Toxicology Reports 14 (2025) 102042

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Safety evaluation of *Akkermansia massiliensis* sp. nov. DSM 33459

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ARTICLE INFO

Keywords:
Subchronic toxicity
Akkermansia massiliensis sp. nov. DSM 33459 (AkkerBalance™)
Safety
Probiotics
Rats

ABSTRACT

A novel strain of *Akkermansia massiliensis* sp. nov., designated as DSM 33459, was isolated from the feces of a healthy human donor. In order to fully assess the safety of this strain, following previously performed full genomic assessment, further *in-vitro* characterization and a combined *in-vivo* subchronic 28-day and 90-day toxicity study is reported herein. *A. massiliensis* DSM 33459 is tolerant to bile, somewhat tolerant to gastric juice pH conditions, and does not exhibit any aspects of virulence. This strain also demonstrates the ability to engraft the gastrointestinal tract of rats, persisting with continuous administration of the strain until the end of the study. Exposure to 2000 mg/kg BW/day *A. massiliensis* DSM 33459 did not produce any evidence of toxicity after either 28- or 90-days of exposure and did not translocate across the gastrointestinal barrier. Therefore, the NOEL for *A. massiliensis* DSM 33459, administered for 28- or 90-days, was determined to be the limit dose at 2000 mg/kg/day in male and female rats, a level which meets or exceeds calculated dose equivalent of 5.62×10^{11} CFU/kg/day.

1. Introduction

The genus *Akkermansia*, belonging to the Verrucomicrobiota phylum of Gram-negative bacteria, was first described by Derrien *et al.* in 2004. After isolation of a mucin-degrading strain from a healthy adult, it was named *Akkermansia muciniphila* and became the type strain MucT (=ATCC BAA-835) for the species [12]. *A. muciniphila* has since been identified as present in the infant gut microbiome [7] and also in breastmilk of lactating women [8,23]. Maternal transfer was specifically demonstrated by Ferretti et al. [19] to the infant via breastmilk, as the same strain of an uncharacterized *Akkermansia* was found in both mother and infant gut microbiomes.

In some studies, *Akkermansia* has been identified as associated with resilience and longevity in animals [2] and with a lean body type in humans [48]. Specifically, the human gut commensal *A. muciniphila* has gained attention in recent years due to its potential role in metabolic health and as a next generation probiotic for human health [4]. Two human studies have demonstrated that *A. muciniphila* ingestion is safe and may improve certain metabolic parameters in overweight and obese human volunteers [9] and in patients with type 2 diabetes undergoing treatment with metformin when administered as part of a mixture with butyrate-producing commensal bacteria and inulin [41]. Furthermore,

Abbreviations: AAALAC, American Association for Accreditation of Laboratory Animal Care; AMas, *Akkermansia massiliensis* sp. nov. DSM 33459; ANOVA, analysis of variance; APTT, Activated partial thromboplastin time; ATCC, American Type Culture Collection.; BHI, brain heart infusion; BMI, body mass index; BW, body weight; CFU, Colony forming unit; DIO, diet induced obesity; DSM, Deutsche Sammlung von Mikroorganismen; EFSA, European Food Safety Authority; EU, European Union; FOB, Functional Observational Battery; GANI, genome-wide Average Nucleotide Identity; GLP, Good Laboratory Practices; NOEL, No Observed Effect Level; NOAEL, No Observed Adverse Effect Level; PBS, Phosphate Buffered Saline; PT, Prothrombin time; rRNA V4, ribosomal RNA variable region 4; YCFAC, yeast casitone fatty acids agar with carbohydrates.

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https://doi.org/10.1016/j.toxrep.2025.102042
Received 30 March 2025; Received in revised form 29 April 2025; Accepted 1 May 2025
Available online 4 May 2025

Table 1
Quantification of D-/L-Lactate in culture supernatant.

	D-Lactate g/L	L-Lactate g/L
***A. massiliensis* DSM 33459**	0.008	0.003
Positive Control 3.75 ug each D/L lactate 1:1	0.048	0.049
Positive Control 7.5 ug each D/L lactate 1:1	0.095	0.088
Negative Control	−0.005	0

Garcia-Gamboa et al. [20] recently assessed the intestinal bacteriota of healthy individuals with a range of BMI levels. Uniquely, the study identified higher levels of *A. muciniphila* in overweight and obese participants compared to those with a healthy weight and lower BMI. Interestingly, of the overweight and obese populations, those with an abundance of *A. muciniphila* demonstrated statistically significant improvements in their glucose and lipid profiles versus those with a lower abundance, indicating the significance of the role of *A. muciniphila* in metabolic health.

Pasteurized *A. muciniphila* has been assessed for safety [13,18] and was subsequently authorised as a Novel Food in the EU [37]. In addition, a safety evaluation including acute and sub-chronic toxicity testing of a live strain of *A. muciniphila* has been reported and a no-observed-adverse-effect level (NOAEL) proposed at 6.4×10^{11} viable bacteria for an average adult individual weighing 70 kg [29].

Further exploration into the genomic diversity of *A. muciniphila* using metagenomic data sets, has revealed diversity within the genus *Akkermansia* in the human gut [21,25]. Species variety within the genus of *Akkermansia* has been identified, which includes *Akkermansia* strain DSM 33459 (AkkerBalance™), isolated from the human feces of a healthy donor and demonstrated to be distinct from *A. muciniphila* [26,27]. Ndongo *et al.* also proposed a new *Akkermansia* species based on a human gut isolate and named it *A. massiliensis* sp. nov., with strain Marseille-P6666T (= CSUR P6666 = CECT 30548) as the type strain [34]. Alignment of the genome of *Akkermansia* strain DSM 33459 showed 99.85 % gANI similarity to the genome of the type strain *A. massiliensis* sp. nov. Marseille-P6666 (NCBI RefSeq: GCF_023516715.1), indicating that strain DSM 33459 also belongs to the proposed new species *A. massiliensis* sp. nov. [24]. Since that time, *Akkermansia massiliensis* sp. nov. has been recognized as the second most prevalent *Akkermansia* species in the human gut with unique properties and potential relevance for human health [26,31]. The strain *A. massiliensis* DSM 33459 has previously been studied as *Akkermansia* sp. DSM 33459 and will be commercialized under the name AkkerBalance™.

Akkermansia strain DSM 33459, belonging to the proposed, but not taxonomically validated species *A. massiliensis* sp. nov., has been characterized to understand its role in human health and safety profile as a potential next generation probiotic. Kumar et al. [27] demonstrated in a pre-clinical animal model of diet-induced obesity, that *A. massiliensis* DSM 33459 oral administration significantly improves body weight, total fat weight, insulin and resistin levels after administration for 12 weeks, particularly when administered as live culture. In addition, the antibiotic resistance profile of *A. massiliensis* DSM 33459 was described and its genomic safety assessment showed no evidence of any known acquired antibiotic resistance genes, virulence factors, hemolysin genes, toxins, or biogenic amines of concern [27].

In order to comprehensively assess the safety of *A. massiliensis* DSM 33459, this strain was further characterized and evaluated for potential hemolytic activity, lactate production and acid/bile tolerance. Any potential sub-chronic activity was also investigated using a combined 28-day and 90-day oral toxicity study in rats and included translocation analysis. The ability of this strain to engraft the gastrointestinal tract has also been assessed in this study. The results of this comprehensive analysis are described herein.

2. Materials and methods

2.1. *A. massiliensis DSM 33459 substance information*

2.1.1. *In vitro testing for characterization and safety analysis*

For *in vitro* testing, media and consumables were deoxygenated for 48 h, and all work was performed in an anaerobic chamber. *A. massiliensis* DSM 33459 was cultured onto Brucella blood agar to test for hemolytic activity. A commercial assay kit was used to determine D-/L-Lactate production from the culture supernatant following the manufacturer's instructions (D-/L-Lactate Rapid Assay kit, Megazyme, Chicago, IL). The survival rate of *Akkermansia* sp. DSM 33459 following gastric acid and bile salt challenge was tested by resuspending the cell pellet in Butterfield's Phosphate Buffer after 48 h of growth in YCFAC broth with mucin. A 1:100 dilution of resuspended cells with modified gastric juice (0.32 % wt/v pepsin + 0.2 % wt/v NaCl, pH 3.5) was made (T0) and then incubated for 1 h (T1). For bile salt tolerance testing, a final concentration 0.3 % Difco™ Oxgall was used (T0). Three serial dilutions (6 replicates) of each with or without gastric acid or bile salt

Table 3
Grip strength and foot splay after 28- and 90-days of exposure, males and females.

Study Day		Control Male	AMas Male	Control Female	AMas Female
Mean Fore Limb Grip Strength (kg)	26 (28-days)	0.72 ± 0.1407	0.71 ± 0.0458	0.56 ± 0.1701	0.65 ± 0.1611
	81 (90-days)	0.69 ± 0.1456	0.94 ± 0.2109*	0.62 ± 0.2301	0.54 ± 0.1463
Mean Hind Limb Grip Strength (kg)	26 (28-days)	0.46 ± 0.0983	0.46 ± 0.0999	0.36 ± 0.0677	0.45 ± 0.1471
	81 28-days	0.64 ± 0.0436	0.67 ± 0.0508	0.39 ± 0.0727	0.47 ± 0.1021
Mean Foot Splay (cm)	26 (28-days)	10.2 ± 1.70	8.8 ± 0.87	9.4 ± 2.90	9.3 ± 1.59
	81 28-days	13.4 ± 1.65	11.2 ± 2.12	8.1 ± 1.62	7.6 ± 2.51

N = 5/sex at each endpoint
* $p < 0.05$

Table 2
Cell survival under acid or bile challenge conditions.

Condition[1]	Average Colony Counts (mean n = 6)			Number colonies per 100 µL	Number colonies per 1 mL	Dilution Factor	% Survival
	10^{-3}	10^{-4}	10^{-5}				
T0, Control	176.33	12.83	0.83	176.33	1763.30	1.76 × 106	100.00
T0, Bile treatment	135.33	11.17	0.83	135.33	1353.3	1.35 × 106	76.7
T1, Acid treatment	17.67	1.5	0.17	17.67	176.7	1.77 × 105	10.1

[1]Conditions: T0, Control = Pour plated with YCFAC + mucin agar; T0, Bile treatment = Pour-plated with YCFAC + mucin agar supplemented with 0.3 % Oxgall bile salt; T1, Acid treatment = 1 hour incubation with modified gastric juice then pour-plated with YCFAC + mucin agar

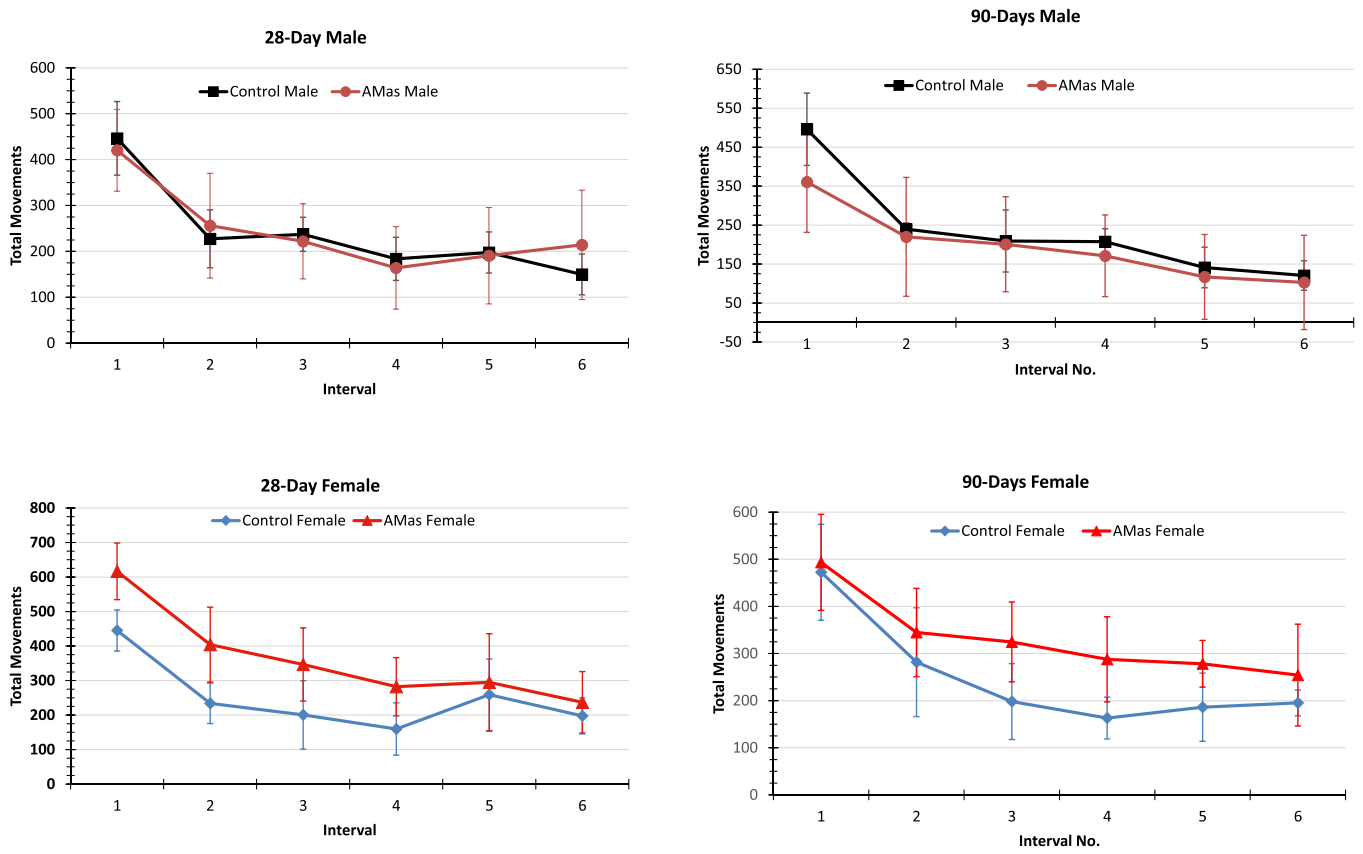

Fig. 1. Motor activity measurements of male and female rats in after 28-Days and 90-days of AMas exposure (N = 5/sex/group).

were pour plated with YCFAC agar with mucin, and colonies were enumerated after 72 h incubation at 37°C.

2.2. 28- and 90-day repeated dose study in rats

The 28- and 90-day study was conducted in accordance with Good Laboratory Practice (GLP) regulations (US FDA, 21 CFR 58; OECD, ENV/MC/CHEM(98)17), except for the translocation analysis. The test facility was accredited by AAALAC International, and the protocol was reviewed and approved by the appropriate test facility animal welfare authorities prior to study initiation.

2.2.1. *A. massiliensis DSM 33459 concentrate*

A. massiliensis DSM 33459 probiotic strain was propagated using anaerobic fermentation. An optimized blend of nutrients, including proteins, carbohydrates, vitamins, and minerals are mixed with water, sterilized, and inoculated with *A. massiliensis* DSM 33459. Following fermentation, the media was removed by centrifugation and the resulting cell slurry was freeze-dried.

2.2.2. *Test system and animal husbandry*

The Sprague-Dawley® rat was the system of choice as historically, it has been the preferred and most commonly used species for oral toxicity tests. Forty-five male and 45 female experimentally naïve Sprague-Dawley CD® IGS rats were obtained from Charles River Laboratories, Inc. (Raleigh, NC USA). The animals were between 6 and 7 weeks of age at study initiation and individual weight variation did not exceed ± 20 % of the mean weight of each sex.

The animals were individually housed in cages which conformed to the size recommendations in the latest *Guide for the Care and Use of Laboratory Animals* [33]. Each animal was given a sequential number in addition to being uniquely identified with a Monel® self-piercing stainless steel ear tag. Litter paper placed beneath the cage was changed at least three times/week. The animal room had a 12-hour light/dark cycle and environmental controls were set to maintain temperature and relative humidity ranges of 21 ± 2°C and 30–70 %, respectively. The animals were acclimated to the housing facilities for at least five days prior to testing and released from quarantine based on acceptable health status. Feed, 2016 Certified Envigo Teklad Global Rodent Diet® (Envigo Teklad, Inc.) and filtered tap water were available *ad libitum* during acclimation and throughout the study. There were no known contaminants found in the food or water that would interfere with the results of this study.

2.2.3. *Study design*

The overall study design was based on the OECD and US FDA guidelines for toxicity studies in rodents [36,45]. While the overall study design followed those standard 90-day guideline studies, additional animals were included in each group for evaluation after 28-days of exposure to *A. massiliensis* DSM 33459 test concentrate. When there are no expectations of toxicity, these guidelines allow a limit-test, which means only one test group at a maximum exposure of 5 % in the diet or equivalent to 1000 mg/kg BW/day is evaluated. However, because of the intended use and that *Akkermansia* is a commensal intestinal bacterium in humans, the maximum dose (only limited by formulation viscosity) of 2000 mg/kg BW/day was used in this study.

Animals were randomized according to stratification by body weight into 2 groups of 15 males and 15 females, with body weights within 20 % of the mean for each sex. Within each group, 5 animals/sex were assigned to the 28-day arm and 10 animals/sex were assigned to the 90-day arm.

2.2.4. *Dose formulation preparation and procedures*

The *A. massiliensis* DSM 33459 test material was supplied in

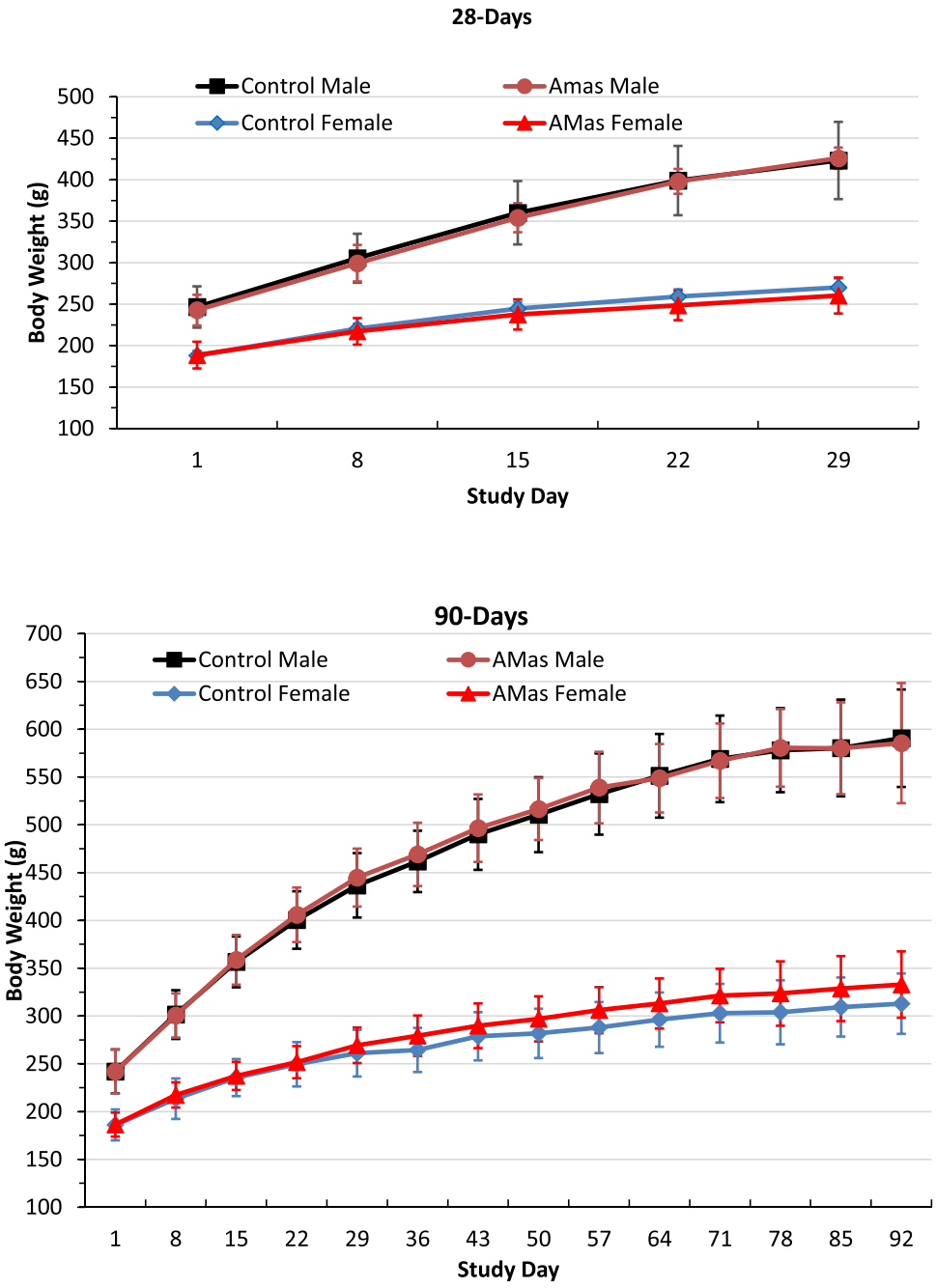

Fig. 2. Body weights of male and female rats after 28-Days (N = 5/sex/group) and 90 Days (N = 10/sex/group) of AMas exposure.

individual sachets, and formulations were made under anaerobic conditions daily. Sachets were allowed to equilibrate to room temperature for approximately 30 minutes. The *A. massiliensis* DSM 33459 test material was mixed weight to volume (w/v) in sterile, anaerobic phosphate buffered saline (PBS, Mediatech, Inc., Manassas, VA), supplemented with 0.05 % L-cysteine (Thermo Fisher Scientific, Ward Hill, MA), under anaerobic conditions. The Control group received PBS supplemented with 0.05 % L-cysteine. The formulations were stirred at ambient temperature until a visually homogeneous mixture was achieved. Individual doses were calculated based on the most recent weekly body weights and were adjusted each week to maintain the targeted dose level for all rats (i.e., mg/kg BW/day). All doses were administered at 10 mL/kg. The control group received the vehicle only, at the same dose volume as the test animals. Each animal was dosed by oral gavage using a stainless-steel ball-tipped gavage needle attached to a syringe. Dose administration occurred daily (7 days/week). The dose solutions and/or individual syringes were maintained in anaerobic conditions during dose administration. The first day of administration was considered Day 1 of the study.

Prior to initial dosing on Day 1 of the study, samples from the dose preparation were collected from the top, middle, and bottom for each concentration. The vehicle control mixture was sampled from the middle of the dose preparation only. Dose preparations were also sampled at the middle and end of the study for verification of dose concentration. Dose preparations were verified for viable CFU content at the beginning, middle and end of the study according to the following method (Section 2.2.5).

2.2.5. *Enumeration of A. massiliensis DSM 33459 dosing solutions*

Anaerobic PBS + L-cysteine (90 mL) were added to a sterile bottle in

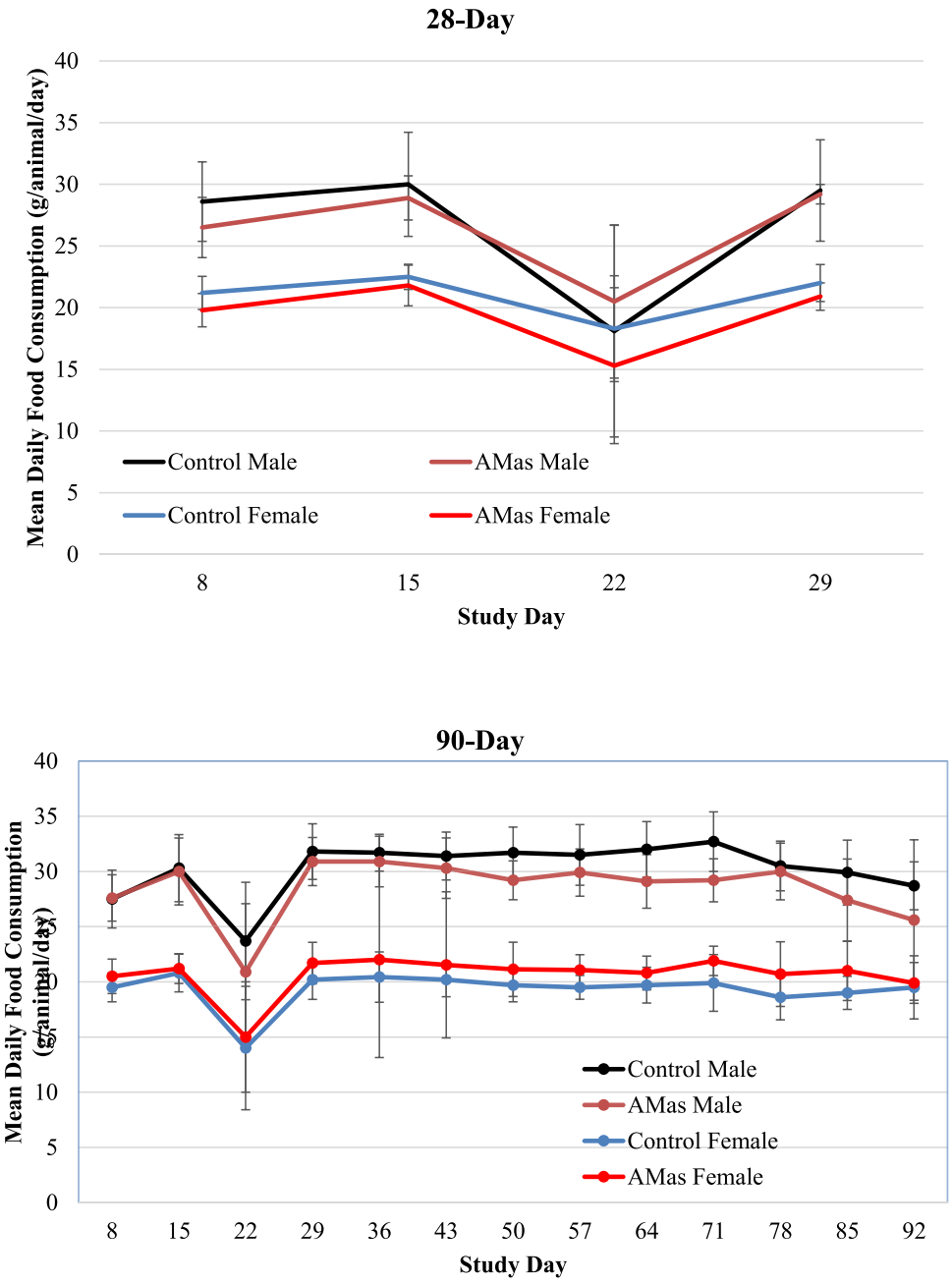

Fig. 3. Food Consumption of male and female rats after 28-Days (N = 5/sex/group) and 90 Days (N = 10/sex/group) of AMas exposure.

a Bactron EZ anaerobic chamber. In the anaerobic chamber, five anaerobic BHI plates were inoculated with 100 µL of the dosing formulation. Plates were inverted and incubated in the anaerobic chamber at 37 ± 2°C for 9 days. Colonies were hand counted and CFU/mL determined using the following formula:

CFU/mL = (Average # of colonies)x(dilution factor)x1000µL/volume per plate)

For homogeneity determination, 0.1 mL of the dosing formulation from the top, middle and bottom of the formulation container was serially diluted with PRAS diluent to final dilutions of 10^{-7}, 10^{-8} and 10^{-9}. 100 µL of each dilution was plated onto 5 anaerobic BHI plates, allowed to dry, inverted and incubated anaerobically in Bactron EZ chamber at 37 ± 1°C for 9 days. CFU/mL were determined as described above.

2.2.6. Experimental variables

Animal Observations: During the acclimation period, the eyes of all rats were examined by focal illumination, indirect ophthalmoscopy and, when indicated, slit-lamp microscopy. Mydriatic eye drops were

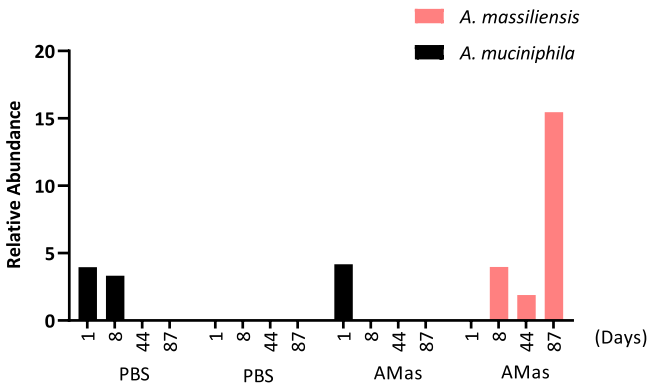

Fig. 4. Engraftment of native *A. muciniphila* in the rat gastrointestinal tract following oral gavage with *A. massiliensis* DSM 33459 (AMas) or PBS.

administered prior to ophthalmoscopy and the eyes were examined in subdued light. These procedures were repeated on all surviving test animals prior to test termination. All animals were observed at least twice daily for viability. Cage-side observations of all animals were performed daily during the study. Potential clinical signs of toxicity included, but were not limited to: changes in skin, fur, eyes, and mucous membranes; occurrence of secretions and excretions; autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern); changes in gait, posture and response to handling as well as the presence of clonic or tonic movements; stereotypies (e.g., excessive grooming, repetitive circling), and bizarre behavior (e.g., self-mutilation, walking backwards).

Functional Observational Battery (FOB): Once at the end of each exposure period, all rats/sex/group for the 28-day time point and a randomly selected 5 rats/sex/group for the 90-day time point received a FOB evaluation. Each rat was evaluated during handling and while in an open field for excitability, autonomic function, gait, and sensorimotor coordination (open field and manipulative evaluations), reactivity and sensitivity (elicited behavior), and other abnormal clinical signs, (e.g., convulsions, tremors unusual or bizarre behavior emaciation, dehydration, and general appearance. The rats were observed in random order and without the observer having knowledge of the treatment group. In addition to the above observations, Forelimb and Hindlimb Grip Strength and Foot Splay measurements were obtained.

Motor activity (MA) was evaluated with FOB assessments for the same animals. Activity was monitored using an automated Photobeam Activity System® (San Diego Instruments, Inc.). The system monitored up to twenty animals during one session. An equal number of animals assigned to Motor Activity assessment from all dose groups were evaluated in each session, when possible. Each animal was placed into a polycarbonate solid bottom cage and the evaluation phase began immediately for that animal. Each animal was evaluated for a single one-hour phase, with photobeam count accumulated over six 10-minute intervals.

Body Weight: Individual body weights were recorded at least two times during acclimation. Animals assigned to the study were weighed on Day 1 (prior to study start) and weekly thereafter. The animals were also weighed immediately prior to necropsy in order to calculate organ-to-body weight ratios. Body weight gain was calculated for selected intervals and for the overall study period.

Food Consumption: Food consumption was measured and recorded corresponding with body weight measurements, e.g., two times during acclimation and weekly thereafter on the same days body weights were recorded.

Clinical Pathology: Hematology, coagulation, clinical chemistry and urinalysis evaluations were performed at the end of their respective points of terminal sacrifice (after 28 days of administration or after 90 days of administration). Animals were fasted overnight prior to blood collection. Blood samples were collected from the sublingual vein or vena cava/abdominal aorta, under isoflurane anesthesia. Hematology samples (~500 µL) were collected in tubes containing K_2EDTA; coagulation samples (~1.8 mL) were collected in tubes containing 3.2 % sodium citrate; clinical chemistry samples (~1000 µL) were collected in tubes containing no preservatives. Where appropriate samples were centrifuged to separate plasma or serum. For urinalysis evaluation, rats were placed in metabolism cages on the day prior to blood collection and urine was collected overnight. The following parameters were evaluated:

Hematology - hematocrit, hemoglobin, mean corpuscular hemoglobin, platelet count, red blood cell count, red cell distribution width, reticulocyte count, white blood cell count (total and absolute differential).

Coagulation - activated partial thromboplastin time and prothrombin time.

Clinical Chemistry - alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, bilirubin (total), creatinine, cholesterol (total), electrolytes (calcium, chloride, potassium, sodium), glucose (fasting), gamma glutamyl transferase, globulin and A/G (albumin/globulin) ratio (calculated), lipoprotein (LD & HD), phosphorus, protein (total), sorbitol dehydrogenase, triglycerides, urea nitrogen.

Urinalysis - bilirubin, blood, color and clarity, glucose, ketones, microscopy of centrifuged sediment, pH, protein (total), quality, specific gravity, volume, urobilinogen.

Anatomic Pathology: At the respective terminal sacrifices, all animals were euthanized by exsanguination under isoflurane anesthesia. Randomly selected animals in the study (5 animals/sex/group at 28-days and 10 animals/sex/group at 90-days, including decedents) were subjected to a full necropsy, which included examination of the external surface of the body, all orifices, musculoskeletal system and the thoracic, abdominal, pelvic and cranial cavities and their contents. The following tissues were weighed as soon as possible after dissection to avoid drying; adrenals (combined), brain, epididymides (combined), heart, kidneys (combined), liver, ovaries with oviducts (combined), testes (combined), spleen, thymus, uterus. The pituitary, thyroid/parathyroid and prostate and seminal vesicles with coagulating gland were weighed after 24 hours in 10 % neutral buffered formalin. The following tissues were preserved in 10 % neutral buffered formalin (exceptions indicated below) for histopathological evaluation: accessory genital organs (prostate and seminal vesicles), adrenals, all gross lesions, aorta, bone (femur), bone marrow (femur and sternum), brain (medulla/pons, cerebellar and cerebral cortex), cecum, cervix, colon, duodenum, esophagus, Harderian gland, heart, ileum with Peyer's patches, jejunum, kidneys, larynx, liver, lungs, lymph node (mandibular and mesenteric), mammary gland, nasal turbinates, nose, ovaries, oviducts, pancreas, parathyroid, peripheral nerve (sciatic), pharynx, pituitary gland, rectum, salivary glands (sublingual submandibular, and parotid), skeletal muscle, skin, spinal cord (cervical, mid-thoracic and lumbar), spleen, sternum, stomach, thymus, thyroid, trachea, urinary bladder, uterus, vagina. The eyes, epididymides, optic nerve and testes were preserved in modified Davidson's fixative and then stored in ethanol.

Histopathology: Histological examination was performed on the preserved organs and tissues of the animals from the control and *A. massiliensis* DSM 33459 test groups on animals necropsied at both 28 and 90 days. The fixed tissues (fixation described above) were trimmed, processed, embedded in paraffin, sectioned with a microtome, placed on glass microscope slides, stained with hematoxylin and eosin and examined by light microscopy. Both gross-to-microscopic correlations, and the incidence of microscopic findings were recorded. Grading criteria for microscopic observations were: Normal, finding not present; Minimal, a focal, subtle, or trivial change; Mild, an easily identifiable change of limited severity and/or distribution; Moderate, an obvious change with normal tissue remaining; Marked, an extensive change that obliterates much of the normal tissue; Severe, a maximal change, and Present, a finding for which grading is not appropriate. The anatomic

Table 4
Clinical pathology after 28- or 90-days of exposure, males and females.

	28-days				90-days			
	Control Male	AMas Male	Control Female	AMas Female	Control Male	AMas Male	Control Female	AMas Female
Absolute Basophils	0.078 ± 0.058	0.060 ± 0016	0.046 ± 0.015	0.034 ± 0.017	0.036 ± 0.025	0.059 ± 0025*	0.027 ± 0.021	0.043 ± 0.025
Absolute Eosinophils	0.110 ± 0.065	0.100 ± 0053	0.062 ± 0.028	0.068 ± 0.038	0.134 ± 0.057	0.167 ± 0073	0.123 ± 0.07	0.097 ± 0.034
Absolute Leucocytes	0.078 ± 0.036	0.076 ± 0049	0.054 ± 0.018	0.058 ± 0.035	0.069 ± 0.032	0.089 ± 0.041	0.061 ± 0.018	0.077 ± 0.041
Absolute Lymphocytes	8.27 ± 3.829	8.054 ± 2.095	6.724 ± 1.741	5.038 ± 1.617	7.52 ± 1.845	8.611 ± 2.924	5.447 ± 1.383	6.372 ± 2.605
Absolute monocytes	0.234 ± 0.190	0.192 ± 0.128	0.152 ± 0.077	0.140 ± 0.122	0.296 ± 0.186	0.394 ± 0.205	0.194 ± 0.114	0.265 ± 0.135
Absolute neutrophils	1.324 ± 0.844	1.004 ± 0.358	0.734 ± 0.356	0.644 ± 0.241	1.407 ± 0.464	1.882 ± 0.604	0.938 ± 0.618	1.011 ± 0.780
Absolute reticulocytes	185.3 ± 28.88	163.5 ± 28.96	124.9 ± 28.26	135.9 ± 13.38	208.2 ± 46.76	182.7 ± 48.67	148.8 ± 13.76	140.0 ± 29.82
Hematocrit	51.6 ± 2.68	53.3 ± 0.89	50.5 ± 1.76	50.8 ± 4.56	50.8 ± 3.09	53.4 ± 3.76	49.4 ± 3.75	50.1 ± 3.35
Hemoglobulin	15.3 ± 0.55	16.14 ± 0.21*	15.28 ± 0.43	15.58 ± 1.15	19.96 ± 0.94	15.27 ± 0.99	14.9 ± 0.92	15.0 ± 0.89
MCV	62.86 ± 1.25	61.42 ± 0.91	61.4 ± 1.40	59.5 ± 2.09	56.61 ± 2.69	57.39 ± 2.04	59.5 ± 1.57	59.1 ± 1.31
MCH	18.66 ± 0.	18.48 ± 0.63	18.6 ± 0.41	18.3 ± 0.70	16.66 ± 0.97	16.80 ± 0.60	17.9 ± 0.65	17.7 ± 0.49
MCHC	29.70 ± 0.60	30.14 ± 0.89	30.3 ± 0.73	30.7 ± 0.68	29.40 ± 0.78	29.15 ± 0.74	30.1 ± 0.63	29.9 ± 0.68
Platelets	781 ± 445.05	796.4 ± 456.17	839 ± 109.4	601 ± 374.5	902.2 ± 86.92	1016.7 ± 301.68	803 ± 317.8	859 ± 121.6
Red Blood Cells	8.206 ± 0.390	8.736 ± 0.319	8.236 ± .033	8.53 ± 0.572	8.996 ± 0.408	9.136 ± 0.584	8.332 ± 0.666	8.486 ± 0.616
RDW	12.64 ± 0.11	12.10 ± 0.51	11.28 ± 0.33	11.38 ± 0.23	14.84 ± 1.29	14.66 ± 1.15	12.73 ± 0.43	12.55 ± 0.29
White Blood Cells	10.09 ± 4.99	9.488 ± 2.561	7.77 ± 1.964	5.982 ± 1.830	9.463 ± 2.476	11.198 ± 3.005	6.786 ± 1.491	7.867 ± 3.276
APTT (seconds)	15.50 ± 1.76	22.58 ± 7.64*	13.54 ± 0.92	17.12 ± 3.04	17.94 ± 1.73	15.47 ± 1.45**	15.58 ± 3.42	16.64 ± 1.32
PT (seconds)	9.68 ± 0.40	9.84 ± 0.30	8.90 ± 0.14	9.28 ± 0.34	10.10 ± 0.27	9.87 ± 0.26	9.41 ± 0.15	9.04 ± 0.38*
Alanine aminotransferase (U/L)	53.5 ± 46.6	31.0 ± 04.4	31.8 ± 8.9	51.2 ± 39.6	29.0 ± 4.8	28.2 ± 0.4.3	41.2 ± 20.1	82.9 ± 86.45
Albumin (g/dL)	3.75 ± 0.25	3.88 ± 0.13	5.06 ± 0.47	4.88 ± 0.42	4.05 ± 0.20	3.86 ± 0.36	5.41 ± 0.56	5.25 ± 0.45
Alkaline Phosphatase (U/L)	168.0 ± 0.38.3	14.47 ± 0.30.0	77.0 ± 16.1	83.8 ± 21.6	83.3 ± 8.5	100.6 ± 50.8	36.3 ± 9.4	34.6 ± 8.5
Aspartime transferase (U/L)	151.5 ± 93.2	97.2 ± 0.16.6	104.0 ± 42.7	152.8 ± 83.1	107.6 ± 0.18.6	100.6 ± 0.20.3	149.7 ± 102.0	318.4 ± 516.0
Calcium (mg/dL)	11.23 ± 0.56	11.10 ± 0.0.61	11.68 ± 0.40	11.52 ± 0.66	11.00 ± 0.57	11.01 ± 0.83	11.95 ± 0.74	11.56 ± 0.61
CL (mmol/L)	99.83 ± 1.86	99.00 ± 1.95	99.04 ± 1.25	100.70 ± 2.26	99.87 ± 0.95	98.89 ± 1.36	98.77 ± 0.93	97.94 ± 2.26
Cholesterol (mg/dL)	70.8 ± 27.0	63.2 ± 0.94	87.2 ± 16.0	94.8 ± 24.3	81.8 ± 18.2	71.6 ± 12.1	105.3 ± 18.6	125.0 ± 45.5
Creatinine (mg/dL)	0.185 ± 0.024	0.192 ± 0.019	0.300 ± 0.052	0.25 ± 0.020	0.253 ± 0.051	0.259 ± 0.055	0.28 ± 0.051	0.273 ± 0.043
GGT (U/L)	3.0 ± 0.000	3.0 ± 0.00	3.0 ± 0.00	3.0 ± 0.00	3.0 ± 0.00	3.0 ± 0.00	3.0 ± 0.00	3.0 ± 0.00
Glob (g/dL)	2.23 ± 0.46	2.16 ± 0.38	2.06 ± 0.19	1.96 ± 0.18	2.31 ± 0.19	2.40 ± 0.42	2.20 ± 0.26	2.14 ± 0.33
Glucose (mg/dL)	162.3 ± 28.2	153.8 ± 50.1	185.6 ± 68.0	126.8 ± 29.1	176.2 ± 28.1	174.0 ± 32.2	170.8 ± 39.8	170.4 ± 21.5
HDL (mmol/L)	1.325 ± 0.591	1.120 ± 0.217	1.808 ± 0.363	1.878 ± 0.519	1.450 ± 0.344	1.250 ± 0.196	2.292 ± 0.331	2.505 ± 0.660
iPHS (mg/dL)	9.75 ± 0.57	10.80 ± 1.32	8.90 ± 0.58	9.34 ± 1.05	7.84 ± 0.58	8.05 ± 0.99	6.89 ± 0.50	6.63 ± 0.95
LDL (mmol/L)	0.275 ± 0.096	0.260 ± 0.055	0.210 ± 0.045	0.252 ± 0.046	0.260 ± 0.107	0.270 ± 0.106	0.190 ± 0.097	0.224 ± 0.085
Potassium (mmol/L)	6.135 ± 0.247	6.888 ± 7.727	7.088 ± 0.492	7.236 ± 0.996	6.529 ± 0.973	7.097 ± 1.421	5.737 ± 0.747	5.695 ± 1.113
Sodium (mmol/L)	143.75 ± 1.71	141.20 ± 1.64	142.40 ± 0.89	143.10 ± 1.79	144.80 ± 1.32	143.10 ± 2.60	145.10 ± 0.74	143.10 ± 1.79*
Sorbitol dehydrogenase (U/L)	10.08 ± 15.05	6.48 ± 10.45	16.08 ± 4.78	16.56 ± 20.89	0.25 ± 0.66	0.91 ± 1.54	13.51 ± 17.21	25.38 ± 0.31.09
Bilirubin (mg/dL)	0.055 ± 0.019	0.052 ± 0.019	0.092 ± 0.022	0.126 ± 0.044	0.084 ± 0.020	0.072 ± 0.036	0.129 ± 0.034	0129 ± 0.039
Total Protein (g/dL)	5.98 ± 0.35	6.04 ± 0.34	7.12 ± 0.62	7.39 ± 0.74	6.36 ± 0.30	6.26 ± 0.31	7.61 ± 0.62	7.39 ± 0.74
Triglycerides (mg/dL)	42.3 ± 22.2	36.0 ± 10.6	46.8 ± 23.9	33.0 ± 14.7	93.1 ± 32.1	90.5 ± 5.2	54.8 ± 15.6	64.2 ± 23.2
BUN (mg/L)	11.3 ± 1.5	12.8 ± 1.3	15.2 ± 3.4	15.4 ± 1.5	13.7 ± 2.6	14.7 ± 1.2	15.3 ± 3.0	16.0 ± 1.8
Urine Volume (mL)	10.20 ± 4.48	9.30 ± 696	10.0 ± 3.94	4.8 ± 2.91	9.95 ± 4.60	6.80 ± 3.11	4.3 ± 4.32	2.1 ± 1.15

(*continued on next page*)

Table 4 (*continued*)

	28-days				90-days			
	Control Male	AMas Male	Control Female	AMas Female	Control Male	AMas Male	Control Female	AMas Female
pH	7.10 ± 0.82	7.20 ± 1.25	6.9 ± 0.42	6.4 ± 0.42	6.90 ± 0.21	6.80 ± 0.54	6.1 ± 1.05	5.8 ± 0.59
Urine Glucose (mg/dL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10.0 ± 0.31.6	10 ± 31.6	40 ± 51.6
Urine Ketone (mmol/L)	8.0 ± 6.7	7.0 ± 7.6	0.0 ± 0.0	1 ± 2.2	6.0 ± 5.2	5.0 ± 5.8	1 ± 2.1	1 ± 2.1
Urine Protein (mg/dL)	43.0 ± 52.4	43.0 ± 52.4	6 ± 5.2	9 ± 13.4	39.5 ± 32.6	76.5 ± 85.8	64 ± 46.6	80 ± 86.7
Specific Gravity	1.022 ± 0.0042	1.017 ± 0.0076	1.02 ± 0.0042	1.02 ± 0.0061	1.022 ± 0.0042	1.026 ± 0.0055	1.02 ± 0.0083	1.03 ± 0.0000
Urobilinogen (EU/dL)	0.20 ± 0.00	0.20 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.20 ± 0.00	0.28 ± 0.25	0.6 ± 0.42	0.4 ± 0.39

For 28-day, N = 4–5/sex/group and for 90-day, N = 10/sex/group. Historical Control range for hemoglobulin in male Sprague-Dawley CD® IGS rats = 12.3–18.5 g/dL, N = 244 animals. Historical Control range for absolute basophils in male Sprague-Dawley CD® IGS rats = 0.01–0.28 × 103/μL, N = 244 animals.

* Statistically different from control at $p < 0.05$

** Statistically different from control at $p < 0.01$

Table 5
Coagulation after 28- or 90-days of exposure, males and females.

	28-days				90-days			
	Control Male	AMas Male	Control Female	AMas Female	Control Male	AMas Male	Control Female	AMas Female
APTT (seconds)	15.5 ± 1.76	22.6 ± 7.64	13.5 ± 0.92	17.1 ± 3.04	17.9 ± 1.73	15.5 ± 1.45**	15.6 ± 3.42	16.6 ± 1.32
PT (seconds)	9.68 ± 0.40	9.84 ± 0.30	8.9 ± 0.94	9.28 ± 0.34	10.1 ± 0.27	9.87 ± 0.26	9.4 ± 0.15	9.0 ± 0.38*

Historical Control range for APTT in male Sprague-Dawley CD® IGS rats = 10.1–30.3 seconds, N = 217 animals. Historical Control range for PT in female Sprague-Dawley CD® IGS rats = 7.9–11.1 seconds, N = 220 animals.

* $p < 0.05$

** $p < 0.01$

pathology findings from the histological slide evaluation were peer reviewed.

Translocation Analysis: Additional samples from five randomly selected animals/sex/group at the end of each exposure period (28 and 90 days) were collected for translocation analysis. Samples of whole blood (0.5 mL), liver and mesenteric lymph nodes (0.2 – 0.5 g of tissue) were excised and placed in deoxygenated PBS + 0.05 % L-cysteine. Tissue samples were homogenized under anaerobic conditions and aliquots of selected homogenized and/or diluted samples were plated on pre-reduced Brain Heart Infusion Agar (BHI) plates and incubated in anaerobic conditions at 37 ± 1°C for 3–9 days to achieve adequate colony growth for counting. Colonies were counted and morphology assessed for seven characteristics, including size, form, elevation, margin, surface, opacity, and pigmentation, for all colonies present. Colonies were selected and replated on fresh BHI plates and regrown for nine days at 37 ± 1°C, under anaerobic conditions, after which DNA was isolated. If there were more than five colonies of a given morphology on plates from a single animal tissue, only five colonies of that morphology were subject to further analysis. The average CFU/gram of tissue was calculated based on the amount of sample collected and dilution factors for each specific sample.

All plates were visually inspected for CFU growth after incubation and individual colonies were counted. The mean CFU/gram of tissue was calculated using the amount of respective sample evaluated, plate colony growth and factoring for applicable dilutions.

The sensitivity of the method was determined for each sample type using the average amount of tissue in each preparation, assuming a minimum detection limit of 1 CFU/plate and factoring for dilution of samples including plated volumes. Dilutions of the sample preparations either prior to inoculation or prior to plating were accounted for in the calculations by additional multiplication of the DF by the appropriate factor.

$$\text{Sensitivity}(\text{CFU/gor mL}) = \frac{\text{Minimum Detectable CFU/plate}(1\ \text{CFU})}{\text{Avg amount of sample}(\text{g or mL})} \text{X DF}$$

Microbial DNA was extracted, subjected to PCR amplification of the 16S ribosomal RNA (rRNA) gene variable region 4 (V4), and sequenced on a MiSeq instrument (Illumina, San Diego, CA, USA) as previously described [5,6]. For each tissue sample of origin, the morphology was assessed and a taxonomic designation was assigned to the sequences by comparison to the Genbank database [3]. Taxonomic assignments based on the V4 16S rRNA gene cannot distinguish strains, and for some taxa, may not differentiate species. However, *A. massiliensis* DSM 33459, can be differentiated from other *Akkermansia* species by at least two base pairs difference to the most homologous *A. muciniphila* match in the Genbank 16S rRNA database.

2.2.7. Statistical analysis

In-Life Data: For all in-life endpoints that were identified as multiple measurements of continuous data over time (e.g., body weight parameters and food consumption), treatment and control groups were compared using a repeated-measures analysis of variance (RMANOVA). Significant interactions observed between treatment and time as well as main effects were further analyzed by a *post hoc* multiple comparisons test (e.g., Dunnett's test) of the individual treated groups to control.

Organ Weight and Enumeration/Translocation Data: When warranted by sufficient sample sizes, all endpoints with single measurements of continuous data within groups (e.g., organ weight and relative organ weight) were evaluated for homogeneity of variances c and normality. Where homogeneous variances and normal distribution were observed, treatment and control groups were compared using a one-way analysis of variance (ANOVA). When one-way ANOVA was significant, a comparison of the treated groups to control was performed with a multiple comparisons test (e.g., Dunnett's test). Where variances were considered significantly different, groups were compared using a non-parametric method (e.g., Kruskal-Wallis non-parametric analysis of variance). When non-parametric analysis of variance was significant, a comparison of treated groups to control was performed (e.g., Dunn's test).

Statistical Methods (Clinical Pathology): Preliminary analysis was conducted using Barlett's test for homogeneity and Shapiro-Wilk test for normality. When an individual observation was recorded as being less than a certain value (e.g., below the lower limit of quantitation), calculations were performed on half the recorded value. For example, if

Table 6
Histopathologic evaluation of tissues from male and female rats treated with AMas for 90-Days. Only tissues with abnormalities are included, tissues without any observations were excluded from the table.

Tissue/Organ	Control Male	AMas Male	Control Female	AMas Female
ADRENALS				
Examined	10	10	10	10
Normal	10	8	10	10
Vacuolation; cortex; multifocal				
…. mild	0	2	0	0
BONE MARROW (FEMUR)	10	10	10	10
Examined				
Normal	10	9	10	10
Cellularity, Increased; myeloid cell				
…. mild	0	1	0	0
Cellularity, Decreased; erythroid cell				
…. mild	0	1	0	0
BONE MARROW (STERNUM)	10	10	10	10
Examined				
Normal	10	9	10	10
Cellularity, Decreased; erythroid cell				
…. mild	0	1	0	0
Cellularity, Increased; myeloid cell				
…. mild	0	1	0	0
BRAIN				
Examined	10	10	10	10
Normal	9	9	10	9
Infiltration; mononuclear cell, multifocal				
…. minimal	1	0	0	0
Infiltration; perivascular; mononuclear cell, focal	0	1	0	1
…. minimal	0	1	0	1
EPIDIDYMIDES	10	10		
Examined				
Normal	10	9		
Sperm, Decreased; lumen; unilateral, diffuse				
…. severe	0	1		
Infiltration; interstitium; mononuclear cell, focal				
…. minimal	0	1		
ESOPHAGUS				
Examined	10	10	10	10
Normal	10	8	10	8
Degeneration/Regeneration; muscularis; focal				
…. mild	0	1	0	0
Degeneration; muscularis				
…. minimal	0	1	0	2
EYES	10	10	10	10
Examined				
Normal	9	10	10	10
Retinal Rosette				
…. minimal	1	0	0	0
HARDERIAN GLAND	10	10	10	10
Examined				
Normal	10	8	9	6
Infiltration; mononuclear cell				
…. minimal	0	1	1	4
…. mild	0	1	0	0
HEART	10	10	10	10
Examined				
Normal	8	8	9	9
Inflammation; epicardium; mixed, multifocal				
…. moderate	0	1	0	0
Cardiomyopathy; progressive				
…. minimal	2	1	1	1
KIDNEYS	10	10	10	10
Examined				
Normal	7	8	4	4
Chronic Progressive Nephropathy				
…. minimal	0	1	0	2
…. mild	1	0	0	0
Mineralization; tubule; multifocal				
…. minimal	0	0	1	1
Infiltration; mononuclear cell, focal				
…. minimal	1	0	2	2
…. mild	0	0	0	1
Infiltration; mononuclear cell, multifocal				
…. minimal	1	1	2	0
Degeneration/Regeneration; cortex; tubular, multifocal				
…. minimal	0	0	0	1
Dilation; pelvis; unilateral				
…. minimal	0	1	0	0
Cyst; tubular, focal				
…. minimal	0	0	0	1
Cyst; multifocal				
…. moderate	0	0	1	0
Nephropathy; bilateral				
…. moderate	0	0	1	0
Exudate; pelvis; neutrophilic				
…. minimal	0	1	0	0
LARYNX	10	10	10	10
Examined				
Normal	7	4	6	6
Inflammation; submucosa; mixed				
…. minimal	2	3	1	3
…. mild	1	3	3	1
Degeneration/Regeneration; myofiber; unilateral, focal				
…. mild	0	1	0	0
Degeneration; cartilage				
…. minimal	1	1	0	0
…. mild	0	1	0	0
LIVER	10	10	10	10
Examined				
Normal	0	2	3	0
Infiltration; mononuclear cell				
…. minimal	10	8	6	10
Infiltration; periportal; mononuclear cell				
…. mild	0	0	1	0
Necrosis				
…. minimal	0	0	1	1
…. mild	0	0	0	2
Vacuolation; hepatocyte; multifocal				
…. minimal	1	0	0	0
Proliferation; biliary				
…. moderate	0	0	1	0
LUNGS	10	10	10	10
Examined				
Normal	7	4	10	10
Infiltration; interstitium; mononuclear cell, multifocal				
…. minimal	1	0	0	0
Infiltration; perivascular; lymphocytic				
…. minimal	0	1	0	0
Infiltration; perivascular; mononuclear cell, multifocal				
…. minimal	0	1	0	0
Inflammation; mixed, focal				
…. minimal	0	1	0	0
Inflammation; mixed, multifocal				
…. minimal	1	0	0	0
…. mild	0	1	0	0

(*continued on next page*)

Table 6 (*continued*)

Tissue/Organ	Control Male	AMas Male	Control Female	AMas Female
ADRENALS				
Inflammation; neutrophilic, multifocal				
…. moderate	0	1	0	0
Inflammation; mononuclear cell, focal				
…. minimal	1	0	0	0
Alveolar Macrophages, Increased				
…. minimal	0	1	0	0
Alveolar Macrophages, Increased; focal				
…. minimal	0	2	0	0
LYMPH NODE, MESENTERIC Examined	10	10	10	9
Normal	9	10	10	9
Infiltration; medulla; sinus; mast cell				
…. minimal	1	0	0	0
MAMMARY GLAND Examined	4	3	7	9
Normal	4	3	6	5
Hyperplasia; lobuloalveolar				
…. minimal	0	0	1	2
…. mild	0	0	0	2
NASAL TURBINATES Examined	10	10	10	10
Normal	10	9	10	10
Hypertrophy/Hyperplasia; olfactory epithelium; multifocal				
…. moderate	0	1	0	0
NOSE				
Examined	10	10	10	10
Normal	10	8	10	10
Infiltration; mononuclear cell, multifocal				
…. minimal	0	1	0	0
Hyperplasia; lymphoid				
…. minimal	0	1	0	0
Inflammation; mixed				
…. moderate	0	1	0	0
Hyperplasia/Metaplasia; goblet cell; multifocal				
…. moderate	0	1	0	0
OVARIES Examined			10	10
Normal			5	6
Atrophy				
…. mild			1	0
…. moderate			2	4
…. marked			1	0
Cysts; follicular, bilateral				
…. mild			1	0
PANCREAS Examined	10	10	10	9
Normal	8	7	10	9
Fibrosis; islet; focal				
…. mild	1	0	0	0
Fibrosis; islet; multifocal				
…. minimal	0	1	0	0
Hyperplasia; islet				
…. minimal	1	1	0	0
Inflammation; lobule; mononuclear cell				
…. mild	0	1	0	0
Apoptosis/Single Cell Necrosis; acinar cell				
…. mild	1	0	0	0
Infiltration; lobule; mononuclear cell				
…. minimal	0	1	0	0
…. mild	1	1	0	0
Accumulation, Pigment; islet; macrophage; multifocal				
…. minimal	0	1	0	0
Degeneration; acinar cell				
…. minimal	0	2	0	0
PROSTATE Examined	10	10		
Normal	8	8		
Infiltration; interstitium; mononuclear cell, multifocal				
…. minimal	2	1		
…. mild	0	1		
SALIVARY GLANDS (PAROTID) Examined	9	9	10	10
Normal	8	8	10	10
Infiltration; mononuclear cell				
…. minimal	1	1	0	0
SALIVARY GLANDS (SUBMANDIBULAR) Examined	10	10	10	10
Normal	10	10	9	10
Infiltration; mononuclear cell				
…. minimal	0	0	1	0
SKINExamined	10	10	10	10
Normal	10	9	10	10
Infiltration; subcutaneous; mononuclear cell, focal				
…. minimal	0	1	0	0
STOMACHExamined	10	10	10	9
Normal	10	9	10	9
Inflammation; non-glandular; mixed, multifocal				
…. severe	0	1	0	0
TESTESExamined	10	10		
Normal	10	9		
Degeneration/Atrophy; seminiferous tubule; unilateral, diffuse				
…. severe	0	1		
THYROIDExamined	10	10	10	10
Normal	7	7	3	3
Infiltration; mononuclear cell, focal				
…. minimal	0	1	0	0
Ectopic Tissue; focal				
…. present	2	1	0	0
Ultimobranchial Cyst				
…. minimal	1	2	7	7

bilirubin was reported as < 0.1 (or ≤ 0.1), 0.05 was used for any calculations performed with that bilirubin data. When an individual observation was recorded as being greater than a certain value (e.g., above the upper limit of quantitation), calculations were performed on the recorded value. For example, if specific gravity was reported as ≥ 1.100 (or ≥ 1.100), 1.100 was used for any calculation performed with that specific gravity data. If preliminary test was not significant, a one-way analysis of variance was performed followed by Dunnett's test. If the preliminary test was significant, log transformations of the data to achieve normality and variance homogeneity were used. If the log transformation failed, a non-parametric method (e.g., Kruskal-Wallis non-parametric analysis of variance) wase used. When non-parametric analysis of variance was significant, a comparison of treated groups to control was performed (e.g., Dunn's test).

2.3. *In vivo Engraftment in rats*

Fecal matter was collected from each animal prior to test substance administration, and once/week for weeks 1–3 and once every three weeks thereafter beginning on Day 42. A single fecal pellet was collected for each animal at the respective time points indicated and stored frozen at −20 ℃ until used for microbiota profiling. Using the same protocol

Table 7
Organ-to-body-weight ratios for 28- or 90-days of exposure, males and females.

	28-days				90-days			
	Control Male	AMas Male	Control Female	AMas Female	Control Male	AMas Male	Control Female	AMas Female
Adrenal	0.208 ± 0.0346	0.1814 ± 0.0381	0.3357 ± 0.0710	0.3392 ± 0.0718	0.1052 ± 0.0180	0.1133 ± 0.0322	0.2461 ± 0.0535	0.1962 ± 0.0552
Brain	5.535 ± 0.587	5.373 ± 0.279	7.883 ± 0.219	7.749 ± 0.493	3.887 ± 0.353	4.070 ± 0.442	6.427 ± 0.677	6.610 ± 0.535
Epididymides	3.0959 ± 0.3764	2.9299 ± 0.6746	NA	NA	2.7955 ± 0.2864	2.6635 ± 0.1962	NA	NA
Heart	3.336 ± 0.223	3.210 ± 0.240	3.558 ± 0.209	3.513 ± 0.265	2.752 ± 0.277	2.641 ± 0.224	3.294 ± 0.257	3.112 ± 0.259
Kidneys	7.494 ± 0.485	7.603 ± 0.418	7.521 ± 0.344	7.848 ± 0.933	5.816 ± 0.466	5.937 ± 0.400	6.927 ± 1.160	6.879 ± 0.399
Liver	30.852 ± 9.887	31.423 ± 2.485	35.843 ± 2.247	32.277 ± 3.518	24.873 ± 2.090	25.312 ± 1.883	28.817 ± 3.183	28.364 ± 3.966
Pituitary	0.0039 ± 0.0004	0.0038 ± 0.0004	0.0085 ± 0.0010	0.0079 ± 0.0013	0.0037 ± 0.0009	0.0037 ± 0.0013	0.0088 ± 0.0026	0.0080 ± 0.0024
Prostate, Seminal Vesicles, Coagulating Glands	0.008 ± 0.001	0.007 ± 0.000	NA	NA	0.007 ± 0.001	0.007 ± 0.001	NA	NA
Spleen	2.027 ± 0.376	2.157 ± 0.182	2.225 ± 0.371	2.291 ± 0.185	1.634 ± 0.172	1.541 ± 0.171	1.793 ± 0.281	1.703 ± 0.319
Testes	9.260 ± 1.344	8.144 ± 1.857	NA	NA	6.511 ± 0.550	6.275 ± 0.565	NA	NA
Thymus	1.9835 ± 0.4045	1.9091 ± 0.2404	1.6767 ± 0.0856	1.6071 ± 0.1503	0.524 ± 0.1032	0.5037 ± 0.2135	0.7725 ± 0.1678	0.7703 ± 0.1431
Thyroid/Parathyroid	0.68447 ± 0.15195	0.67153 ± 0.10957	0.99733 ± 0.21221	0.85415 ± 0.20952	0.50708 ± 0.13181	0.45300 ± 0.15306	0.91694 ± 0.25138	0.90473 ± 0.14796
Ovary with oviducts	NA	NA	0.6239 ± 0.1248	0.554 ± 0.1682	NA	NA	0.3709 ± 0.0565	0.3433 ± 0.0766
Uterus	NA	NA	2.189 ± 0.373	2.024 ± 0.179	NA	NA	2.534 ± 1.236	2.340 ± 0.770

Table 8
Number of animals with bacterial colonies found in each sample type (blood, liver, mesentery).

Group	Blood (number of animals with any bacteria)	Liver(number of animals with any bacteria)	Mesentery (number of animals with any bacteria)	Number of animals showing any bacteria
28-Day Male control	0/5	2/5	1/5	3/5
28-Day Female control	0/5	3/5	4/5	5/5
28-Day Male *A. massiliensis*	0/5	1/5	4/5	4/5
28-Day Female *A. massiliensis*	0/5	2/5	4/5	5/5
90-Day Male control	3/10	2/10	2/10	4/10
90-Day Female control	1/10	4/10	2/10	4/10
90-Day Male *A. massiliensis*	2/10	3/10	2/10	4/10
90-Day Female *A. massiliensis*	1/10	1/10	2/10	3/10
Total all groups				
Control	4	11	9	16
A. massiliensis	3	8	12	16

Incidence indicates isolation of at least one bacterial colony on plates from animal tissues by treatment and sex. Identified bacteria included the following Genera: *Adlercreutzia, Bacillus, Bacteroides, Bifidobacterium, Bilophila, Blautia, Butyricicoccus, Clostridium, Enterococcus, Escherichia, Koinonema, Lactobacillus, Limosillactobacillus, Luteipulveratus, Odoribacter, Parabacteroides, Phocaeicola, Porphyromonas, Proteus, Roseburia, Staphylococcus and Streptococcus*.

described in Kumar et al. [27] the 16S rRNA profiling was performed on rat fecal pellet samples collected from days pre-dose, 8, 44, and 87. Using sterile forceps, rat fecal pellets were placed into the wells of a Bead Plate from the DNeasy PowerSoil HTP 96 Kit (Qiagen, Hilden, Germany). DNA was extracted according to the manufacturer's protocol and then subjected to 16S rRNA amplicon sequencing as previously described [27]. Briefly, the variable V4 region (515 F/806 R primers) was amplified and sequenced for 2 × 250 cycles on the MiSeq (Illumina, San Diego, CA, USA). The resulting reads were clustered at 99 % similarity, and those with abundance > 0.1 % were assigned taxonomy against the RDP 16S rRNA taxonomy training set no. 18 [46].

3. Results

3.1. In Vitro probiotic properties and strain safety

3.1.1. Hemolytic and D-/L-lactic acid

No hemolytic activity was observed for *A. massiliensis* DSM 33459 after 48 h incubation on Brucella blood agar, and no hemolysin-related toxin genes were identified in the genome. Therefore, this strain is not known to have any hemolytic properties.

The level of D-/L-lactic acid measured in the supernatant of *A. massiliensis* DSM33459 was close to zero, and below the minimum level that could be accurately detected by the kit (Table 1).

3.1.2. Acid and bile tolerance

In comparison to the control media, *A. massiliensis* DSM 33459 had a 76.7 % cell survival when grown with 0.3 % Oxgall bile salt and 10.1 % survival after 1 hour incubation with modified gastric juice (Table 2).

3.2. 28- and 90-day repeated dose study in rats

3.2.1. Dose formulation

Stability: The percent change in the CFU content of the test substances over the course of the study (Day 1, 46 and 92) was 1.41 % and the overall stability was determined to be 101.41 %.

Homogeneity: Analysis of the Day 1 dose preparations resulted in a relative standard deviation (RSD) of 0.83. The test substance was

considered to be homogenously distributed in the dose mixtures at all study concentrations.

Concentration Verification: The analysis of Day 1 samples resulted in 318.2, 313.6, and 313.6 % of the target concentration. The Day 46 samples resulted in 123.1 % and the Day 92 samples resulted in 155.8 % of target concentrations.

3.2.2. Animal observations

There were no test substance-related mortalities or clinical signs of toxicity noted during the study. All animals included in the study were normal upon ophthalmic exam.

Functional Observational Battery: There were no treatment-related adverse changes or biologically relevant changes in any of the parameters evaluated in the functional observational battery, including grip strength (Table 3), motor activity (Fig. 1) and additional detailed clinical observations (data not shown). The only statistically different finding was mean forelimb grip strength which was significantly increased in the male *A. massiliensis* DSM 33459 group at 90-days of exposure.

Body weight and body weight gain: There were no adverse test substance-related or biologically relevant changes in body weight parameters for both male and female rats attributable to the administration of *A. massiliensis* DSM 33459 test material (Fig. 2).

Food consumption: There were no adverse test substance-related changes in food consumption (Fig. 3 Fig. 4) for male or female rats attributable to the administration of *A. massiliensis* DSM 33459.

3.2.3. Clinical pathology

There were no adverse treatment-related changes in hematology, coagulation, clinical chemistry, or urinalysis parameters. Two hematology endpoints absolute basophils at 28-days and hemoglobin at 90-days were significantly increased in the *A. massiliensis* DSM 33459 treated males. Because these values were within historical control ranges (Table 4), occurred only in a single sex and did not occur at both evaluation time points, they were not considered to be biologically relevant nor related to *A. massiliensis* DSM 33459 treatment. Activated partial thromboplastin time (APTT) was statistically increased in males at 28-days and statistically decreased in males at 90-days. Prothrombin time (PT) was statistically decreased at 90-days in females (Table 5). As the coagulation changes were not consistent across timepoints (28-day and 90-days) or sexes and were within historical control ranges (Table 5), these effects were not considered treatment-related or biologically relevant. Sodium (Na^+) levels were significantly decreased ($p < 0.05$) at 90-days for *A. massiliensis* DSM 33459-treated females (Table 4). As the Na^+ value was within the historical control range (126–149 mmol/L), the effect did not occur in both sexes and did not occur at both timepoints, the difference was not considered biologically relevant nor treatment related.

3.2.4. Anatomic pathology and histopathology

There were no macroscopic findings observed at necropsy; spontaneous macroscopic observations in the 28 and 90-day animals at necropsy included small left seminal vesicle, flaccid or small left testis and epididymis, with microscopic correlations of decreased luminal secretions, bilateral marked seminiferous tubule degeneration/atrophy, respectively. No microscopic findings were noted that were considered to be attributed to test substance administration (Table 6). No test substance-related organ weight differences were identified at necropsy for at either 28-days or 90-days of exposure (Table 7).

3.2.5. Translocation analysis

All isolates generated DNA from which the16S rRNA V4 region were successfully amplified and sequenced. There were no statistically significant ($p < 0.05$) differences in translocation of bacteria in blood, liver or mesentery samples between vehicle control and treatment group for both sexes and both time points across both 28- and 90-days of exposure. (Table 8). A total of 206 isolates were subject to molecular characterization. These represented 22 distinguishable genera based on 16S rRNA V4 sequences. Three of these were novel uncultured organisms, although the sequences have been noted in culture-independent molecular surveys. None of the isolates identified were determined to be *Akkermansia* spp (Table 7).

3.2.6. In vivo engraftment

As shown in Fig. 3, rats in the PBS-control group harbored *A. muciniphila* at baseline and day 8. Rats gavaged with *A. massiliensis* DSM 33459, while housing equivalent abundance of *A. muciniphila* at day 1, showed a faster decline in native *A. muciniphila* abundance when compared to the control group. Interestingly, *A. massiliensis* DSM 33459 was able to replace this native population of *A. muciniphila* and showed significant presence in the samples from days 8–87.

4. Discussion

We previously reported the antibiogram of *A. massiliensis* DSM 33459, and genomic sequencing and bioinformatics safety analyses [27] and did not identify any virulence or toxin genes according to the criteria outlined by the European Food Safety Authority (EFSA Panel on Additives and Products or Substances used in [14,17]). The genome of *A. massiliensis* DSM 33459 does not harbor any known antibiotic resistance genes that would be defined by EFSA guidelines as being acquired (not present in the vast majority of strains representative of the species), or as a safety concern [16]. This strain does not contain the genes required to produce the biogenic amines histamine and tyramine, hemolysis-related toxins or known bacteriocins. Furthermore, we have now confirmed that no hemolytic activity or lactate production (neither L- nor D- isomers) was observed based upon *in vitro* laboratory testing. We have shown that *A. massiliensis* DSM 33459 has a favorable tolerance of bile salts, which is a desirable probiotic property for survival in gastrointestinal passage; however, it was sensitive to artificial gastric fluid with low pH when directly exposed. This phenomenon of acid sensitivity has been observed for strains of *A. muciniphila* isolated from human feces as well [23].

Animal safety studies for *A. muciniphila* using a pasteurized [13] or live strain [29] have been conducted following OECD test guidelines, however, to our knowledge, this is the first report of an OECD guideline study evaluating sub-chronic toxicity using a strain of the species *A. massiliensis* sp. nov., namely *A. massiliensis* DSM 33459. Analogous to the Druart et al. [13] and the [29] studies, the current study established the lack of toxicity associated with consuming *Akkermansia massiliensis* sp. nov. As reported, there were some differences within the treatment groups, including forelimb grip strength increase in 90-day males, absolute basophil increase in 28-day males, hemoglobin increase in 90-day males, APTT increase in 28-day males and decrease in 90-day males, PT decrease in 90-day females, and Na decrease in 90-day females. As explained above, each of these endpoints were within historical control ranges, were not observed in both sexes and did not occur at both timepoints, and these differences were not considered biologically relevant, nor treatment related. Spontaneous macroscopic and microscopic observations noted in the pathology findings were considered to be background and/or incidental findings, based on their low frequency of occurrence. Therefore, the NOEL for exposure periods (28-day and 90-day) was greater than 2000 mg/kg body weight/day (5.6×10^{11} CFU/kg body weight/day). Applying a 100-fold minimum required safety factor when applying animal study data to humans, this is equivalent to a daily dose of 3.9×10^{11} CFU/day for a 70 kg human adult.

To date, *Akkermansia massiliensis* sp. nov. is identified as the second most prevalent *Akkermansia* species in humans, after *A. muciniphila*. In a pangenomic analysis human GI tract across health outcomes (n = 1088), Mueller et al. [31] reported a diverse prevalence of *Akkermansia* species, including *A. muciniphila* as the most predominant (32.5 %), followed by

A. massiliensis (11.9 %) and *A. biwaensis* (5.2 %). Similarly, in screening of the American Gut Project samples [30] for the prevalence of *Akkermansia*, Kumar et al. [26] reported that the prevalence for *A. massiliensis* sp. nov. (14.7 %) was lower than *A. muciniphila* (47.5 %). Interestingly, a further segmentation of lean versus obese cohorts demonstrated that *A. muciniphila* prevalence remained high (87.7 %; 82 %) but the prevalence of *A. massiliensis* was only present in the lean cohorts (26.3 %) and absent in the obese (0 %) within the Next Generation Probiotics for Metabolic Health (NGP for MH) Project cohort of 96 subjects (ClinicalTrials.gov identifier NCT04229082). Previously, studies have indicated that *A. massiliensis* DSM 33459 has the genetic capability to produce beneficial metabolites, such as Vitamin B12, propionate and agmatine, and to improve metabolic health in mice using a DIO model. This strain has also been demonstrated to remove extracellular ATP which is known to cause inflammation [27]. These strain-specific *in vitro* and *in vivo* analyses of *A. massiliensis* DSM 33459 have demonstrated benefits that align with the prevalence of *A. massiliensis* in a lean cohort.

Interestingly, the above GI tract analyses show that only a small percentage of individuals were co-colonized with both *A. muciniphila* and *A. massiliensis* sp. nov., suggesting that these two species may be somewhat mutually exclusive [26,31]. From this study, the *in vivo* engraftment results as shown in Fig. 3 support the observation of mutual exclusivity of the two *Akkermansia* species. Rat fecal pellets from the sub-chronic 90-day toxicity study were analyzed for the presence of *A. muciniphila* in both the PBS control group and the *A. massiliensis* DSM 33459 treatment group. While *A. muciniphila* was present in the PBS control group at days 1 and 8, it was no longer detected at days 44 and 87, indicating a decline with age. In the *A. massiliensis* DSM 33459 treatment group, *A. muciniphila* was detected only at day 1 (baseline) and was replaced by *A. massiliensis* at days 8, 44 and 87. These results are supported by a previous *in vivo* study in mice which investigated the effects of *A. massiliensis* DSM 33459 on metabolic health using a diet induced obesity (DIO) model. A similar competitive exclusion was observed where *A. muciniphila* was identified early in the study but was replaced by *A. massiliensis* DSM 33459 and *A. muciniphila* also decreased in the control groups with age in mice [27]. While these studies in rodents support a possible engraftment of *A. massiliensis* it is not known if *A. massiliensis* will persist without continuous administration, or if the observed engraftment is transient.

The possibility of competitive exclusion between these two Akkermansia species needs to be further explored. While the abundance of *A. muciniphila* and *A. massiliensis* have both generally been associated with health, some observational studies have reported increased abundance of *A. muciniphila* in patients with certain neurological conditions; however, these observations are inconsistent and do not indicate causality ([4]; EFSA Panel on Nutrition et al., 2021). Regardless of the lack of causality or understanding of this phenomenon, *A. massiliensis* has not been specifically identified in these populations.

Concerning safety, it has been suggested that *Akkermansia* species with the capability to utilize mucins for growth may have undesirable effects in relation to human health, based on speculations that degradation of mucin may lead to a loss of mucosal integrity, increase gut permeability and induce local inflammation [15]. However, this speculation has not been supported scientifically. In fact, it has been demonstrated that mucin-degrading microbes, including *Bifidobacterium* spp., are identified in infants soon after birth [35], and this ability is considered advantageous in selection of desirable, beneficial microbes [10]. *In silico* analysis indicates that 86 % of commensals have genetic capability to utilize mucin glycans, which is believed to be employed in the absence of dietary fiber [38,42]. In further support of the safety of mucin-degrading microbes, Wolter et al. [47] have recently demonstrated that susceptibility to the mucosal pathogen *Citrobacter rodentium* in mice is both microbiome- and diet-dependent, where susceptibility to the pathogen is associated solely with a fiber-free diet. Conversely, a fiber-rich diet allows *A. muciniphila* to convey resistance to the pathogen. This suggests that with adequate dietary fiber, commensals, including *A. muciniphila,* do not have any undesirable effects on the mucus layer and may provide pathogen exclusivity as a protective effect. As *A. massiliensis* DSM 33459 houses genetic regions that would indicate it also has the ability to utilize mucin as a carbon source, this has not been demonstrated *in vivo*. However, it is believed that as indicated through scientific studies for the safety of *A. muciniphila, Bifidobacterium,* and other mucin-utilizing commensals, that this attribute alone does not indicate virulence [44].

Furthermore, as described by Atuma et al. [1], there is a continuous mucin layer that lines the entire gastrointestinal track, thinnest in the jejunum to thickest in the colon. As a strict anaerobe, *A. massiliensis* is known to colonize the colon, which houses two distinct mucin layers. The outer layer is approximately 700–800 microns in thickness and is described as mobile or non-adherent layer, where continuous removal and renewal of mucin acts as a lubricant to remove microbes and viruses. In fact, it has been suggested that *A. muciniphila* can actually increase the number of mucus-producing goblet cells and restore or increase mucus layer thickness [39]. Furthermore, the outer mucin layer is believed to actually provide a protective effect, acting as a prebiotic also with binding sites similar to the epithelial layer, thereby removing bacteria that potentially may translocate [11]. In this study, we investigated the translocation potential of *A. massiliensis* DSM 33459, which was isolated from the stool of a healthy adult [27]. Despite the high dose tested (5.6×10^{11} CFU/kg BW/day) and an increase in relative abundance of the strain within the GI tract of the rats approaching more than 15 % over the course of the study, we did not see any signs of translocation or any other signs of increased gut permeability. While the data from the translocation analysis shows that molecular characterization of the small numbers of isolates obtained from liver, kidney and mesentery samples were in both control and treatment groups, it is important to note that none of the identified microbes were of the genus *Akkermansia*, and that there was no statistically significant difference in the number of colonies isolated between control and treatment groups. Translocation studies often result in low numbers of colonies per rodent in both control and test substance animals and this has been demonstrated even for probiotic strains with a very long history of safe use [32,49]. Low levels of bacteria have been identified in extra-intestinal sites of healthy rodents without evidence of pathology [22,28,40,43]. The absence of higher bacterial counts in extra-intestinal locations coupled with a lack of adverse hematology and microscopic findings indicates that *A. massiliensis* DSM 33459 will not translocate from the gastrointestinal tract in healthy populations, as indicated by this study.

Isolated from the feces of a healthy human adult, *A. massiliensis* DSM 33459 presents a promising next generation probiotic that has been established as safe for human ingestion given the comprehensive genomic, phenotypic, *in vitro*, and *in vivo* data presented herein and previously by [27]. Human clinical studies are warranted to understand the association of this strain and human health, the potential mutual exclusivity with *A. muciniphila*, absence in obese populations, and relevance of its ability to metabolize mucin. This study has summarized the comprehensive safety profile of *A. massiliensis* DSM 33459 and supports the use of this commensal microbe as a potential for a next generation probiotic.

CRediT authorship contribution statement

David Elisabeth: Investigation, Formal analysis, Data curation. **Bry Stéphanie:** Investigation, Formal analysis, Data curation. **Auzanneau Isabelle:** Investigation, Formal analysis, Data curation. **Wang Qiong:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Kane Helene:** Writing – review & editing, Methodology, Investigation, Data curation. **Hibberd Ashley:** Writing – review & editing, Writing – original draft, Resources, Conceptualization. **Hasselwander Oliver:** Writing – review & editing, Writing – original draft, Resources, Conceptualization. **Kumar Ritesh:** Writing – review & editing, Investigation, Formal analysis, Data curation,

Conceptualization. **Bauter Mark:** Writing – review & editing, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Smith Amy:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Conceptualization. **Pitt Jeffrey:** Writing – review & editing, Writing – original draft, Resources, Methodology, Conceptualization. **Burns Frank:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Seguinot Pauline:** Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Author and co-authors are/were employed either by International Flavors & Fragrances Inc. (IFF) or were employed by Contract Research Organization that conducted the experiments for IFF. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The data that has been used is confidential.

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