±0.527	3.570 ±0.373
Liver (g)	15.197 ±1.931	15.045 ±2.368	15.934 ±2.730	14.079 ±1.771
Pituitary gland (g)	0.0145 ±0.0027	0.0148 ±0.0042	0.0149 ±0.0047	0.0144 ±0.0040
Prostate, SV, CG (combined) (g)	4.430 ±0.587	4.662 ±1.040	4.476 ±0.504	4.052 ±0.840
Spleen (g)	0.944 ±0.110	0.979 ±0.158	1.029 ±0.129	0.973 ±0.127
Testes (g)	3.909 ±0.383	3.836 ±0.333	3.967 ±0.283	3.960 ±0.313
Thymus (mg)	0.3094 ±0.0614	0.3639 ±0.1487	0.2883 ±0.0636	0.3199 ±0.0766
Thyroid-parathyroid (g)	0.0286 ±0.0061	0.0246 ±0.0030	0.0269 ±0.0048	0.0244 ±0.0075
Organ-weight-to-body-weight ratios				
Adrenals/BW	0.1183 ±0.0265	0.1135 ±0.0129	0.1064 ±0.0234	0.1218 ±0.0206
Brain/BW	3.966 ±0.283	4.030 ±0.347	3.908 ±0.456	4.157 ±0.430
Epididymides/BW	2.9789 ±0.4531	3.0455 ±0.6776	2.9574 ±0.3733	3.1237 ±0.3820
Heart/BW	2.921 ±0.169	2.828 ±0.247	2.897 ±0.205	2.935 ±0.181
Kidneys/BW	6.307 ±0.393	6.492 ±0.795	6.504 ±0.551	6.450 ±0.452
Liver/BW	26.633 ±2.599	26.395 ±2.994	26.733 ±2.644	25.402 ±2.175
Pituitary/BW	0.0026 ±0.0005	0.0026 ±0.0008	0.0025 ±0.0008	0.0026 ±0.0008
Prostate, SV, CG (combined)/BW	0.008 ±0.001	0.008 ±0.002	0.008 ±0.001	0.007 ±0.001
Spleen/BW	1.660 ±0.191	1.729 ±0.299	1.744 ±0.263	1.783 ±0.372
Testes/BW	6.858 ±0.460	6.809 ±1.024	6.731 ±0.744	7.190 ±0.809
Thymus/BW	0.5458 ±0.1156	0.6266 ±0.1781	0.4921 ±0.1289	0.5768 ±0.1249
Thyroid-parathyroid/BW	0.50188 ±0.10032	0.43481 ±0.06454	0.45954 ±0.10907	0.45226 ±0.17229
Organ-weight-to-brain-weight ratios				
Adrenals/BrW	0.0298 ±0.0058	0.0282 ±0.0030	0.0272 ±0.0051	0.0293 ±0.0035
Epididymides/BrW	0.7530 ±0.1163	0.7516 ±0.1267	0.7578 ±0.0482	0.7514 ±0.0457
Heart/BrW	0.740 ±0.075	0.704 ±0.051	0.749 ±0.087	0.711 ±0.066
Kidneys/BrW	1.596 ±0.129	1.611 ±0.128	1.678 ±0.175	1.560 ±0.123
Liver/BrW	6.741 ±0.763	6.608 ±1.052	6.913 ±0.964	6.153 ±0.659
Pituitary/BrW	6.4279 ±1.1206	6.5253 ±1.9543	6.4800 ±2.0446	6.3088 ±1.7326

(*continued on next page*)

Table 5 (*continued*)

Parameter	Group 1, 0 mg/kg BW/day AMUC	Group 2, 500 mg/kg BW/day AMUC	Group 3, 1000 mg/kg BW/day AMUC	Group 4, 2000 mg/kg BW/day AMUC
Prostate, SV, CG (combined)/ BrW	1.961 ±0.199	2.055 ±0.489	1.945 ±0.171	1.777 ±0.398
Spleen/BrW	0.420 ±0.055	0.430 ±0.073	0.447 ±0.049	0.426 ±0.057
Testes/BrW	1.736 ±0.151	1.685 ±0.161	1.727 ±0.118	1.732 ±0.111
Thymus/BrW	0.1376 ±0.0277	0.1589 ±0.0614	0.1252 ±0.0256	0.1395 ±0.0305
Thyroid-parathyroid/ BrW	0.01271 ±0.00270	0.01081 ±0.00142	0.01175 ±0.00238	0.01068 ±0.00330

AMUC = viable *Akkermansia muciniphila* bacteria lyophilized into powder form; BrW = brain weight; BW = body weight; CG = coagulating gland; SV = seminal vesicles.

decreased in male rats of Group 4 (p<0.05) (Table 8) while no changes in hematology or coagulation values were observed in female rats for any treatment groups compared to the respective control. Further, the coagulation parameter prothrombin time (PT) was significantly increased (p<0.001) in males of Groups 3 and 4 (Table 8). Clinical chemistry analysis (Table 9) revealed a significant decrease in albumin and triglycerides (p<0.01) in males of Group 4 administered AMUC. Further, total cholesterol and high-density lipoprotein (HDL) were decreased in males of Group 4 (p<0.01) and in females of Group 3 (p<0.05).

Despite their statistical significance, the findings in hematology, coagulation, and clinical chemistry parameters were not observed in a dose-dependent manner, not associated with any corresponding histopathological observations, and within historical control range, and thus considered to be not adverse and unrelated to the administration of AMUC. Urinalysis was unremarkable and not significantly different (p>0.05) from the control groups.

3.4.6. Translocation analysis

Translocated bacteria were found among both males and females in each group, including vehicle control animals (Supplementary Table S6). The bacteria isolates were characterized and differentiated from the test item *A. muciniphila* isolates by sequence of the 16S V4 region. The following genera were identified: *Bacillus* (69 isolates), *Proteus* (35 isolates), *Staphylococcus* (9 isolates), *Bifidobacterium* (6 isolates), *Enterococcus* (6 isolates), *Bacteroides* (4 isolates), *Lactococcus* (3 isolates), *Escherichia* (3 isolates), *Ligilactobacillus* (2 isolates), *Cutibacterium* (1 isolate), *Phocaeicola* (1 isolate), *Streptococcus* (1 isolate), and *Aerococcaceae* (1 isolate). Importantly, none of the translocated bacteria were of the *Akkermansia* genus, and thus no treatment-administered bacteria were found to be translocated in this study.

The numbers of isolates from blood or mesenteric lymph nodes were not significantly different (p>0.05) across groups in both male and female rats. For liver tissue, although no significant differences (p>0.05) were reported in male rats, the number of isolates was significantly higher (p<0.01) in the female group administered 1000 mg/kg body weight/day of AMUC (Group 3). This finding was not observed in male rats and in the female high-dose group, and was therefore considered not treatment-related.

4. Discussion

The safety of AMUC was evaluated in several toxicity studies to assess its potential genotoxicity and subchronic toxicity. The results of the bacterial reverse mutation assay in *S. Typhimurium* and *E. coli* confirmed that nonviable AMUC was not mutagenic under the

Table 6
Absolute and relative organ weights of female rats administered AMUC for 90 days.

Parameter	Group 1, 0 mg/kg BW/day AMUC	Group 2, 500 mg/kg BW/day AMUC	Group 3, 1000 mg/kg BW/day AMUC	Group 4, 2000 mg/kg BW/day AMUC
Female terminal body weight (g)	335.2±32.9	319.4±43.6	318.9±33.5	324.8±34.6
Absolute organ weights				
Adrenals (g)	0.0631 ±0.0102	0.0645 ±0.0136	0.0708 ±0.0135	0.0678 ±0.0142
Brain (g)	2.045 ±0.079	2.040±0.084	2.033 ±0.067	2.088±0.084
Heart (g)	1.107 ±0.102	1.089±0.118	1.048 ±0.105	1.065±0.112
Kidneys (g)	2.174 ±0.179	2.094±0.137	2.124 ±0.249	2.067±0.182
Liver (g)	9.177 ±0.490	8.875±1.086	8.674 ±1.453	8.582±1.389
Ovaries with oviducts (g)	0.1415 ±0.0212	0.1359 ±0.0152	0.1294 ±0.0255	0.1385 ±0.0323
Pituitary Gland (g)	0.0187 ±0.0049	0.0176 ±0.0035	0.0183 ±0.0028	0.0199 ±0.0059
Spleen (g)	0.588 ±0.072	0.635±0.117	0.595 ±0.099	0.593±0.093
Thymus (g)	0.2437 ±0.0597	0.2412 ±0.0611	0.2233 ±0.0436	0.2540 ±0.1036
Thyroid-parathyroid (g)	0.0163 ±0.0026	0.0244 ±0.0040**	0.0188 ±0.0049	0.0218 ±0.0055*
Uterus (g)	0.773 ±0.227	0.754±0.316	0.729 ±0.286	0.931±0.311
Organ-weight-to-body-weight ratios				
Adrenals/BW	0.1902 ±0.0366	0.2076 ±0.0584	0.2257 ±0.0570	0.2090 ±0.0405
Brain/BW	6.169 ±0.801	6.512±1.029	6.444 ±0.762	6.488±0.677
Heart/BW	3.312 ±0.225	3.433±0.322	3.290 ±0.125	3.284±0.209
Kidneys/BW	6.520 ±0.597	6.627±0.670	6.669 ±0.473	6.387±0.459
Liver/BW	27.609 ±3.056	28.174±4.653	27.091 ±2.626	26.379 ±2.846
Ovaries with oviducts/BW	0.4229 ±0.0535	0.4322 ±0.0732	0.4088 ±0.0807	0.4260 ±0.0862
Pituitary/BW	0.0056 ±0.0015	0.0056 ±0.0012	0.0058 ±0.0009	0.0061 ±0.0018
Spleen/BW	1.760 ±0.188	2.023±0.489	1.882 ±0.362	1.828±0.235
Thymus/BW	0.7335 ±0.1986	0.7593 ±0.1925	0.6998 ±0.1112	0.8088 ±0.3925
Thyroid-parathyroid/ BW	0.49626 ±0.12677	0.76645 ±0.10667***	0.60103 ±0.19377	0.67452 ±0.18204*
Uterus/BW	2.318 ±0.649	2.334±0.772	2.267 ±0.763	2.969±1.237
Organ-weight-to-brain-weight ratios				
Adrenals/BrW	0.0310 ±0.0057	0.0315 ±0.0059	0.0349 ±0.0072	0.0324 ±0.0066
Heart/BrW	0.543 ±0.061	0.536±0.079	0.516 ±0.056	0.511±0.058
Kidneys/BrW	1.064 ±0.087	1.029±0.091	1.045 ±0.120	0.991±0.087
Liver/BrW	4.495 ±0.327	4.362±0.603	4.276 ±0.778	4.109±0.634
Ovaries with oviducts/BrW	0.0694 ±0.0114	0.0666 ±0.0067	0.0635 ±0.0118	0.0662 ±0.0153
Pituitary/BrW	9.1268 ±2.3128	8.6166 ±1.6818	9.0352 ±1.6288	9.5339 ±2.8072

(*continued on next page*)

Table 6 (*continued*)

Parameter	Group 1, 0 mg/kg BW/day AMUC	Group 2, 500 mg/kg BW/day AMUC	Group 3, 1000 mg/kg BW/day AMUC	Group 4, 2000 mg/kg BW/day AMUC
Spleen/BrW	0.288 ±0.037	0.312±0.059	0.293 ±0.051	0.284±0.040
Thymus/BrW	0.1197 ±0.0320	0.1184 ±0.0299	0.1099 ±0.0216	0.1219 ±0.0488
Thyroid-parathyroid/BrW	0.00798 ±0.00126	0.01202 ±0.00231***	0.00924 ±0.00230	0.01045 ±0.00261
Uterus/BrW	0.382 ±0.126	0.374±0.173	0.359 ±0.140	0.447±0.148

AMUC = viable *Akkermansia muciniphila* bacteria lyophilized into powder form; BrW = brain weight; BW = body weight.
* $p<0.01$; ** $p<0.01$; *** $p<0.001$.

conditions of the study. These results are consistent with the summary of an unpublished study submitted to the European Food Safety Authority (EFSA) for the safety evaluation of pasteurized *A. muciniphila*, in which the pasteurized *A. muciniphila* was not mutagenic at up to 5000 μg/plate in *S.* Typhimurium TA100, TA102, TA1535, and TA1537 in the absence and presence of S9 metabolic activation [21]. Similarly, Ma et al. [42] found that soluble components of *A. muciniphila* PROBIO were not mutagenic in *S.* Typhimurium TA97a, TA98, TA100, and TA1535, and *E. coli* WP2 uvrA when analyzed at 15.81, 50, 158.1, 500, 1581, and 5000 μg/plate. Treatment of human peripheral blood lymphocytes in the *in vitro* chromosomal aberration study showed that the irradiated AMUC did not induce clastogenicity, and that the viable *A. muciniphila* cells administered to CD-1 mice in the *in vivo* micronucleus assay did not promote micronucleus formation, confirming a lack of genotoxicity under the study conditions. EFSA [21] evaluated the effect of pasteurized *A. muciniphila* in an *in vitro* mammalian cell micronucleus test in human peripheral blood lymphocytes, when treated at up to 750 μg/mL for 3 hours (in the absence and presence of S9 mix) and up to 375 μg/mL for 24 hours, and concluded that the pasteurized *A. muciniphila* preparation was not clastogenic or aneugenic under the conditions of the OECD-compliant test. The results of the present *in vitro* chromosomal aberration test in human peripheral lymphocytes are supported by the *in vivo* mammalian cell micronucleus test conducted in Kunming mice that were administered AKK PROBIO (*A. muciniphila*) bacterial suspensions at doses of 222.2, 666.7, and 2000 mg/kg body weight [42]. Bone marrow samples from the femurs of the mice were evaluated and Ma et al. [42] concluded that AKK PROBIO "*did not display genotoxic activity*," as there was no difference in micronuclei formation in polychromatic erythrocytes in the test groups ($p>0.05$), when compared to the negative control group. The work by Ma et al. [42] is in agreement with the current *in vivo* mammalian micronucleus test of the peripheral blood of male and female CD-1 mice administered viable AMUC that showed a lack of micronucleus formation and thus a lack of genotoxic potential under the conditions of the study.

No treatment-related adverse effects were observed when AMUC was administered *via* oral gavage to male and female CRL Sprague-Dawley CD IGS rats for 90 days at 0, 500, 1000, or 2000 mg/kg body weight/day, corresponding to 0, 4.1×10^{10}, 8.2×10^{10}, and 1.64×10^{11} CFU/kg body weight/day of viable *A. muciniphila*, respectively. There were no mortality, food consumption, body weight, body weight gain, ophthalmological and clinical observations, absolute or relative organ weights, clinical pathology, hematology, coagulation, urinalysis, or histopathology changes attributable to the administration of AMUC. Significant changes reported in experimental animals compared with basal controls were deemed incidental and without toxicological significance as they were either within historical control values, not dose-dependent, or not correlated with significant changes in clinical pathology or

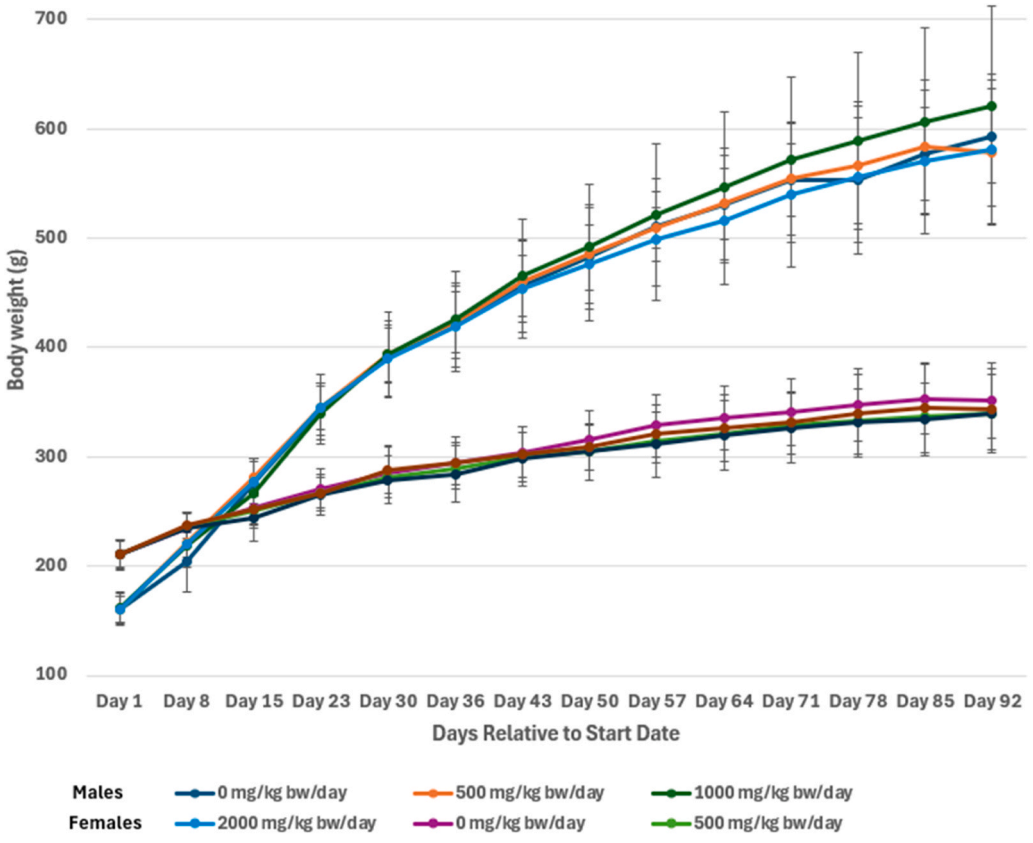

Fig. 1. Mean body weights of male and female rats administered AMUC for 90 days. AMUC = viable *Akkermansia muciniphila* bacteria lyophilized into a powder form; bw = body weight.

Table 7
Incidence thyroid follicular cell hypertrophy of control and high-dose rats following 90-day oral administration of AMUC.

	Males		Females	
	Group 1 (control) 0 mg/kg BW/day AMUC	Group 4 2000 mg/kg BW/day AMUC	Group 1 (control) 0 mg/kg BW/day AMUC	Group 4 2000 mg/kg BW/day AMUC
Number of rats examined	10	10	10	10
Hypertrophy				
Number unremarkable	0	1	2	1
Mild	1	1	2	1
Moderate	2	0	3	4
Marked	6	1	3	3
Severe	1	7	0	1
Total finding incidence	10	9	8	9

AMUC = viable *Akkermansia muciniphila* bacteria lyophilized into powder form; BW = body weight.

histopathology.

Minimal liver and pancreatic infiltration of mixed cells, mild pancreatic fibrosis, minimal chronic progressive nephropathy, and mild to severe thyroid follicular cell hypertrophy were reported in male and female rats in the control and high-dose groups. Follicular cell hypertrophy is generally reported with low incidence in Sprague-Dawley CD rats. For instance, Isobe et al. [33] assessed the historical control background incidence of spontaneous thyroid lesions of Sprague-Dawley CD rats used in 104-week carcinogenicity studies and reported that incidence of spontaneous thyroid follicular cell hypertrophy was observed in 2 of the 935 male rats evaluated (0.21 %) while this change was not observed in the 942 female rats evaluated. Similarly, the incidence of mild thyroid follicular cell hypertrophy/hyperplasia was reported to be 0.94 % in male Sprague-Dawley CD rats used as control animals in the studies evaluated by Giknis and Clifford [28]. Nevertheless, diffuse thyroid follicular cell hypertrophy/hyperplasia are often observed in control animals, especially in male rats, and the diffuse nature of the thyroid change, as was seen in this study, typically indicates reversibility [31]. The changes were considered not toxicologically significant and not related to the oral administration of AMUC, as no patterns, trends, or test substance–dependent responses were detected.

RDW was significantly decreased in male rats of the high-dose group while PT was significantly increased in male rats of the mid- and high-dose groups. Although a non-statistically significant trend was noted in males, the values of RDW and PT were within historical control range values and were not correlated to histopathological changes. Further, these changes were not observed in female rats. For these reasons, the increase in RDW and the decrease in PT were not considered to be of toxicological concern. Similarly, the decrease of triglycerides levels in males of the high-dose groups and of total cholesterol and HDL levels in males of the high-dose group and females of the mid-dose group were not considered of toxicological significance as they were not associated with any other correlating histopathological alteration. Previous studies reported that administration of 2 $\times 10^8$ to 5 $\times 10^9$ viable *A. muciniphila* for 6–24 weeks decreased levels of triglycerides [3,55,58,59] and total cholesterol [3,30,55,56,58] in diet-induced obese mice, while levels of HDL were found to be increased in diet-induced obese mice following administration of 1 $\times 10^9$ for four to five weeks [3,58]. Slight decreases in blood triglyceride and total cholesterol concentrations are not typically viewed as adverse, however increased levels of blood triglyceride and total cholesterol concentrations may be of more toxicological significancewhen occurring for long periods of time [47,48].

Based on the toxicological data from the 90-day oral administration

Table 8
Hematology/coagulation data for male and female rats following 90-day oral administration of AMUC.

Parameter	Group 1, 0 mg/kg BW/day	Group 2, 500 mg/kg BW/day	Group 3, 1000 mg/kg BW/day	Group 4, 2000 mg/kg BW/day
Males				
Hematology				
Hematocrit (%)	52.40±2.20	52.58±1.57	51.93±2.33	51.70 ±1.51
Hemoglobin (g/dL)	15.53±0.45	15.61±0.64	15.47±0.72	15.52±0.34
MCV (fL)	56.57±1.58	57.22±1.46	56.94±1.66	55.30±1.56
MCH (pg)	16.73±0.59	17.00±0.61	16.97±0.69	16.59±0.49
MCHC (g/dL)	29.57±0.60	29.69±0.69	29.82±0.63	30.07±0.37
Platelet count (10^3/μL)	973.60 ±128.50	877.60 ±138.28	975.00 ±92.68	969.70 ±151.48
RBC (10^6/μL)	9.289 ±0.473	9.192 ±0.253	9.116±0.270	9.347±0.271
RDW (%)	15.13±1.17	14.10±0.93	14.01±1.16	13.75 ±0.88*
WBC (10^3/μL)	10.407 ±1.739	9.825 ±2.484	11.028 ±2.177	11.321 ±3.540
ABAS (10^3/μL)	0.076 ±0.025	0.062 ±0.023	0.065±0.031	0.073±0.029
AEOS (10^3/μL)	0.134 ±0.046	0.113 ±0.043	0.118±0.034	0.142±0.060
ALUC (10^3/μL)	0.091 ±0.028	0.114 ±0.061	0.114±0.031	0.123±0.057
ALYM (10^3/μL)	8.362 ±1.619	7.598 ±2.469	8.561±2.110	8.678±2.384
AMON (10^3/μL)	0.378 ±0.069	0.313 ±0.116	0.381±0.125	0.380±0.103
ANEU (10^3/μL)	1.367 ±0.211	1.634 ±0.719	1.788±0.728	1.927±1.222
ARET (10^3/μL)	225.870 ±49.718	183.780 ±48.671	199.680 ±32.534	172.640 ±43.933
Coagulation				
APTT (seconds)	16.62±1.56	17.00±1.10	18.16±2.90	19.58±3.32
PT (seconds)	9.69±0.26	9.90±0.16	10.10 ±0.24**	10.24 ±0.21**
Females				
Hematology				
Hematocrit (%)	50.72±2.93	49.40±1.47	48.49±3.67	49.20±2.98
Hemoglobin (g/dL)	15.29±0.72	15.06±0.37	14.83±0.95	15.14±0.77
MCV (fL)	59.31±0.97	58.99±0.93	58.83±1.61	58.96±1.71
MCH (pg)	17.87±0.34	17.98±0.45	17.96±0.39	18.12±0.47
MCHC (g/dL)	30.12±0.58	30.63±0.54	30.50±0.84	30.80±0.54
Platelet count (10^3/μL)	845.90 ±116.65	882.67 ±96.43	894.60 ±132.39	930.80 ±107.77
RBC (10^6/μL)	8.555 ±0.468	8.382 ±0.347	8.263±0.520	8.369 ±0.492
RDW (%)	11.91±0.32	12.30±0.96	11.93±0.30	12.07±0.40
WBC (10^3/μL)	6.021 ±1.183	5.717 ±0.895	6.308±2.559	6.667±1.835
ABAS (10^3/μL)	0.037 ±0.021	0.028 ±0.015	0.033±0.028	0.047±0.027
AEOS (10^3/μL)	0.078 ±0.024	0.090 ±0.027	0.081±0.046	0.083±0.045
ALUC (10^3/μL)	0.060 ±0.024	0.076 ±0.076	0.062±0.039	0.058±0.026
ALYM (10^3/μL)	4.861 ±1.152	4.527 ±0.592	5.025±1.921	5.542±1.409
AMON (10^3/μL)	0.188 ±0.071	0.241 ±0.123	0.233±0.179	0.166±0.079
ANEU (10^3/μL)	0.798 ±0.346	0.760 ±0.419	0.876±0.586	0.772±0.404
ARET (10^3/μL)	147.990 ±31.463	158.689 ±67.190	136.280 ±33.564	127.310 ±17.559
Coagulation				
APTT (seconds)	16.62±3.35	19.34±6.04	18.96±6.05	17.89±1.57
PT (seconds)	8.96±0.46	9.31±0.22	9.13±0.26	9.32±0.22

ABAS = absolute basophils; AEOS = absolute eosinophils; ALUC = absolute leukocytes; ALYM = absolute lymphocytes; AMON = absolute monocytes; AMUC = viable *Akkermansia muciniphila* bacteria lyophilized into powder form;

ANEU = absolute neutrophils; APTT = activated partial thromboplastin time; ARET = absolute reticulocytes; BW = body weight; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; PT = prothrombin time; RBC = red blood cells; RDW = red cell distribution width; SD = standard deviation; WBC = white blood cells. * $p<0.05$; ** $p<0.001$. All data are presented as mean values ± SD with n=9–10 animals/group.

study in male and female rats, a NOAEL of 2000 mg/kg body weight/day AMUC, equivalent to an intake of approximately 1.64×10^{11} CFU/kg body weight/day of viable *A. muciniphila*, was concluded. Similarly, Druart et al. [13] have previously established a NOAEL of 1500 mg/kg body weight/day for pasteurized *A. muciniphila*, equivalent to 9.6×10^{10} cells/kg body weight/day based on the findings of a comprehensive safety assessment, which included genotoxicity studies, unpublished acute and 14-day toxicity studies reviewed by EFSA [21], as well as a 90-day toxicity study evaluating concentrations of pasteurized *A. muciniphila* up to 1500 mg/kg body weight/day.

Hou et al. [29] evaluated the acute toxicity of two strains of *A. muciniphila*, isolated from fecal matter or breast milk, in female SPF NIH mice (n=6/group). No mortality or visible signs of toxicity and no significant differences in body weight and hematological parameters (*i.e.*, red blood cell count, hemoglobin, platelet count) were observed following oral gavage of a suspension containing 1×10^9 CFU/day for three days. An additional acute study was conducted in immunodeficient BALB/c nude mice which were gavaged with saline (control) or a bacterial suspension containing 1×10^9 CFU/day of the *A. muciniphila* strain isolated from breast milk for three days. No mortality and no adverse effects were reported and there were no *A. muciniphila*–related effects on body weight, hematological parameters, and histological observations in the colon [29].

In a four-week study by the same authors, female SPF BALB/c mice (n=10/group) were gavaged with saline (control) or a bacterial suspension containing 1×10^9 to 1×10^{10} CFU/day of the *A. muciniphila* strain isolated from breast milk. Results indicated no visible sign of toxicity, no abnormal behavior, and no mortality, and no difference between the control and *A. muciniphila* groups with respect to body weight; hematological parameters including red blood cell count, platelet count, white blood cell count, neutrophil count, lymphocytes count, monocytes count, eosinophiles count, basophiles count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean platelet volume, and red cell distribution width; and histopathological alterations in the colon.

Similarly, in a study where mice were gavaged with PBS as control vehicle or a suspension of 1.0×10^9 viable *A. muciniphila* MucT (ATCC BAA-835) for five weeks (n=10/group), no mortalities and no clinical or neurobehavioral signs of toxicity were observed [2]. No significant differences in food consumption were indicated while the mice receiving *A. muciniphila* had significantly reduced body weight and adipose weight compared with the basal control group. Further, no histological alterations were reported in the colon, liver, and adipose tissues while the liver weight was significantly decreased in mice following oral administration of *A. muciniphila*. Analysis of biochemical parameters and inflammatory biomarkers showed that *A. muciniphila* significantly decreased glucose, triglyceride, and aspartate aminotransferase levels along with a reduction in concentrations of proinflammatory cytokines (*i.e.*, interleukin [IL]-6 and tumor necrosis factor-*alpha*) and an increase in the anti-inflammatory cytokine IL-10 levels. The authors did not indicate that these changes were adverse in nature and concluded that *A. muciniphila* improved body weight as well as plasma biochemical and inflammatory markers [2].

Various studies have evaluated the potential health benefits associated with the oral administration of *A. muciniphila*, including amelioration of metabolic inflammation, effects on immune response and metabolic parameters, and effects on intestinal barrier integrity [2,3,30,35,38,40,56,74]. Although these studies were not designed specifically

Table 9
Clinical chemistry and urinalysis data for male and female rats following 90-day oral administration of AMUC.

Parameter	Group 1 0 mg/kg BW/day AMUC	Group 2 500 mg/kg BW/day AMUC	Group 3 1000 mg/kg BW/day AMUC	Group 4 2000 mg/kg BW/day AMUC
Males				
Clinical Chemistry				
ALT (U/L)	32.2±7.1	30.6±12.3	34.8±8.7	26.9±5.7
Albumin (g/dL)	4.04±0.14	3.77±0.25	3.81±0.32	3.62±0.24**
Alkaline phosphatase (U/L)	87.9±25.9	86.3±20.2	85.2±17.4	88.1±17.9
AST (U/L)	97.0±20.1	96.8±25.8	98.4±28.1	96.5 ±25.9
GGT (U/L)	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00
Globulin (g/dL)	2.53±0.32	2.59±0.32	2.53±0.26	2.53±0.28
Bilirubin (mg/dL)	0.089 ±0.016	0.079 ±0.019	0.074±0.030	0.073±0.18
BUN (mg/dL)	12.1±1.6	13.3±1.8	11.9±1.2	13.4±2.1
Blood creatine (mg/dL)	0.249 ±0.036	0.270 ±0.048	0.243±0.038	0.241±0.026
Cholesterol (mg/dL)	84.6±20.1	78.8±23.1	72.6±21.0	52.7±15.3**
SDH (U/L)	9.47±7.51	6.57±5.60	7.85±5.64	4.93±4.49
LDL (mmol/L)	0.270 ±0.082	0.340 ±0.190	0.270±0.125	0.210±0.088
HDL (mmol/L)	1.500 ±0.374	1.320 ±0.374	1.290±0.420	0.910 ±0.303**
Triglycerides (mg/dL)	90.1±36.3	65.9±38.0	61.0±20.4	52.8±38.2**
Fasting glucose (mg/dL)	182.3±31.7	173.5±56.4	195.0±38.8	186.5±29.5
Total protein (g/dL)	6.57±0.37	6.36±0.38	6.34±0.33	6.15±0.32
Calcium (mg/dL)	11.53±0.64	11.45±0.99	11.31±0.36	10.99±0.38
IPHS (mg/dL)	9.16±1.02	9.10±0.89	9.26±0.92	8.91±0.63
Chloride (mmol/L)	99.85±1.36	100.10 ±1.84	99.56±0.99	100.61±1.64
Potassium (mmol/L)	7.867 ±1.783	8.078 ±1.161	7.825±1.161	7.778±0.840
Sodium (mmol/L)	142.70 ±1.34	142.70 ±1.64	142.10±1.66	142.20±1.14
Urinalysis				
Urine volume (mL)	11.60±8.74	12.35±8.98	8.60±3.12	13.25±7.82
Urine pH	7.30±0.63	6.95±0.37	6.90±0.39	7.00±0.41
Urine glucose (mg/dL)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Urine ketones (mmol/L)	14.0±10.7	10.5±6.0	14.0±3.2	11.0±5.2
Urine protein (mg/dL)	45.0±90.0	18.0±9.5	31.0±25.4	15.0±12.2
Specific gravity	1.0210 ±0.0066	1.0225 ±0.0072	1.0250 ±0.0047	1.0210 ±0.0070
Urobilinogen (EU/dL)	0.20±0.00	0.20±0.00	0.20±0.00	0.20±0.00
Females				
Clinical Chemistry				
ALT (U/L)	24.0±3.1	29.9±12.2	27.3±8.4	28.3±4.4
Albumin (g/dL)	4.96±0.46	4.73±0.54	4.60±0.54	4.54±0.35
Alkaline phosphatase (U/L)	35.3±4.2	42.9±17.7	31.2±10.0	43.8±13.1
AST (U/L)	80.0±11.5	112.2±40.9	98.1±28.2	86.2 ±11.1
GGT (U/L)	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00
SDH (U/L)	10.10±4.99	7.17±6.09	10.34±4.20	11.24±4.97
Globulin (g/dL)	2.18±0.12	2.13±0.36	2.03±0.19	2.28±0.24
Bilirubin (mg/dL)	0.107 ±0.039	0.115 ±0.035	0.095±0.020	0.091±0.026
BUN (mg/dL)	14.2±1.9	15.3±2.2	15.0±1.5	14.5±2.0
Blood creatine (mg/dL)	0.294 ±0.039	0.284 ±0.065	0.288±0.056	0.286±0.029

(*continued on next page*)

Table 9 (*continued*)

Parameter	Group 1 0 mg/kg BW/day AMUC	Group 2 500 mg/kg BW/day AMUC	Group 3 1000 mg/kg BW/day AMUC	Group 4 2000 mg/kg BW/day AMUC
Cholesterol (mg/dL)	108.1±31.3	97.4±20.5	81.5±17.1*	86.6±20.1
LDL (mmol/L)	0.203 ±0.074	0.200 ±0.086	0.160±0.037	0.173±0.053
HDL (mmol/L)	2.208 ±0.546	1.981 ±0.415	1.701 ±0.338*	1.787±0.392
Triglycerides (mg/dL)	56.0±13.4	56.9±22.9	46.8±23.7	38.5±15.5
Fasting glucose (mg/dL)	158.7±29.9	151.1±26.3	169.6±60.2	157.5±52.2
Total protein (g/dL)	7.14±0.49	6.86±0.45	6.63±0.61	6.82±0.36
Calcium (mg/dL)	11.56±0.64	11.43±0.66	11.15±1.40	11.57±0.64
IPHS (mg/dL)	7.64±1.30	8.03±1.58	7.66±1.83	8.56±1.19
Chloride (mmol/L)	99.92±1.38	100.73 ±1.88	95.80±8.94	101.18±1.52
Potassium (mmol/L)	6.588 ±2.422	6.321 ±1.530	6.018±1.363	7.559±2.505
Sodium (mmol/L)	142.40 ±1.65	142.80 ±1.81	146.80 ±10.28	142.50±2.76
Urinalysis				
Urine volume (mL)	6.60±6.84	2.42±1.73	2.57±1.85	6.70±4.20
Urine pH	6.95±0.93	6.70±0.67	6.55±0.96	6.85±0.34
Urine glucose (mg/dL)	0.0±0.0	30.0±48.3	20.0±42.2	0.0±0.0
Urine ketones (mmol/L)	2.5±4.9	3.5±2.4	3.5±2.4	1.5±2.4
Urine protein (mg/dL)	42.0±91.6	80.5±86.7	76.0±90.4	15.0±14.1
Specific gravity	0.9728 ±0.1652	1.0275 ±0.0035	1.0270 ±0.0067	1.0225 ±0.0059
Urobilinogen (EU/dL)	0.28±0.25	0.36±0.34	0.36±0.34	0.20±0.00

ALT = alanine aminotransferase; AMUC = viable *Akkermansia muciniphila* bacteria lyophilized into powder form; AST = aspartate aminotransferase; BUN = blood urea nitrogen; BW = body weight; EU = Erlich units; GGT = *gamma*-glutamyl trans-peptidase; HDL = high-density lipoprotein; IPHS = inorganic phosphorous; LDL = low-density lipoprotein; SD = standard deviation; SDH = sorbitol dehydrogenase.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$. All data are presented as mean values ± SD with n=9–10 animals/group.

to evaluate safety, daily administration of 1.0×10^8 to 5.0×10^9 CFU of *A. muciniphila* for up to 40 weeks was shown to be well tolerated and no mortality, adverse effects, or safety concerns were reported.

After 90 days of repeated administration of viable *A. muciniphila*, no bacteria of the *Akkermansia* genus were found in blood, liver, or mesenteric lymph nodes. Although *A. muciniphila* is closely associated with the host mucosa and intestinal epithelial cells, *A. muciniphila* does not cross the intestinal barrier and is not absorbed into the systemic circulation under normal physiological conditions. Conversely, the presence of *Akkermansia*-like sequences was detected in blood samples of individuals with diseases such as cirrhosis and septicemia [14,27,60,67]. The presence of translocated bacteria in male and female rats across control and treatment groups and the genera isolated from the liver and the mesenteric lymph nodes are consistent with previous studies [49]. Bacterial translocation has been previously reported in animals that are subject to stress (either physical or chronic psychological stress) [65,76,8] and is not unexpected in rats subject to daily gavage over an extended period of time. Although a significant increase in the number of isolates in the liver tissue was observed in female rats administered the mid dose of AMUC in the present study, no dose-dependent relationship was identified. Further, the absence of significant changes in pathological findings or treatment-related toxicity suggests that AMUC does not present a safety concern. This translocation study was limited to detecting bacteria able to grow in an anaerobic atmosphere at 37°C in nine days. Additional limitations include the length of the incubation, which resulted in plates that had dried out and could not be evaluated, and the culture conditions and growth time, which favor the overgrowth of *Proteus*, creating mixed colonies. Therefore, to identify the genus of the underlying discreet colony, *Proteus* contribution had to be eliminated from the sequencing data. Further, only five representative colonies were selected for taxa assignments when more than five colonies of a single morphological type were identified on plates from a sample.

Altogether, the findings of the 90-day oral administration study and the genotoxicity studies indicate no safety concern regarding the consumption of viable *A. muciniphila* up to approximately 1.64×10^{11} CFU/kg body weight/day, and support the use of this AMUC preparation as a novel ingredient in human food.

5. Conclusion

The safety of viable *A. muciniphila* was evaluated in the 90-day toxicology study conducted according to GLP. No treatment-related adverse effects were observed when AMUC was administered *via* oral gavage for 90 days to male and female CRL Sprague-Dawley CD IGS rats at 0, 500, 1000, or 2000 mg/kg body weight/day, yielding targeted dose-equivalents of viable *A. muciniphila* of 0, 4.1×10^{10}, 8.2×10^{10}, and 1.64×10^{11} CFU/kg body weight/day, respectively. Under the conditions of the 90-day study, and based on the toxicological endpoints evaluated, the NOAEL for AMUC was established at the highest dose tested of 2000 mg/kg body weight/day, corresponding to approximately 1.64×10^{11} CFU/kg body weight/day of viable *A. muciniphila*. Further, negative results were observed in the *in vitro* bacterial reverse mutation assay and genotoxicity studies. *Gamma*-irradiated AMUC was non-mutagenic in *S.* Typhimurium and *E. coli* test strains at concentrations of up to 5000 μg/plate and did not exhibit clastogenicity when evaluated in the *in vitro* chromosomal aberration study conducted according to OECD Test Guidelines. In addition, AMUC administered to CD-1 mice was not genotoxic when evaluated under the conditions of the OECD protocol for assessing micronucleus formation.

Taken together, these data support the safety of viable *A. muciniphila* (AMUC) at up to approximately 1.64×10^{11} CFU/kg body weight/day for use as an ingredient in human food.

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CRediT authorship contribution statement

Esther Yu: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Andrew Cheng:** Writing – review & editing, Conceptualization. **John Eid:** Writing – review & editing, Conceptualization. **Mark Bauter:** Writing – review & editing, Investigation, Conceptualization. **Barry Lynch:** Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All research reported was funded by Pendulum Therapeutics Inc. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.101790.

Data Availability

Data will be made available on request.

References

[1] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410. <https://doi.10.1016/s0022-2836(05)80360-2>.

[2] F. Ashrafian, S. Keshavarz Azizi Raftar, A. Shahryari, A. Behrouzi, R. Yaghoubfar, A. Lari, H.R. Moradi, S. Khatami, M.D. Omrani, F. Vaziri, A. Masotti, S.D. Siadat, Comparative effects of alive and pasteurized *Akkermansia muciniphila* on normal diet-fed mice, Sci. Rep. 11 (2021) 17898. <https://doi.10.1038/s41598-021-95738-5>.

[3] F. Ashrafian, S. Keshavarz Azizi Raftar, A. Lari, A. Shahryari, S. Abdollahiyan, H. R. Moradi, M. Masoumi, M. Davari, S. Khatami, M.D. Omrani, F. Vaziri, A. Masotti, S.D. Siadat, Extracellular vesicles and pasteurized cells derived from *Akkermansia muciniphila* protect against high-fat induced obesity in mice, Microb. Cell. Fact. 20 (2021) 219. <https://doi.10.1186/s12934-021-01709-w>.

[4] M.S. Bartlett, Properties of sufficiency and statistical tests, Proc. R. Soc. Lond. A Math. Phys. Sci. 160 (1937) 268–282. <https://doi.10.1098/rspa.1937.0109>.

[5] C. Chelakkot, Y. Choi, D.-K. Kim, H.T. Park, J. Ghim, Y. Kwon, J. Jeon, M.-S. Kim, Y.-K. Jee, Y.S. Gho, H.-S. Park, Y.-K. Kim, S.H. Ryu, *Akkermansia muciniphila*-derived extracellular vesicles influence gut permeability through the regulation of tight junctions, Exp. Mol. Med. 50 (2018) e450. <https://doi.10.1038/emm.2017.282>.

[6] S.F. Clarke, E.F. Murphy, O. O'Sullivan, A.J. Lucey, M. Humphreys, A. Hogan, P. Hayes, M. O'Reilly, I.B. Jeffery, R. Wood-Martin, D.M. Kerins, E. Quigley, R. P. Ross, P.W. O'Toole, M.G. Molloy, E. Falvey, F. Shanahan, P.D. Cotter, Exercise and associated dietary extremes impact on gut microbial diversity, Gut 63 (2014) 1913–1920. <https://doi.10.1136/gutjnl-2013-306541>.

[7] M.C. Collado, M. Derrien, E. Isolauri, W.M. De Vos, S. Salminen, Intestinal integrity and *Akkermansia muciniphila*, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly, Appl. Environ. Microbiol. 73 (2007) 7767–7770. <https://doi.10.1128/AEM.01477-07>.

[8] K.A. Costa, A.D.N. Soares, S.P. Wanner, R. das Graças Carvalho dos Santos, S.O. A. Fernandes, F. dos Santos Martins, J.R. Nicoli, C.C. Coimbra, V.N. Cardoso, L-arginine supplementation prevents increases in intestinal permeability and bacterial translocation in male swiss mice subjected to physical exercise under environmental heat stress, J. Nutr. 144 (2014) 218–223. <https://doi.10.3945/jn.113.183186>.

[9] M.C. Dao, A. Everard, J. Aron-Wisnewsky, N. Sokolovska, E. Prifti, E.O. Verger, B. D. Kayser, F. Levenez, J. Chilloux, L. Hoyles, M.I.-O. Consortium, M.-E. Dumas, S. W. Rizkalla, J. Doré, P.D. Cani, K. Clément, *Akkermansia muciniphila* and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology, Gut 65 (2016) 426–436. <https://doi.10.1136/gutjnl-2014-308778>.

[10] C. Depommier, A. Everard, C. Druart, H. Plovier, M. Van Hul, S. Vieira-Silva, G. Falony, J. Raes, D. Maiter, N.M. Delzenne, M. De Barsy, A. Loumaye, M. P. Hermans, J.-P. Thissen, W.M. De Vos, P.D. Cani, Supplementation with *Akkermansia muciniphila* in overweight and obese human volunteers: a proof-of-concept exploratory study, Nat. Med. 25 (2019) 1096–1103. <https://doi.10.1038/s41591-019-0495-2>.

[11] M. Derrien, M.C. Collado, K. Ben-Amor, S. Salminen, W.M. De Vos, The mucin degrader *Akkermansia muciniphila* is an abundant resident of the human intestinal tract, Appl. Environ. Microbiol. 74 (2008) 1646–1648. <https://doi.10.1128/AEM.01226-07>.

[12] M. Derrien, E.E. Vaughan, C.M. Plugge, W.M. De Vos, *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium, Int. J. Syst. Evol. Microbiol 54 (2004) 1469–1476. <https://doi.10.1099/ijs.0.02873-0>.

[13] C. Druart, H. Plovier, M. Van Hul, A. Brient, K.R. Phipps, W.M. De Vos, P.D. Cani, Toxicological safety evaluation of pasteurized *Akkermansia muciniphila*, J. Appl. Toxicol. 41 (2021) 276–290. <https://doi.10.1002/jat.4044>.

[14] G. Dubourg, F. Cornu, S. Edouard, A. Battaini, M. Tsimaratos, D. Raoult, First isolation of *Akkermansia muciniphila* in a blood-culture sample, Clin. Microbiol. Infect. 23 (2017) 682–683. <https://doi.10.1016/j.cmi.2017.02.031>.

[15] O.J. Dunn, Multiple comparisons using rank sums, Technometrics 6 (1964) 241–252. <https://doi.10.1080/00401706.1964.10490181>.

[16] C.W. Dunnett, New tables for multiple comparisons with a control, Biometrics 20 (1964) 482. <https://doi.10.2307/2528490>.

[17] C.W. Dunnett, Pairwise multiple comparisons in the unequal variance case, J. Am. Stat. Assoc. 75 (1980) 796–800. <https://doi.10.1080/01621459.1980.10477552>.

[18] Earth Microbiome Project, 2024., 16s Illumina Amplicon Protocol. <⟨https://earthmicrobiome.org/protocols-and-standards/16s/⟩>.

[19] P.B. Eckburg, E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K.E. Nelson, D.A. Relman, Diversity of the human intestinal microbial flora, Science 308 (2005) 1635–1638. <https://doi.10.1126/science.1110591>.

[20] R.M.R.A. Effendi, M. Anshory, H. Kalim, R.F. Dwiyana, O. Suwarsa, L.M. Pardo, T. E.C. Nijsten, H.B. Thio, *Akkermansia muciniphila* and faecalibacterium prausnitzii in immune-related diseases, Microorganisms 10 (2022) 2382. <https://doi.10.3390/microorganisms10122382>.

[21] EFSA, Scientific Opinion on the safety of pasteurised *Akkermansia muciniphila* as a novel food pursuant to Regulation (EU) 2015/2283, EFSA J. 19 (2021) 6780. <https://doi.10.2903/j.efsa.2021.6780>.

[22] A. Everard, C. Belzer, L. Geurts, J.P. Ouwerkerk, C. Druart, L.B. Bindels, Y. Guiot, M. Derrien, G.G. Muccioli, N.M. Delzenne, W.M. De Vos, P.D. Cani, Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity, Proc. Natl. Acad. Sci. USA 110 (2013) 9066–9071. <https://doi.10.1073/pnas.1219451110>.

[23] V. Fernandes Rodrigues, J. Elias-Oliveira, Í.S. Pereira, J.A. Pereira, S.C. Barbosa, M. S.G. Machado, D. Carlos, *Akkermansia muciniphila* and gut immune system: a good friendship that attenuates inflammatory bowel disease, obesity, and diabetes, Front. Immunol. 13 (2022) 934695. <https://doi.10.3389/fimmu.2022.934695>.

[24] R. Filardi, G. Gargari, D. Mora, S. Arioli, Characterization of antibiotic-resistance traits in *Akkermansia muciniphila* strains of human origin, Sci. Rep. 12 (2022) 19426. <https://doi.10.1038/s41598-022-23980-6>.

[25] E. Fröhlich, R. Wahl, Microbiota and thyroid interaction in health and disease, Trends Endocrinol. Metab. 30 (8) (2019) 479–490, doi: 10.1016/j.tem.2019.05.008. Epub 2019 Jun 27. PMID: 31257166.

[26] D. Gatehouse, Bacterial mutagenicity assays: test methods, in: J.M. Parry, E. M. Parry (Eds.), Genetic Toxicology: Principles and Methods, Humana Press (Springer Scienc+Business Media, LLC), New York, 2012. Methods in Molecular Biology, Vol. 817, pp., 21-34. <https://doi.org/10.1007/978-1-61779-421-6_2>.

[27] S.Y. Geerlings, I. Kostopoulos, W.M. De Vos, C. Belzer, *Akkermansia muciniphila* in the human gastrointestinal tract: when, where, and how?, in: Microorganisms, 6, 2018, p. 75. <https://doi.10.3390/microorganisms6030075>.

[28] M.L. Giknis, C.B. Clifford, Histopathology Findings In 4-26 Week Old Crl: CD(SD) Rats, Charles River Laboratories, Wilmington, MA, 2012. <https://www.criver.com/sites/default/files/resources/doc_a/HistopathologyFindingsin4-26WeekOldCrlCDSDRats.pdf>.

[29] F. Hou, J. Tang, Y. Liu, Y. Tan, Y. Wang, L. Zheng, D. Liang, Y. Lin, L. Wang, Z. Pan, R. Yang, Y. Bi, F. Zhi, Safety evaluation and probiotic potency screening of *Akkermansia muciniphila* strains isolated from human feces and breast milk, Microbiol. Spectr. 11 (2023) e03361-03322. <https://doi.10.1128/spectrum.03361-22>.

[30] D. Huang, J. Gao, C. Li, C. Nong, W. Huang, X. Zheng, S. Li, Y. Peng, A potential probiotic bacterium for antipsychotic-induced metabolic syndrome: mechanisms underpinning how *Akkermansia muciniphila* subtype improves olanzapine-induced glucose homeostasis in mice, Psychopharmacology 238 (2021) 2543–2553. <https://doi.10.1007/s00213-021-05878-9>.

[31] M. Huisinga, L. Bertrand, R. Chamanza, I. Damiani, J. Engelhardt, S. Francke, A. Freyberger, T. Harada, J. Harleman, W. Kaufmann, K. Keane, J. Köhrle, B. Lenz, M.S. Marty, S. Melching-Kollmuss, X. Palazzi, G. Pohlmeyer-Esch, A. Popp, T. J. Rosol, V. Strauss, H. Van den Brink-Knol, C.E. Wood, M. Yoshida, Adversity considerations for thyroid follicular cell hypertrophy and hyperplasia in nonclinical toxicity studies: results from the 6th ESTP International Expert Workshop, Toxicol. Pathol. 48 (2020) 920–938. <https://doi.10.1177/0192623320972009>.

[32] ICH, 2011. Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use: S2(R1). International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), Geneva, Switzerland. ICH Harmonised Tripartite Guideline - Current Step 4 version [Combines S2A & S2B]. <⟨https://database.ich.org/sites/default/files/S2%28R1%29%20Guideline.pdf⟩>.

[33] K. Isobe, J. Baily, S. Mukaratirwa, C. Petterino, A. Bradley, Historical control background incidence of spontaneous pituitary gland lesions of Han-Wistar and Sprague-Dawley rats and CD-1 mice used in 104-week carcinogenicity studies, J. Toxicol. Pathol. 30 (2017) 339–344. <https://doi.10.1293/tox.2017-0030>.

[34] C.L.J. Karlsson, J. Önnerfält, J. Xu, G. Molin, S. Ahrné, K. Thorngren-Jerneck, The microbiota of the gut in preschool children with normal and excessive body weight, Obesity 20 (2012) 2257–2261. <https://doi.10.1038/oby.2012.110>.

[35] S. Kim, Y.-C. Shin, T.-Y. Kim, Y. Kim, Y.-S. Lee, S.-H. Lee, M.-N. Kim, E. O, K.S. Kim, M.-N. Kweon, Mucin degrader *Akkermansia muciniphila* accelerates intestinal stem cell-mediated epithelial development, Gut. Microbes 13 (2021), 1892441. <https://doi.10.1080/19490976.2021.1892441>.

[36] J. Knezevic, C. Starchl, A. Tmava Berisha, K. Amrein, Thyroid-gut-axis: how does the microbiota influence thyroid function? Nutrients 12 (6) (2020) 1769, doi: 10.3390/nu12061769. PMID: 32545596; PMCID: PMC7353203.

[37] W.H. Kruskal, W.A. Wallis, Use of ranks in one-criterion variance analysis, J. Am. Stat. Assoc. 47 (1952) 583–621. <https://doi.10.1080/01621459.1952.10483441>.

[38] T. Li, X. Lin, B. Shen, W. Zhang, Y. Liu, H. Liu, Y. Wang, L. Zheng, F. Zhi, *Akkermansia muciniphila* suppressing nonalcoholic steatohepatitis associated tumorigenesis through CXCR6+ natural killer T cells, Front. Immunol. 13 (2022), 1047570. <https://doi.10.3389/fimmu.2022.1047570>.

[39] M.-J. Liu, J.-Y. Yang, Z.-H. Yan, S. Hu, J.-Q. Li, Z.-X. Xu, Y.-P. Jian, Recent findings in *Akkermansia muciniphila*-regulated metabolism and its role in intestinal diseases, Clin. Nutr. 41 (2022) 2333–2344. <https://doi.10.1016/j.clnu.2022.08.029>.

[40] J. Liu, H. Liu, H. Liu, Y. Teng, N. Qin, X. Ren, X. Xia, Live and pasteurized *Akkermansia muciniphila* decrease susceptibility to Salmonella Typhimurium infection in mice, J. Adv. Res. 52 (2023) 89–102. <https://doi.10.1016/j.jare.2023.03.008>.

[41] Y. Luo, C. Lan, H. Li, Q. Ouyang, F. Kong, A. Wu, Z. Ren, G. Tian, J. Cai, B. Yu, J. He, A.G. Wright, Rational consideration of *Akkermansia muciniphila* targeting intestinal health: advantages and challenges, Npj. Biofilms. Micro 8 (2022) 81. <https://doi.10.1038/s41522-022-00338-4>.

[42] X. Ma, M. Tian, X. Yu, M. Liu, B. Li, D. Ren, W. Wang, Characterization and preliminary safety evaluation of *Akkermansia muciniphila* PROBIO, Foods 13 (2024) 442. <https://doi.10.3390/foods13030442>.

[43] A.C. Midtvedt, B. Carlstedt-Duke, T. Midtvedt, Establishment of a mucin-degrading intestinal microflora during the first two years of human life, J. Pediatr. Gastroenterol. Nutr. 18 (1994) 321–326. <https://doi.10.1002/j.1536-4801.1994.tb11182.x>.

[44] K. Mortelmans, E. Zeiger, The Ames Salmonella/microsome mutagenicity assay, Mutat. Res. 455 (2000) 29–60. <https://doi.10.1016/S0027-5107(00)00064-6>.

[45] H. Motulsky, Intuitive Biostatistics: A Nonmathematical Guide to Statistical Thinking, Oxford University Press, Cary, NC, 2014.

[46] NCBI Resource Coordinators, Database resources of the National Center for Biotechnology Information, Nucleic Acids Res 44 (2016) D7–D19. <https://doi.10.1093/nar/gkv1290>.

[47] NHLBI, 2022. Cholesterol & Your Heart: What You Need to Know Fact Sheet. National Institutes of Health (NIH), National Heart, Lung, and Blood Institute (NHLBI), Bethesda, MD. <⟨https://www.nhlbi.nih.gov/health/blood-cholesterol⟩>.

[48] NHLBI, 2023. High Blood Triglycerides. National Institutes of Health (NIH), National Heart, Lung, and Blood Institute (NHLBI), Bethesda, MD. <⟨https://www.nhlbi.nih.gov/health/high-blood-triglycerides⟩>.

[49] V.I. Nikitenko, A.A. Stadnikov, V.A. Kopylov, Bacterial translocation from the gastrointestinal tract in healthy and injured rats, J. Wound Care 20 (2011) 114–122. <https://doi.10.12968/jowc.2011.20.3.114>.

[50] OECD, 1998. OECD Principles of Good Laboratory Practice. Organisation for Economic Co-Operation & Development (OECD), Environment Directorate, Chemicals Group and Management Committee, Paris, France. Series on Principles of Good Laboratory Practice and Compliance Monitoring, no. 1 [ENV/MC/CHEM (98)17]. <https://dx.doi.org/10.1787/9789264078536-en>.

[51] OECD, 2016a. *In Vitro* mammalian chromosome aberration test, in: OECD Guidelines for the Testing of Chemicals. Organisation for Economic Co-operation and Development (OECD), Paris, France. OECD Guideline, no. 473 [updated & adopted: 29 July 2016]. <https://dx.doi.org/10.1787/9789264224223-en>.

[52] OECD, 2016b. Mammalian erythrocyte micronucleus test, in: OECD Guidelines for the Testing of Chemicals. Organisation for Economic Co-operation and Development (OECD), Paris, France OECD Guideline, no. 474 [updated & adopted: 29 July 2016]. <⟨https://www.oecd-ilibrary.org/environment/test-no-474-mammalian-erythrocyte-micronucleus-test_9789264264762-en⟩>.

[53] OECD, 2018. Repeated dose 90-day oral toxicity study in rodents, in: OECD Guidelines for the Testing of Chemicals. Organisation for Economic Co-operation and Development (OECD), Paris, France. OECD Guideline no. 408 [updated & adopted: 27 June 2018]. <https://dx.doi.org/10.1787/9789264070707-en>.

[54] OECD, 2020. Bacterial reverse mutation test, in: OECD Guidelines for the Testing of Chemicals. Organisation for Economic Co-operation and Development (OECD), Paris, France. OECD Guideline no. 471 [updated & adopted: 26 June 2020]. <https://dx.doi.org/10.1787/9789264071247-en>.

[55] Z. Ou, L. Deng, Z. Lu, F. Wu, W. Liu, D. Huang, Y. Peng, Protective effects of *Akkermansia muciniphila* on cognitive deficits and amyloid pathology in a mouse model of Alzheimer's disease, Nutr. Diabetes 10 (2020) 12. <https://doi.10.1038/s41387-020-0115-8>.

[56] H. Plovier, A. Everard, C. Druart, C. Depommier, M. Van Hul, L. Geurts, J. Chilloux, N. Ottman, T. Duparc, L. Lichtenstein, A. Myridakis, N.M. Delzenne, J. Klievink, A. Bhattacharjee, K.C.H. Van Der Ark, S. Aalvink, L.O. Martinez, M.-E. Dumas, D. Maiter, A. Loumaye, M.P. Hermans, J.-P. Thissen, C. Belzer, W.M. De Vos, P. D. Cani, A purified membrane protein from *Akkermansia muciniphila* or the pasteurized bacterium improves metabolism in obese and diabetic mice, Nat. Med. 23 (2017) 107–113. <https://doi.10.1038/nm.4236>.

[57] C.W. Png, S.K. Lindén, K.S. Gilshenan, E.G. Zoetendal, C.S. McSweeney, L.I. Sly, M. A. McGuckin, T.H.J. Florin, Mucolytic bacteria with increased prevalence in ibd mucosa augment in vitro utilization of mucin by other bacteria, Am. J. Gastroenterol. 105 (2010) 2420–2428. <https://doi.10.1038/ajg.2010.281>.

[58] S.K.A. Raftar, F. Ashrafian, A. Yadegar, A. Lari, H.R. Moradi, A. Shahriary, M. Azimirad, H. Alavifard, Z. Mohsenifar, M. Davari, F. Vaziri, A. Moshiri, S. D. Siadat, M.R. Zali, The protective effects of live and pasteurized *Akkermansia muciniphila* and its extracellular vesicles against hfd/ccl4-induced liver injury, Microbiol. Spectr. 9 (2021) e00484-00421. <https://doi.10.1128/Spectrum.00484-21>.

[59] Y. Rao, Z. Kuang, C. Li, S. Guo, Y. Xu, D. Zhao, Y. Hu, B. Song, Z. Jiang, Z. Ge, X. Liu, C. Li, S. Chen, J. Ye, Z. Huang, Y. Lu, Gut *Akkermansia muciniphila* ameliorates metabolic dysfunction-associated fatty liver disease by regulating the metabolism of L-aspartate via gut-liver axis, Gut. Microbes 13 (2021), 1927633. <https://doi.10.1080/19490976.2021.1927633>.

[60] A. Santiago, M. Pozuelo, M. Poca, C. Gely, J.C. Nieto, X. Torras, E. Román, D. Campos, G. Sarrabayrouse, S. Vidal, E. Alvarado-Tapias, F. Guarner, G. Soriano, C. Manichanh, C. Guarner, Alteration of the serum microbiome composition in cirrhotic patients with ascites, Sci. Rep. 6 (2016) 25001. <https://doi.10.1038/srep25001>.

[61] S.S. Shapiro, M.B. Wilk, An analysis of variance test for normality (Complete samples), Biometrika 52 (1965) 591–611. <https://doi.10.1093/biomet/52.3-4.591>.

[62] J.D.A. Sharpen, B. Dolan, E.E.L. Nyström, G.M.H. Birchenough, L. Arike, B. Martinez-Abad, M.E.V. Johansson, G.C. Hansson, C.V. Recktenwald, Transglutaminase 3 crosslinks the secreted gel-forming mucus component Mucin-2 and stabilizes the colonic mucus layer, Nat. Commun. 13 (2022) 45. <https://doi.10.1038/s41467-021-27743-1>.

[63] Q. Shu, C. Kang, J. Li, Z. Hou, M. Xiong, X. Wang, H. Peng, Effect of probiotics or prebiotics on thyroid function: a meta-analysis of eight randomized controlled trials, PLoS One 19 (1) (2024) e0296733 doi: 10.1371/journal.pone.0296733. PMID: 38206993; PMCID: PMC10783727.

[64] J. Si, H. Kang, H.J. You, G. Ko, Revisiting the role of *Akkermansia muciniphila* as a therapeutic bacterium, Gut. Microbes 14 (2022), 2078619. <https://doi.10.1080/19490976.2022.2078619>.

[65] J.D. Söderholm, M.H. Perdue, II. Stress and intestinal barrier function, Am. J. Physiol. Gastrointest. Liver Physiol. 280 (2001) G7–G13. <https://doi.10.1152/ajpgi.2001.280.1.G7>.

[66] T.F.S. Teixeira, Ł.M. Grześkowiak, S. Salminen, K. Laitinen, J. Bressan, M.D. C. Gouveia Peluzio, Faecal levels of *Bifidobacterium* and *Clostridium coccoides* but not plasma lipopolysaccharide are inversely related to insulin and HOMA index in women, Clin. Nutr. 32 (2013) 1017–1022. <https://doi.10.1016/j.clnu.2013.02.008>.

[67] D. Traykova, B. Schneider, M. Chojkier, M. Buck, Blood microbiome quantity and the hyperdynamic circulation in decompensated cirrhotic patients, PLoS. One 12 (2017) e0169310. <https://doi.10.1371/journal.pone.0169310>.

[68] U.S. FDA, 2000a. C. Guidelines for specific toxicity studies. IV.C.1.a. Bacterial reverse mutation test. In: Toxicological Principles for the Safety Assessment of Food Ingredients: Redbook 2000 (Updated to July, 2007). U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Silver Spring, MD. <⟨https://www.fda.gov/regulatory-information/search-fda-guidance-documents/redbook-2000-ivc1a-bacterial-reverse-mutation-test⟩> (July 2000).

[69] U.S. FDA, 2000b. C. Guidelines for specific toxicity studies. IV.C.1.d. Mammalian erythrocyte micronucleus test, in: Toxicological Principles for the Safety Assessment of Food Ingredients: Redbook 2000: Guidance for Industry and Other Stakeholders [Updated to July 2007]. U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Silver Spring, MD. <⟨https://www.fda.gov/regulatory-information/search-fda-guidance-documents/redbook-2000-ivc1d-mammalian-erythrocyte-micronucleus-test⟩> (July 2000).

[70] U.S. FDA, 2003. C. Guidelines for specific toxicity studies. IV.C.4.a. Subchronic toxicity studies with rodents, in: Toxicological Principles for the Safety Assessment of Food Ingredients: Redbook 2000: Guidance for Industry and Other Stakeholders [Updated to July 2007]. U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Silver Spring, MD. <⟨https://www.fda.gov/regulatory-information/search-fda-guidance-documents/redbook-2000-ivc4a-subchronic-toxicity-studies-rodents⟩>. (November 2003).

[71] F. Van Herreweghen, K. De Paepe, M. Marzorati, T. Van de Wiele, Mucin as a functional niche is a more important driver of in vitro gut microbiota composition and functionality than supplementation of *Akkermansia muciniphila*, Appl. Environ. Microbiol. 87 (2021) e02647-02620. <https://doi.10.1128/aem.02647-20>.

[72] C. Virili, I. Stramazzo, M.F. Bagaglini, A.L. Carretti, S. Capriello, F. Romanelli, P. Trimboli, M. Centanni, The relationship between thyroid and human-associated microbiota: a systematic review of reviews, Rev. Endocr. Metab. Disord. 25 (1) (2023) 215–237, doi: 10.1007/s11154-023-09839-9. Epub 2023 Oct 12. PMID: 37824030; PMCID: PMC10808578.

[73] M. Wang, S. Ahrné, B. Jeppsson, G. Molin, Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes, Fems Microbiol. Ecol. 54 (2005) 219–231. <https://doi.10.1016/j.femsec.2005.03.012>.

[74] F. Wu, X. Guo, M. Zhang, Z. Ou, D. Wu, L. Deng, Z. Lu, J. Zhang, G. Deng, S. Chen, S. Li, J. Yi, Y. Peng, An *Akkermansia muciniphila* subtype alleviates high-fat diet-induced metabolic disorders and inhibits the neurodegenerative process in mice, Anaerobe 61 (2020) 102138. <https://doi.10.1016/j.anaerobe.2019.102138>.

[75] H.S. Yoon, C.H. Cho, M.S. Yun, S.J. Jang, H.J. You, J.-h Kim, D. Han, K.H. Cha, S. H. Moon, K. Lee, Y.-J. Kim, S.-J. Lee, T.-W. Nam, G. Ko, *Akkermansia muciniphila* secretes a glucagon-like peptide-1-inducing protein that improves glucose homeostasis and ameliorates metabolic disease in mice, Nat. Microbiol. 6 (2021) 563–573. <https://doi.10.1038/s41564-021-00880-5>.

[76] M. Zareie, K. Johnson-Henry, J. Jury, P.C. Yang, B.Y. Ngan, D.M. McKay, J. D. Soderholm, M.H. Perdue, P.M. Sherman, Probiotics prevent bacterial translocation and improve intestinal barrier function in rats following chronic psychological stress, Gut 55 (2006) 1553–1560. <https://doi.10.1136/gut.2005.080739>.

[77] X. Zhang, D. Shen, Z. Fang, Z. Jie, X. Qiu, C. Zhang, Y. Chen, L. Ji, Human gut microbiota changes reveal the progression of glucose intolerance, PLoS. ONE 8 (2013) e71108. <https://doi.10.1371/journal.pone.0071108>.

[78] T. Zhang, Q. Li, L. Cheng, H. Buch, F. Zhang, *Akkermansia muciniphila* is a promising probiotic, Microb. Biotechnol. 12 (2019) 1109–1125. <https://doi.10.1111/1751-7915.13410>.

[79] K. Zhou, Strategies to promote abundance of *Akkermansia muciniphila*, an emerging probiotics in the gut, evidence from dietary intervention studies, J. Funct. Foods 33 (2017) 194–201. <https://doi.10.1016/j.jff.2017.03.045>.