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RESEARCH ARTICLE

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Toxicological safety evaluation of live *Anaerobutyricum soehngenii* strain CH106

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Abstract

The gut commensal *Anaerobutyricum soehngenii* is an anaerobe that can produce both propionate and butyrate, metabolites that have been shown to have a positive effect on gut and overall health. Murine and human dose finding studies have shown that oral intake of *A. soehngenii* has a positive influence on peripheral insulin resistance, thereby reducing the risk of type 2 diabetes. A recent human intervention provided support for the mode of action of *A. soehngenii* as it affected gene expression in the duodenum, stimulated the secretion of GLP-1 and improved insulin sensitivity. For these reasons *A. soehngenii* has been proposed as a food ingredient. Before introducing this bacterium to the food chain, however, it must be established that oral intake of live *A. soehngenii* bacteria does not pose any health risk. As part of the safety analysis of *A. soehngenii* strain CH106, we performed genotoxicity assays to determine its mutagenic potential (bacterial reverse mutation and in vitro mammalian cell micronucleus tests) and a 90-day subchronic toxicity study in rats to determine overall toxicity potential. The results of both genotoxicity studies were negative, showing no genotoxic effects. For the 90-day subchronic toxicity study, no adverse events were registered that could be attributed to the feeding with *A. soehngenii* strain CH106. Even at the highest dose, which exceeds the expected daily human intake more than 100-fold, no adverse events were observed. These result support the conclusion that the use of *A. soehngenii* strain CH106 as a food ingredient is safe.

KEYWORDS

Anaerobutyricum soehngenii, beneficial microorganism, food ingredient, genotoxicity, microbiota, safety, subchronic toxicity

1 | INTRODUCTION

Our intestinal tract is colonized since birth by a myriad of microbes belong to over 1000 species that mainly belong to the Gram-positive and anaerobic Firmicutes (Rajilić-Stojanović & De Vos, 2014). *Anaerobutyricum soehngenii* L2-7^T is a commensal non-spore forming bacterium that was isolated from the stool of an 11 month old healthy infant and was originally described as *Eubacterium hallii* (Barcenilla et al., 2000; Shetty et al., 2018). It is a metabolically versatile Gram-positive bacterium that is able to produce the short chain fatty acids butyrate and propionate that are linked to many health beneficial traits (Louis & Flint, 2017).

It has been well established that the intestinal microbiota contributes to host metabolism and affects body weight and insulin resistance (Koh & Bäckhed, 2020). Indeed, the microbiota of prediabetic and type 2 diabetes patients are distinct from those of healthy people and in general are characterized by a reduced level of butyrate-producing bacteria (Larsen et al., 2010). Various fecal microbiota transplantation (FMT) studies have shown the microbiota of lean donors could recover the insulin sensitivity in prediabetic subjects highlighting the causal effect of gut microbiota (Kootte et al., 2017; Vrieze et al., 2012). However, what role specific bacteria play in this process is still the subject of many studies. Moreover, most information that we have on features of the microbiota in correlation with host metabolism come from studying colonic fractions. A large part of the metabolic processes (degradation and uptake of simple carbohydrates, fat, and protein), however, occurs in the small intestine. Information on the role of the microbiota in these processes is more difficult to come by as studying the microbiota of the small intestine is hampered by its accessibility. However, the use of FMT has provided new insight and in a first controlled study fecal matter from a lean donor was transferred through a nasal duodenal tube to the duodenum of subjects that had been diagnosed with metabolic syndrome (Vrieze et al., 2012). Insulin sensitivity was measured before and 6 weeks after gut microbiota infusion. Also, small intestinal biopsies were taken before and 6 weeks after gut microbiota infusion. The results showed that improved peripheral insulin sensitivity was correlated with increased small intestinal levels of *Anaerobutyricum* spp. The causal role in the observed insulin sensitivity improvement was confirmed in a db/db mouse model in which live cells of *A. soehngenii* L2-7^T were found to increase insulin sensitivity and affect intestinal and liver gene expression (Udayappan et al., 2016). Encouraged by these results, a dose finding study in prediabetic subjects was performed with a daily intake of a frozen formulation of *A. soehngenii* L2-7^T for 4 weeks (Gilijamse et al., 2020). While the primary aim of this study was to show the safety of the bacterium for human consumption, the results showed that oral consumption of *A. soehngenii* L2-7^T could indeed affect peripheral insulin sensitivity. A recent study aiming to elucidate the mode of action corroborated these findings and showed that a single duodenal administration of *A. soehngenii* L2-7^T affected gene expression in the duodenum, stimulated the secretion of GLP-1 and improved insulin sensitivity (Koopen et al., n.d.).

These results altogether show that *A. soehngenii* L2-7^T has great potential to serve as a next generation therapeutic product for people that are at risk of developing type 2 diabetes and show increased levels of insulin resistance. For this purpose, it is important to show that intake of this bacterium at the recommended dosages is safe. Following the US Food and Drug Administration (FDA) and European Food Safety Authority (EFSA) guidance on toxicity testing required for safety assessment of a new nonabsorbable food ingredient (European Food Safety Authority EFSA, 2012; US FDA, 2007) lyophilized live cells of *A. soehngenii* CH106, a derivate strain of L2-7^T, was subjected to non-clinical studies assessing the potential for genotoxicity (bacterial reverse mutation and in vitro micronucleus test on mammalian cells) and subchronic toxicity (90-day oral toxicity study in rats). The objective of this research article is to describe the methodology and results for these nonclinical studies conducted with lyophilized live *A. soehngenii* strain CH106 as part of a comprehensive assessment to demonstrate its safety for use as a food ingredient.

2 | MATERIALS AND METHODS

2.1 | Regulatory guidance and quality assurance

The genotoxicity studies were performed at the Good Laboratory Practice (GLP) certified Eurofins BioPharma Product Testing facilities in Munich. The 90-day sub-chronic toxicity study in rats was performed at BSL BIOSERVICE Scientific Laboratories Munich GmbH. Both facilities are regularly audited and inspected for GLP compliance. All studies have been performed according to the highest GLP standards, following the OECD principles for Good Laboratory Practice. The in vitro mammalian micronucleus assay was carried out in human lymphocytes, following the OECD guidelines for testing of chemicals, section 4, number 487 (OECD, 2016). The Reverse Mutation Assay was carried out according to the OECD guidelines for testing of chemicals section 4, number 471 (OECD, 2020). The 90-day sub chronic toxicity study in rats was carried out according to OECD guidelines for testing of chemicals section 4, number 408 (OECD, 2018). Procedures and facilities complied with the requirements of Directive 2010/63/EU of September 22, 2010 (European Union, 2010) and the German legislation defined in the animal protection law concerning the protection of animals used for experimental and other scientific procedures (Tierschutzgesetz, July 1972 last modified June 2020).

2.2 | Test materials

A. soehngenii CH106 was derived from the *A. soehngenii* type strain L2-7^T (Shetty et al., 2017) and batches containing live cells of this strain were supplied by Caelus Health as an off-white powder. The batch that was used for the genotoxicity studies contained 5×10^9 CFU/gram. The batch that was used for the 90-day subchronic toxicity study in rats contained 2×10^{11} CFU/gram.

The vehicle for the 90-day study was phosphate buffered saline (GIBCO), supplemented with 0.05% L-cysteine (Merck). This is also used as control item. Preparations were made fresh each day. The test material for the genotoxicity studies was prepared similarly as for the 90-day tox study (dissolve in PBS, supplemented with L-cysteine). PBS supplemented with L-cysteine was used as solvent control, distilled water was used as negative control.

Sodium azide, 4-nitro-o-phenylene-diamine, methyl methanesulfonate were used as positive controls for the bacterial reverse mutation test without metabolic activation and were obtained from Sigma. 2-aminoanhracene was used as a positive control for the bacterial reverse mutation test with metabolic activation and was obtained from Alfa Aesar. Tester strains TA98, TA1535 and *Escherichia coli* were obtained from MOLTOX, INC., NC 28607, USA. Tester strains TA100 and TA1537 were obtained from Xenometrix AG, Switzerland.

Positive clastogenic controls (methylmethanesulfonate and cyclophosphamide) and aneugenic positive control (colchicine) for the micronucleus test (MNT) were obtained from Sigma. Stock solutions were prepared by dissolving in 0.9% NaCl (cyclophosphamide) and PBS (colchicine) and stored below −20°C. Dilutions were made in RPI medium on the day of testing. Cell culture medium (RPMI) was used as negative control for MNT studies.

2.3 | Genotoxicity

2.3.1 | Bacterial reverse mutation test (AMES test)

A. soehngenii was investigated for its potential to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) as previously described (Ames et al., 1975; Maron & Ames, 1983) using *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and tester strain *E. coli* WP2 uvrA (pKM101). In line with the OECD test guideline No. 471 (OECD, 2020) for the plate assay, amounts of 31.6, 100, 316, 1000, 2500, and 5000 μg of rehydrated lyophilizate/plate were used in the absence or presence of metabolic activation (S9 mix).

For the direct plate incorporation method, 0.1-ml suspensions of pasteurized *A. soehngenii* were mixed with 0.5 ml of S9 mix or substitution buffer (depending on whether metabolic activation was required), 0.1 ml of a suspension of each bacterial strain and 2 ml of overlay agar containing Agar-Agar (Becton Dickinson), NaCl (Merck), D (+) Biotin (Roth) and L-Histidine (Roth) or Tryptophan (Sigma). After vortexing, the mixture was overlaid on to a Petri dish containing minimal glucose agar (Eurofins BioPharma Product Testing Munich GmbH). The same quantities of test item suspensions (0.1 ml), S9 mix (0.5 ml), and bacterial suspensions (0.1 ml) were used for the pre-incubation method, but the mixture was then pre-incubated for 60 minutes at 37°C (under shaking) before overlay agar was added and the mixture was poured on to the surface of a minimal glucose agar plate. For both methods, after 48–72 h of incubation at 37°C, the number of revertants per plate were scored for each strain and for each experimental point using a ProtoCOL counter (Meintrup DWS Laborgeräte GmbH). Tester strains with a low spontaneous mutation frequency like TA1535 and TA1537 were counted manually.

Following confirmation of criteria of validity, mutagenicity was evaluated. A positive result for mutagenicity was defined as a reproducible two-(TA98, TA100) and *E.coli* WP2 uvra (pKM101) or three-fold increase (TA1535 and TA1537) in the number of revertant colonies, compared with vehicle controls, and/or a reproducible dose–response relationship.

2.3.2 | In vitro mammalian cell micronucleus test

The in vitro mammalian cell micronucleus test was conducted using the cytokinesis-block method (Fenech, 2000). For each experiment, cell cultures were prepared from whole blood samples (obtained from a single healthy, nonsmoking donor), which were added to a final volume of 5 ml of culture medium containing phytohemagglutinin (PHA, a mitogen to stimulate the lymphocytes to divide; supplied by PAN Biotech). The cultures were incubated at 37°C for 44–48 hr. Next, the cultures were exposed to cells of *A. soehngenii* and incubated for 4 h in the absence or presence of metabolic activation (S9 mix) or for 44 h in the absence of metabolic activation. Cytochalasin B was added to block cytokinesis. As no concentration showed precipitation or cytotoxicity at the end of the exposure periods, in accordance with OECD Test Guideline No. 487 the highest concentrations (1000, 2500, and 5000 μg/ml) were selected for micronucleus analysis for both 4- and 44-h exposure times.

At harvest, the cells were collected by centrifugation (200 g for 10 min) and subjected to a hypotonic treatment to induce cell swelling (i.e., incubation for 30 min at room temperature in cold 0.075 M potassium chloride). The cells were then fixed in methanol + glacial acetic acid (3 + 1). Cells were resuspended gently, and the suspension was dropped onto clean glass slides. Consecutively, the cells were dried on a heating plate. The cells were stained with acridine orange solution.

The slides were coded to allow for blind scoring. Micronuclei were analyzed in 1000 binucleated cells per culture when possible (in total, 2000 binucleated cells per concentration level in all conditions) according to the criteria of Fenech (Fenech, 2000), that is, clearly surrounded by a nuclear membrane, having an area of less than one-third of that of the main nucleus, being located within the cytoplasm of the cell and not linked to the main nucleus via nucleoplasmic bridges.

For statistical analysis, cell cultures exposed to the test item were compared with that of the vehicle control cell cultures using the χ^2 test, in which $P = 0.05$ was used as the lowest level of significance. To assess the dose–response trend, the χ^2 test for trend was performed.

A positive result for clastogenicity or aneugenicity was defined as a statistically significant increase in the frequency of micronucleated binucleated cells in comparison with the corresponding vehicle controls, obtained at one or more dose levels, associated with a dose–response relationship, and for at least one concentration, the frequency of micronucleated binucleated cells being above the corresponding vehicle historical range.

2.4 | Subchronic toxicity

2.4.1 | Animal and housing conditions

In total, 40 male and 40 female specific pathogen-free Crl:WI (Han) rats were supplied by Charles River for the study. The animals were 5–6 weeks old at the start of the acclimatization period. This period comprised of an initial 5 days of acclimatization after which then the animals were assigned to the groups. In the following 8 days a neurological evaluation was performed and body weight, food intake, and body temperature, was assessed. At the start of the intervention males weight on average 265 g and females weight on average 177 g. The study took place in an animal room supplied with filtered air (ventilated to give 10 air changes per hour) and maintained at a

temperature of 22 ± 3°C and relative humidity of 55 ± 10%. A 12-h light/dark cycle was controlled automatically. The rats were kept in IVC cages (type IV, polysulphonite cages) on Altromin saw fibre bedding with five animals per group per cage, and males and females separated. Pelleted rodent diet (Altromin 1324 maintenance diet for rats and mice, Altromin Spezialfutter GmbH & Co. KG) was available ad libitum. Domestic quality drinking water was freely available throughout the study.

2.4.2 | Test item formulation, administration and dosing schedule

The test item was provided in sachets, each containing sufficient material for preparing daily dosages for all animals. Briefly, on each administration day a new sachet was opened and dissolved in the vehicle (sterilized anaerobic PBS, supplemented with 0.05% L-cysteine). Dilutions were made in the vehicle and the resultant suspensions were administered by oral gavage to groups of 10 males and 10 females at 40, 200, or 1000 mg per animal, at a dose volume of 1 ml once daily for 90 days, until the day before necropsy. In mg/kg/day this translates to 150–225, 750–1100, and 3750–5500 mg/kg/day at the start of the trial and 95–170, 450–850, and 2350–4250 mg/kg/day at the end of the trial where the higher numbers reflect the average dosage for the female rats and the lower numbers reflect the average dosage for the male rats. This is equivalent to 8×10^9, 4.0×10^{10} and 2.0×10^{11} live *A. soehngenii* cells per animal per day and translates to $3.0–4.5 \times 10^{10}$, $1.5–2.2 \times 10^{11}$, and $7.5–11 \times 10^{11}$ live *A. soehngenii* cells/kg body weight/day at the start of the trial and $1.9–3.4 \times 10^{10}$, $9.5–17 \times 10^{10}$, and $4.7–8.5 \times 10^{11}$ live *A. soehngenii* cells/kg body weight/day at the end of the trial (where the lower number reflects the relative amount for male and the higher number the relative amount for female rats).

The high dose, equivalent to 3750–5500 mg/kg/day (or $7.5 \times 10^{11}–1.1 \times 10^{12}$ live *A. soehngenii* cells/kg body weight/day) provided a margin of safety of at least 1,000-fold compared with anticipated maximum exposure in foods. The five-fold descending intervals for the mid and low dose were included to demonstrate any dose-related response in effects.

The main stability parameter of the powder is the viable cell count (the active ingredient). All animals were dosed within 1 h of formulation preparation, which was within the stability period (1 h when stored at room temperature protected from excessive exposure to oxygen), confirmed as part of a separate validation study (data not shown). Dosing of the rehydrated *A. soehngenii* lyophilized powder was verified by periodical determination of the total protein concentration in the suspension (week 1, 5, 9, and 13). Protein concentrations for all dilutions were within expected range. On occasion a higher amount was measured for the higher dosage which could be explained by a higher viscosity of the test item, resulting in possible pipetting errors. The results confirmed that all animals received at least the minimal indicated dosage and samples were prepared correctly (data not shown).

2.4.3 | Clinical examinations, body weights, and food and water consumption

All animals were observed at least once daily for changes in clinical condition, with standard arena observations that were more comprehensive and conducted once weekly. Once before the first dose administration and once in week 13 both eyes of each animal were examined with an indirect ophthalmoscope and a portable slit-lamp microscope after application of 0.5% Atropin-POS (URSAPHARM Arzneimittel GmbH). On days 86 and 87 of dosing, all animals were subjected to a modified Irwin test. The Irwin test included a functional observation battery with assessment of sensory reactivity to different stimuli, grip strength and motor activity. Body weight was measured once before the start of the study to assign the animals to the groups, creating homogeneous weight groups. The body weight of each animal was recorded on the first day of dosing and weekly thereafter, until necropsy. Food consumption was recorded weekly throughout the dosing period.

2.4.4 | Hematology, coagulation and clinical biochemistry

At the end of the dosing period (just before necropsy), blood samples were collected from all surviving animals for hematology, coagulation and clinical chemistry assessments. For hematology, 400 µl of blood was collected into K3 ethylenediaminetetraacetic acid tubes (Sarstedt AG & Co.) for analysis of the following parameters using the ADVIA 120 (Siemens): hematocrit, hemoglobin, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, platelet count, white blood cell count, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and large unstained cells. Values for coagulation parameters (activated partial thromboplastin time, prothrombin time) were determined from 500-µl blood samples collected into 1-ml tubes containing tri-sodium-citrate solution (Sarstedt AG & Co.) using an ACL 7000 (Instrumentation Laboratories).

For clinical biochemistry, 750 µl of blood was collected into 1-ml serum tubes (Sarstedt AG & Co); values for alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, creatinine, total protein, albumin, urea, bilirubin, total bile acids, cholesterol (total, high-density lipoprotein, and low-density lipoprotein), triglycerides, glucose, sodium, and potassium, were determined using an Olympus AU480 (Beckman Coulter), values for triiodothyronine (T3) and thyroxine (T4) and thyroid-stimulating hormone using xMAP technology (MagPIX, Luminex).

A urinalysis was performed with samples collected from all animals prior to or as part of the sacrifice of the animals. Values for specific gravity, nitrite, pH-value, protein, glucose, ketone bodies, urobilinogen, bilirubin, erythrocytes, and leukocytes were measured using Henry Schein Urine Stripes (URI 10SL). Additionally, urine color/appearance was recorded.

2.4.5 | Macroscopic examinations, organ weights and histopathology

One day after the last administration (day 91) all surviving animals of the treatment period were sacrificed using anesthesia (ketamine/xylazin) and were subjected to a detailed gross necropsy which included careful examination of the external surface of the body, all orifices and the cranial, thoracic and abdominal cavities and their contents. Vaginal smears were examined on the day of necropsy to determine the stage of estrous cycle.

A macroscopic examination was performed by opening the cranial, thoracic and abdominal cavities and observing the appearance of organs and tissues in situ. For animals in all groups, specified organs (liver, kidneys, adrenal glands, testes, epididymides, prostate, seminal vesicles and coagulating glands, ovaries, uterus with cervix, thymus, and thyroid/parathyroid glands, spleen, brain, pituitary gland, and heart) were weighed. A full list of organs and tissues (adrenal glands, aorta, brain, caecum, colon, duodenum, epididymides, eyes [with optic nerve and Harderian gland], femur with knee joint, heart, ileum (including Peyer's patches), jejunum, kidneys, liver, lungs, lymph nodes [mandibular, mesenteric and axillary], mammary gland area [male and female], esophagus, ovaries, oviducts, pancreas, pituitary, prostate, and seminal vesicles with coagulating glands as a whole, rectum, salivary glands [sublingual, submandibular, parotis], sciatic nerve, skeletal muscle, skin, spinal cord [cervical, thoracic and lumbar segments], spleen, sternum [with bone marrow], stomach, testes, thymus, thyroid gland including parathyroid glands, tongue, trachea, ureters, urinary bladder, and uterus with cervix and vagina) was examined microscopically for animals in the vehicle control and high-dose groups only. All tissues were fixed in phosphate-buffered neutral 4% formaldehyde (Hounissen), except for the eyes and testes and epididymites, which were fixed in Modified Davidsons's fixative (prepared by BSL Munich from water, ethanol, formaldehyde and glacial acetic acid). After fixation, the organs and tissues from vehicle control and high dose animals were trimmed and representative specimens were taken for histological processing. The specimens were embedded in paraffin wax and cut at a nominal thickness of 2–4 μm, before being stained with hematoxylin and eosin and examined under a light microscope (Leica DM3000).

All pathological findings were entered directly into the validated computerized Pathology Data Systems Ltd. system (version 6.2e2). Histological findings were graded on a five-level scale (minimal, mild, moderate, marked, and severe).

2.4.6 | Evaluation of results and statistical analysis

The findings of this study were evaluated in terms of the observed effects. Parameters like body weight gain and food consumption were calculated for each animal as the difference in weight measured from 1 week to the next. The relative organ weights were calculated in relation to the brain weight and in relation to the body weight (measured at necropsy) and were presented as percentage. All results are reported in tabular form (summarized in mean or summary tables and/or listed in individual data tables).

Toxicology and pathology data were captured on paper according to appropriate standard operating procedures (SOPs) and using the validated computerized system Ascentos® System (version 1.3.4, Pathology Data Systems Ltd.). A statistical assessment of the results of the body weight, parameters of hematology, blood coagulation, and clinical biochemistry and absolute and relative organ weights were performed for each gender by comparing values of dosed with control animals using either a parametric one-way ANOVA and a post-hoc Dunnett Test or a non-parametric Kruskal–Wallis Test and a post hoc Dunn's Test, based on the results of homogeneity and normality tests. These statistics were performed with Ascentos 1.3.4 software or GraphPad Prism V.6.01 software. For all tests, the level of significance was $P < 0.05$.

3 | RESULTS

3.1 | Genotoxicity

3.1.1 | Bacterial reverse mutation test (AMES)

Mean revertant colony counts of the *Salmonella typhimurium* strains in the AMES test are provided in Table 1. The freeze-dried powder, containing *A. soehngenii* was investigated for its potential to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and tester strain *E. coli* WP2 uvrA (pKM101).

All tester strains demonstrated their typical response to ampicillin (TA98, TA100) and *E.coli* WP2 uvra (pKM101). Spontaneous reversion frequencies in the presence of the vehicle with and without the S9 mix were within historical range of the laboratory, while the positive controls showed a distinct enhancement of revertant rates over the control plate. Background growth was similar on both the negative control as on test plates. Thereby all criteria of validity were met.

No precipitation of the test item was observed in any tester strain used in experiment I and II (with and without metabolic activation). No toxic effects of the test item were noted in any of the five tester strains used up to the highest dose group evaluated with and without metabolic activation in experiment I and experiment II. No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with *A. soehngenii* at any concentration level, neither in the presence nor absence of metabolic activation in experiment I and experiment II.

3.1.2 | In vitro mammalian cell micronucleus test

Results for the micronucleus analysis are provided in Table 2. Micronucleated binucleated cell frequencies for vehicle control groups were within vehicle control historical ranges (data not shown) and the

TABLE 1 Bacterial reverse mutation test with rehydrated lyophilized *A. soehngenii*

	Revertant colonies per plate (mean ± standard deviation)									
	Without metabolic activation (-S9)					With metabolic activation (+S9)				
	S. Typhimurium					S. Typhimurium				
Concentration (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA (pKM101)	TA98	TA100	TA1535	TA1537	WP2 uvrA (pKM101)
Plate incorporation assay (experiment 1)										
0 (water)	52 ± 6.1	105 ± 9.6	8 ± 3.5	10 ± 2.3	231 ± 8.7	39 ± 4.7	86 ± 6.1	8 ± 2.9	20 ± 6.1	248 ± 21.4
Vehicle	45 ± 14.5	91 ± 6.2	8 ± 0.6	13 ± 4.4	200 ± 6.2	45 ± 4.5	98 ± 17.0	8 ± 4.0	16 ± 3.0	214 ± 13.5
31.6	46 ± 6.1	92 ± 5.6	14 ± 4.7	14 ± 2.1	155 ± 2.9	47 ± 0.6	99 ± 12.1	10 ± 3.1	14 ± 3.8	152 ± 14.5
100.0	54 ± 17.2	106 ± 7.1	12 ± 4.0	20 ± 12.5	195 ± 24.7	45 ± 9.8	105 ± 10.5	10 ± 3.1	15 ± 1.5	211 ± 21.8
316.0	46 ± 7	89 ± 4.9	6 ± 1.5	12 ± 1.5	220 ± 15.7	45 ± 4.4	109 ± 13.6	12 ± 3.8	16 ± 1.0	195 ± 6.1
1000.0	51 ± 13.2	97 ± 3.6	6 ± 1.5	17 ± 4.5	168 ± 26.1	48 ± 7.8	105 ± 4.5	7 ± 1.5	19 ± 4.0	209 ± 15.9
2500.0	38 ± 7	103 ± 15.1	11 ± 5.5	13 ± 7.0	192 ± 18.0	49 ± 9.1	99 ± 17.5	10 ± 3.6	20 ± 4.5	194 ± 33.3
5000.0	37 ± 7.5	99 ± 5.0	9 ± 1.5	14 ± 3.0	186 ± 18.3	48 ± 3.0	99 ± 2.1	6 ± 3.1	14 ± 8.1	190 ± 20.5
Positive Control[a]	439 ± 48.6	319 ± 60.3	703 ± 34.9	83 ± 5.6	844 ± 37.4	1466 ± 94.9	1248 ± 151.6	177 ± 26.2	167 ± 8.1	460 ± 29.3
Pre-incubation test (experiment 2)										
0 (water)	42 ± 4.6	104 ± 13.1	9 ± 3.5	21 ± 3.0	178 ± 21.0	35 ± 9.0	112 ± 7.2	8 ± 1.5	16 ± 7.8	249 ± 6.0
Vehicle	50 ± 9.5	127 ± 9.0	10 ± 4.4	22 ± 1.0	173 ± 26.9	49 ± 8.4	122 ± 3.5	7 ± 1.5	23 ± 2.3	270 ± 8.1
31.6	39 ± 10.8	131 ± 3.5	8 ± 3.1	16 ± 8.9	196 ± 8.0	35 ± 8.1	116 ± 18.2	9 ± 2.9	24 ± 2.5	191 ± 46.7
100.0	40 ± 2.1	132 ± 12.5	12 ± 4.6	16 ± 4.9	177 ± 15.0	30 ± 5.6	92 ± 3.8	12 ± 1.0	25 ± 9.9	167 ± 16.3
316.0	52 ± 7.2	132 ± 27.6	12 ± 3.2	19 ± 3.5	204 ± 10.1	34 ± 1.5	134 ± 11.8	10 ± 1.5	25 ± 9.1	208 ± 34.6
1000.0	61 ± 16.0	102 ± 14.8	10 ± 2.3	18 ± 3.2	191 ± 13.8	39 ± 2.6	108 ± 17.1	11 ± 3.5	27 ± 2.1	215 ± 12.5
2500.0	56 ± 7.6	95 ± 4.2	11 ± 2.1	23 ± 5.3	166 ± 19.1	51 ± 2.6	111 ± 13.9	8 ± 3.5	25 ± 5.6	173 ± 8.7
5000.0	55 ± 7.6	93 ± 7.4	7 ± 1.5	29 ± 4.7	140 ± 22.7	38 ± 2.5	118 ± 7.6	10 ± 4.0	18 ± 7.4	170 ± 15.5
Positive Control[a]	510 ± 64.6	374 ± 84.6	1008 ± 67.8	113 ± 4.6	1571 ± 276.1	833 ± 53.1	729 ± 91.1	90 ± 13.7	114 ± 7.5	744 ± 304.2

[a]Positive controls; without metabolic activation 4-nitro-o-phenylene-diamine (TA-98, TA1537) sodium azide (TA100, TA 1535) and methyl methanesulfate (WP2 uvrA (pKM101)); with metabolic activation 2-aminoanhracene.

TABLE 2 In vitro mammalian cell micronucleus test with rehydrated lyophilized *A. soehngenii*

Concentration (ug/ml)	Relative cell growth (%)	Cytostasis (%)	Micronucleated cells frequency (%)	Statistical significant increase (a)
4-h exposure without metabolic activation				
C	104	0[a]	0.70	/
S	100	0	0.70	/
1000	87	13	0.85	−
2500	94	6	0.60	−
5000	88	12	0.90	−
MMS	105	0[a]	2.65	+
ColC	55	45	1.6	+
44-h exposure without metabolic activation				
C	100	0	0.35	/
S	100	0	0.35	/
1000	89	11	0.65	−
2500	82	18	0.50	−
5000	101	0[a]	0.55	−
MMS	94	6	3.15	+
ColC	7	93	4.05	+
4-h exposure with metabolic activation				
C	116	0[a]	0.60	/
S	100	0	1.10	/
1000	96	4	0.40	(+)
2500	104	0[a]	0.60	−
5000	117	0[a]	0.60	−
CPA	69	31	4.50	+

Note: C: negative control (culture medium). S: solvent control (PBS 10% v/v/in culture medium). (a): statistically significant increase compared to solvent control (Chi-square test, $p < 0.05$)
+: significant increase; −: not significant; (+): significant decrease. MMS: Methylmethanosulfate, Postivie control (without metabolic activation). ColC: Colchicine, Postive control (without metabolic activation). CPA: Cyclophosphamide, Positive control (with metabolic activation). Relative cell growth: 100 × ((CBPI Test control − 1)/(CBPI control − 1)). Cytostasis (%): = 100 − Relative cell growth (%).
[a]Cytostasis is defined 0 when relative cell growth exceeds 100%.

positive control compounds induced biologically relevant and statistically significant increases in the frequency of micronucleated binucleated cells under appropriate conditions, demonstrating the validity of the assay. Following the 4- and 44-h exposure periods, lyophilized *A. soehngenii* did not induce any statistically significant or dose-related increases in the frequency of micronucleated binucleated cells, compared with vehicle controls. Moreover, the frequencies of micronucleated binucleated cells under all conditions after exposure to the test item were within the corresponding vehicle control historical ranges. These results met the criteria for a negative response.

3.2 | Subchronic toxicity

3.2.1 | Clinical examinations, body weights, and food consumption

There were no test item-related deaths or clinical signs (data not shown). Two mortalities have been observed till study day 9 in the study that were most likely not test item related since all other animals in the mid dose (MD) and the high dose (HD) group showed no clinical signs. One female in the MD group was found dead on study day 4 in the cage and was already partly cannibalized by the other animals in the cage. On study day 9 one female in the HD group was in a moribund condition showing clinical signs like abnormal breathing, piloerection, half eyelid closure and reduced spontaneous activity. The following necropsy showed a marbled liver, and the adrenal glands were marbled and enlarged. The thorax was filled with a dark fluid and the lung was normal, but with a black mass located at the right median lobe. Macroscopic findings and microscopic findings showed that these were either due to natural events (for animal found dead on day four) or due to a handling error with gavage (for animal found in moribund condition). Therefore, these deaths were unrelated to the nature of the test item. There were no ocular changes and no test item-related findings observed during arena observations or in the modified Irwin test.

Body weights and food consumption throughout the dosing period are presented in Figures 1 and 2, respectively. An interesting

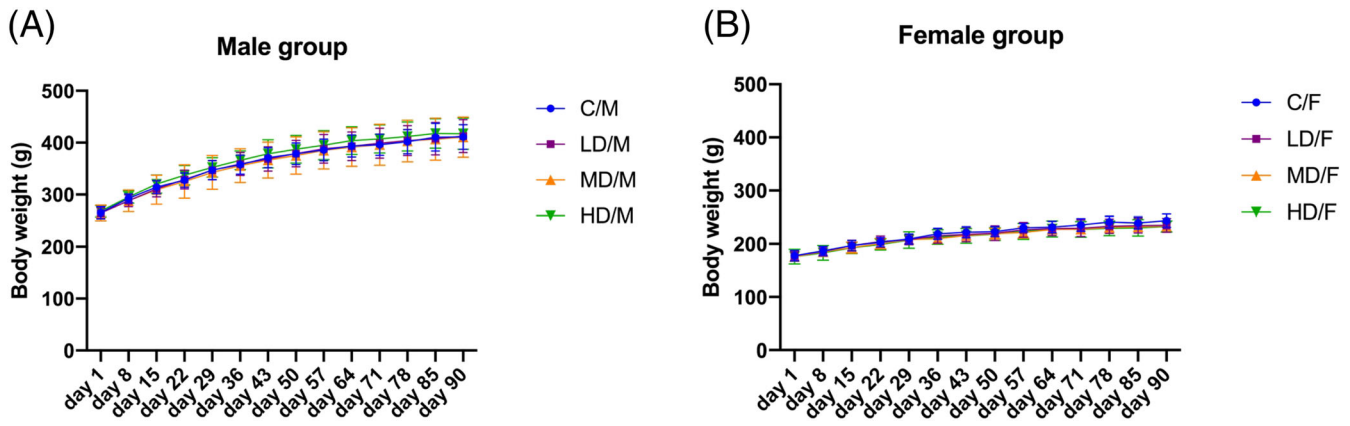

FIGURE 1 Body weights of male (A) and female (B) rats during the 90-day study

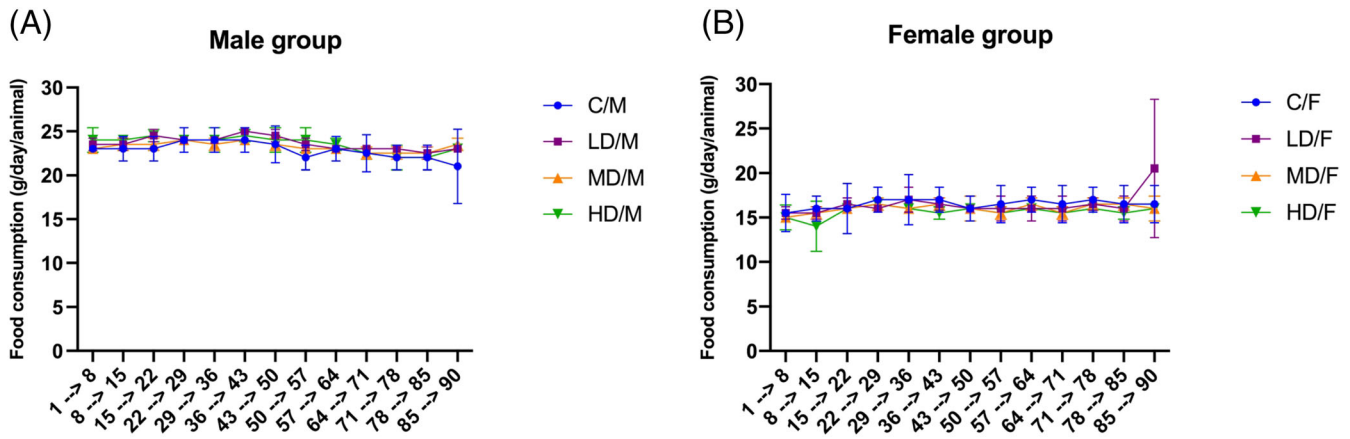

FIGURE 2 Food consumption of male (A) and female (B) rats during the 90-day study

observation was that for the female group a slight but significantly lower body weight change was observed in all dose groups when compared to the control. Nonetheless, the body weight increase falls within historical control data and since there are no other treatment related findings, this effect is considered test item related but not adverse.

The high dose, equivalent to 4.7–8.5 $\times$ 10^{11} live *A. soehngenii* cells/kg body weight/day did not induce any test-item related adverse events, establishing a NOAEL of at least 2 $\times$ 10^{11} cells per day.

3.2.2 | Hematology, coagulation and clinical chemistry

No test item-related differences were observed in values for hematology, coagulation and clinical chemistry. A statistically significant lower white blood cell count was observed in the female HD group, while females in all groups showed slight but significantly increased red blood cell count, hemoglobin content and hematocrit values (Table 3). All values, however, were within historical control range (historical data not shown) and no histopathological observations were made that would raise suspicion in related adverse events. A decrease in the amount of bile acids was observed for all groups when compared to controls, although only significant for the male MD and HD groups (Table 4). Also, the amount of creatinine was significantly decreased in the female MD group (28.3%) as well as the glucose level (27.3% below control) in the female HD group. All values were within historical control range and did not appear to be dose related.

3.2.3 | Macroscopic examinations, organ weights and histopathology

Organ weights relative to body weight are provided in Table 5. There were no differences in organ weights between controls and lyophilized *A. soehngenii*-dosed groups. There were also no test item-related macroscopic or microscopic findings (data not shown). The only findings observed being incidental and generally consistent with changes encountered in rats of this age and strain kept under laboratory conditions (data not shown). Histopathological examination revealed no aberrations in any of the organs.

TABLE 3 Hematology and coagulation values for rats administered rehydrated lyophilized *A. soehngenii* by oral gavage for 90 days

	Hematology and coagulation values (mean + standard deviation)							
	Males ($n = 10$ per group)				Females ($n = 10$ per group except group MD and HD with $n = 9$)			
		A. soehngenii dose				*A. soehngenii* dose		
Parameter	0 (vehicle control)	LD	MD	HD	0 (vehicle control)	LD	MD	HD
WBC ($\times 10^9$/L)	4.14 ± 1.08	4.13 ± 0.97	4.62 ± 0.90	4.68 ± 1.14[a]	3.071 ± 0.766	2.821 ± 1.127	3.068 ± 0.941	1.993 ± 0.575[a]
RBC ($\times 10^{12}$/L)	9.01 ± 0.40	9.47 ± 0.55	9.24 ± 0.57	9.21 ± 0.41	8.009 ± 0.349	8.544 ± 0.387[b]	8.636 ± 0.513[b]	8.528 ± 0.379[b]
Hb (g/dl)	15.53 ± 0.74	16.02 ± 0.81	15.91 ± 0.55	15.62 ± 0.38	13.93 ± 0.46	14.72 ± 0.57[b]	14.97 ± 0.45[b]	14.67 ± 0.46[b]
HCT (%)	47.35 ± 1.89	48.92 ± 2.48	48.67 ± 2.07	47.85 ± 1.21	42.85 ± 1.65	45.23 ± 1.41[b]	45.96 ± 1.81[b]	45.17 ± 1.31[b]
MCV (fL)	52.52 ± 1.26	51.66 ± 1.40	52.72 ± 2.20	51.96 ± 1.87	53.54 ± 1.69	52.99 ± 1.55	53.27 ± 1.46	53.03 ± 1.30
MCH (fmol)	17.19 ± 0.43	16.91 ± 0.39	17.23 ± 0.64	16.97 ± 0.59	17.40 ± 0.59	17.24 ± 0.36	17.37 ± 0.54	17.21 ± 0.42
MCHC (g/dl)	32.80 ± 0.61	32.75 ± 0.51	32.68 ± 0.77	32.65 ± 0.56	32.51 ± 0.36	32.56 ± 0.64	32.58 ± 0.47	32.47 ± 0.53
Platelets ($\times 10^9$/L)	658.10 ± 66.75	618.40 ± 57.01	638.00 ± 52.34	640.70 ± 74.68	678.00 ± 93.43	698.50 ± 48.89	701.88 ± 53.65	709.89 ± 44.72
Retic (%)	1.384 ± 0.202	1.488 ± 0.237	1.342 ± 0.223	1.464 ± 0.258	1.752 ± 0.331	1.691 ± 0.244	1.488 ± 0.271	1.634 ± 0.423
Neutrophils (%)	21.00 ± 6.63	20.39 ± 5.71	20.60 ± 6.83	19.09 ± 5.16	14.85 ± 3.80	20.70 ± 14.49	13.75 ± 4.26	17.02 ± 4.08
Lymphocytes (%)	75.44 ± 6.72	75.36 ± 6.19	75.92 ± 7.47	76.86 ± 5.41	82.03 ± 4.36	75.57 ± 14.15	82.92 ± 4.49	80.07 ± 4.38
Monocytes (%)	2.86 ± 0.71	3.20 ± 0.91	2.84 ± 1.01	3.03 ± 1.17	2.41 ± 1.19	2.82 ± 1.00	2.67 ± 0.50	2.28 ± 0.55
Eosinophils (%)	0.310 ± 0.152	0.590 ± 0.387	0.250 ± 0.126	0.450 ± 0.406	0.230 ± 0.176	0.410 ± 0.172	0.262 ± 0.176	0.277 ± 0.156
Basophils (%)	0.120 ± 0.042	0.160 ± 0.117	0.120 ± 0.078	0.120 ± 0.147	0.120 ± 0.122	0.130 ± 0.094	0.125 ± 0.070	0.111 ± 0.060
LUC (%)	0.770 ± 1.563	0.310 ± 0.119	0.290 ± 0.128	0.420 ± 0.370	0.310 ± 0.159	0.360 ± 0.283	0.275 ± 0.128	0.233 ± 0.206
PT (s)	24.26 ± 0.96	24.38 ± 1.26	24.40 ± 2.46	23.11 ± 1.40	26.78 ± 1.86	26.58 ± 1.53	27.27 ± 1.39	27.21 ± 1.45
aPTT (s)	14.72 ± 2.96	13.26 ± 2.66	14.72 ± 2.58	14.49 ± 2.43	12.13 ± 2.88	12.54 ± 2.41	12.32 ± 2.57	11.03 ± 1.31

Note: LD, low dose; MD, middle dose; HD, high dose.

Abbreviations: Hb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; n, number of animals; RBC, red blood cell count; Retic, absolute reticulocyte count; WBC, white blood cell count; LUC, large unstained cells; PT, prothrombin time; aPTT, activated partial thromboplastin time.

[a]Statistically significant decrease.

[b]Statistically significant increase.

TABLE 4 Clinical chemistry values for rats administered rehydrated lyophilized *A. soehngenii* by oral gavage for 90 days

	Clinical chemistry values (mean + standard deviation)							
	Males ($n = 10$ per group)				Females ($n = 10$ per group except group MD and HD with $n = 9$)			
		A. soehngenii dose				*A. soehngenii* dose		
Parameter	0 (vehicle control)	LD	MD	HD	0 (vehicle control)	LD	MD	HD
Sodium (mmol/L)	117.80 ± 19.34	126.20 ± 22.42	123.20 ± 24.92	128.40 ± 17.84	132.30 ± 9.82	131.70 ± 16.26	128.00 ± 27.46	119.11 ± 28.53
Potassium (mmol/L)	3.596 ± 0.646	3.793 ± 0.643	3.760 ± 0.595	3.703 ± 0.426	3.634 ± 0.323	3.480 ± 0.408	3.472 ± 0.547	3.926 ± 1.173
AP (U/L)	63.696 ± 23.796	67.347 ± 30.871	60.026 ± 17.411	70.468 ± 20.067	46.741 ± 15.118	37.003 ± 11.420	43.465 ± 22.407	40.694 ± 18.822
ASAT (U/L)	72.49 ± 21.13	72.73 ± 18.85	66.57 ± 38.63	63.63 ± 15.92	76.70 ± 14.59	72.90 ± 10.95	63.38 ± 21.12	58.06 ± 19.40
ALAT (U/L)	27.49 ± 7.54	33.17 ± 10.74	33.32 ± 34.30	24.77 ± 5.99	30.08 ± 8.20	27.51 ± 5.34	24.85 ± 4.19	21.64 ± 6.19
Urea (mmol/L)	7.682 ± 1.241	7.141 ± 1.119	7.015 ± 0.818	6.680 ± 0.747	7.060 ± 0.254	6.918 ± 0.768	6.447 ± 0.693	6.341 ± 1.350
Creatinine (μmol/L)	16.60 ± 5.52	14.80 ± 5.12	13.20 ± 4.24	14.50 ± 4.40	20.30 ± 2.31	17.70 ± 3.77	14.56 ± 6.15[a]	14.78 ± 7.79
Total protein (g/L)	54.59 ± 5.67	57.74 ± 7.33	58.28 ± 7.50	59.18 ± 6.09	60.08 ± 3.63	60.36 ± 4.32	58.93 ± 8.60	58.00 ± 7.86
Albumin (g/L)	27.786 ± 4.704	30.015 ± 5.210	29.544 ± 5.805	30.544 ± 4.662	32.966 ± 2.307	33.480 ± 3.396	31.795 ± 7.411	30.914 ± 6.778
Total bilirubin (μmol/L)	1.99 ± 0.28	2.16 ± 0.27	2.05 ± 0.23	2.11 ± 0.24	2.41 ± 0.33	2.48 ± 0.26	2.70 ± 0.47	2.61 ± 0.53
Total bile acids (μmol/L)	27.791 ± 15.469	15.690 ± 8.816	15.027 ± 10.037[a]	12.125 ± 3.618[a]	22.002 ± 13.448	13.736 ± 6.495	19.035 ± 9.339	19.353 ± 17.362
Triglyceride (mmol/L)	0.639 ± 0.265	0.724 ± 0.215	0.671 ± 0.323	0.666 ± 0.290	0.278 ± 0.110	0.309 ± 0.126	0.297 ± 0.068	0.217 ± 0.073
HDL (mmol/L)	1.157 ± 0.137	1.231 ± 0.181	1.136 ± 0.158	1.138 ± 0.113	0.734 ± 0.213	0.715 ± 0.159	0.834 ± 0.092	0.780 ± 0.150
LDL (mmol/L)	0.236 ± 0.126	0.413 ± 0.273	0.298 ± 0.251	0.281 ± 0.140	0.146 ± 0.103	0.156 ± 0.106	0.184 ± 0.050	0.166 ± 0.088
Total cholesterol (mmol/L)	1.473 ± 0.247	1.647 ± 0.435	1.556 ± 0.351	1.552 ± 0.238	0.936 ± 0.305	0.913 ± 0.257	1.078 ± 0.080	0.990 ± 0.228
Glucose (mmol/L)	9.219 ± 1.517	10.383 ± 2.549	10.821 ± 2.452	9.698 ± 1.717	7.197 ± 1.542	6.524 ± 1.409	5.742 ± 1.282	5.230 ± 1.444[a]

Abbreviations: A/G ratio, albumin/globulin ratio; AP, alkaline phospahtase; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; bw, body weight; *n*, number of animals.
[a]Statistically significant decrease.

TABLE 5 Organ weights relative to body weight for rats administered rehydrated lyophilized *A. soehngenii* by oral gavage for 90 days

	Organ weights relative to body weight (mean + standard deviation)							
	Males ($n = 10$ per group)				Females ($n = 10$ per group except group 4 with $n = 9$)			
		Lyophilized *A. soehngenii* (mg/day)				Lyophilized *A. soehngenii* (mg/day)		
Organ	0 (vehicle control)	13.2	66	330	0 (vehicle control)	13.2	66	330
Adrenals	0.0188 ± 0.0030	0.0178 ± 0.0029	0.0158 ± 0.0026	0.0153 ± 0.0019	0.0382 ± 0.064	0.0416 ± 0.0088	0.0371 ± 0.0065	0.0416 ± 0.0052
Brain	0.5585 ± 0.0295	0.5817 ± 0.0502	0.5703 ± 0.0564	0.5413 ± 0.0353	0.8456 ± 0.0510	0.8747 ± 0.0609	0.8719 ± 0.0441	0.8453 ± 0.0507
Epididymides	0.4062 ± 0.0625	0.4497 ± 0.0567	0.4302 ± 0.0831	0.3916 ± 0.0659	NA	NA	NA	NA
Heart	0.3097 ± 0.0296	0.2955 ± 0.0324	0.3005 ± 0.0304	0.2846 ± 0.0252	0.3570 ± 0.0365	0.3731 ± 0.0225	0.3603 ± 0.0462	0.3620 ± 0.0181
Kidneys	0.6488 ± 0.0554	0.6538 ± 0.0526	0.6519 ± 0.0622	0.9557 ± 0.0346	0.7199 ± 0.0400	0.7928 ± 0.1133	0.7366 ± 0.0322	0.7233 ± 0.0494
Liver	2.4037 ± 0.2328	2.3894 ± 0.2637	2.5033 ± 0.1859	2.3228 ± 0.1680	2.5079 ± 0.1582	2.6168 ± 0.1235	2.4857 ± 0.1372	2.5058 ± 0.1813
Ovaries	NA	NA	NA	NA	0.0729 ± 0.0263	0.0686 ± 0.0147	0.0675 ± 0.0140	0.0642 ± 0.0174
Pituitary	0.0029 ± 0.0015	0.0025 ± 0.0007	0.0023 ± 0.0007	0.0021 ± 0.0003	0.0051 ± 0.0015	0.0055 ± 0.0013	0.0051 ± 0.0007	0.0056 ± 0.0005
Prostate	0.7663 ± 0.0491	0.8538 ± 0.0776	0.7656 ± 0.0826	0.7519 ± 0.1076	NA	NA	NA	NA
Spleen	0.2090 ± 0.0227	0.2179 ± 0.0301	0.1970 ± 0.0224	0.2163 ± 0.0317	0.2647 ± 0.0245	0.2825 ± 0.0200	0.2787 ± 0.0492	0.2853 ± 0.0453
Testes	0.9275 ± 0.0356	0.9435 ± 0.1041	0.9294 ± 0.0963	0.9150 ± 0.0701	NA	NA	NA	NA
Thymus	0.1188 ± 0.0221	0.1141 ± 0.0142	0.1132 ± 0.0164	0.1068 ± 0.0200	0.1639 ± 0.0336	0.1635 ± 0.0229	0.1616 ± 0.0251	0.1495 ± 0.0216
Uterus	NA	NA	NA	NA	0.4536 ± 0.1181	0.4373 ± 0.1477	0.4363 ± 0.1180	0.3845 ± 0.1213

Abbreviations: *n*, number of animals; NA, not applicable.

4 | DISCUSSION

In a study to evaluate the influence of microbiota on metabolic processes, *A. soehngenii* (previously known as *E. hallii*; Shetty et al., 2018) emerged as a bacterial species capable of converting sugars as well as lactate and acetate into butyrate that has the potential to positively influence insulin sensitivity. These findings prompted the development of *A. soehngenii* as a novel type of probiotic. Traditionally probiotic strains belong to the genera *Lactobacillus* and *Bifidobacterium* and have since long been part of our food and their health beneficial role has been widely recognized (Aryana & Olson, 2017). More recently, the findings that specific gut microbiota related bacteria show health beneficial traits has spurred interest in further development and use of these bacteria (Douillard & De Vos, 2019). Although most of these bacteria reside in the gut of many human individuals, they are not part of our traditional food chain. Notably, *Anaerobutyricum* spp. have been identified as bacteria that are found in early life and persist in the colon of adults (Duncan et al., 2004). Therefore, the safety of these microorganisms for human consumption needs to be carefully evaluated (Brodmann et al., 2017).

With the current technological state of the art the first analysis to determine the safety of a strain would be a full analysis of the genome to show absence of virulence and pathogenic factors and toxins and the absence of genetically transmissible traits such as antibiotic resistance. The genome of *A. soehngenii* CH106 has been fully sequenced and shown to be free of any virulence or pathogenic factors and genetically transmissible traits. Other important factors that need to be considered are the origin of the strain, if it has been genetically modified and expected exposure levels. By following a comprehensive check list it can be assessed if the strain is suited for safe use for human consumption (Pariza et al., 2015). If the strain has not previously been part of the food chain the absence of undesirable physiological effects needs to be evaluated through appropriately designed safety studies. These studies are the subject of this paper and include potential genotoxicity and subchronic toxicity upon prolonged exposure.

We consume live bacteria through a variety of products (mainly yoghurts, but also other fermented products such as kimchi, sauerkraut, kombucha, and kefir). Since these bacteria have been part of our daily food for a long time and thus were in use as food additive or supplement before May 15, 1997 when the EU regulation on novel food came into force (European Union, 1997), they do not require registration as novel food. More importantly, EFSA established a framework to classify bacteria as safe and bacteria that are placed on this list of Qualified Presumption of Safety (QPS) can be introduced into the food chain without prior novel food application. In 2011 EFSA considered *Clostridium butyricum* for an updated QPS list but based on the observation that some strains contained a gene coding for botulinum neurotoxin concluded that the safety of *C. butyricum* is a strain-related property and the species should therefore not be recommended for the QPS list. The Japanese company Miyarisan was considering introduction of their proprietary *C. butyricum* strain CBM588 "to be marketed as viable spores in tablet form (with a maximum dosing of 5.10^7 spores per tablet), intended for use as a probiotic food supplement or support, maintain or restore healthy gut flora physiology and/or function." Although, *C. butyricum* appears to have been marketed as a probiotic in Asia for half a century, it was ruled that QPS did not apply and introduction of this bacterium to the European market required a full novel food assessment. A novel food application was filed shortly after and *C. butyricum* spores were approved as a novel food in 2014 (European Union, 2014). This the first, and to date the only live bacterium that has been approved for use as a novel food under the EU regulation 258/97 (superseded by EU regulation 2015/2283; European Union, 2015).

A. soehngenii CH106 is derived from the type strain L2-7^T, isolated from a healthy infant (Barcenilla et al., 2000), and is intended to be introduced as a food ingredient or food supplement in a live format to support glucose metabolism in people that are at risk of developing type 2 diabetes. A first clinical study was performed to show that prolonged daily intake of *A. soehngenii* L2-7^T was safe (Gilijamse et al., 2020). Three groups of 9 volunteers that had been diagnosed with metabolic syndrome received a daily dosage for 4 weeks, each group receiving a different concentration. Even at the highest daily dose of 10^{10} live bacteria per day no adverse events were recorded. In this study *A. soehngenii* L2-7^T was provided in a frozen format and had to be thawed before being ingested with milk. This results in exposure to oxygen and leaves the bacteria unprotected against the harsh conditions of the stomach.

In a recent study a thawed solution of *A. soehngenii* L2-7^T was administered directly to the duodenum of volunteers that had been diagnosed with metabolic syndrome through a nasal duodenal tube. The most remarkable outcome of this trial was that a single dose of *A. soehngenii* L2-7^T resulted in an increased postprandial GLP-1 response and altered small intestinal gene expression, resulting in improved human glucose metabolism and giving some indication toward the mode of action (Koopen et al., n.d.). Also, in this trial no treatment related adverse events were recorded.

A. soehngenii CH106 is intended to be used as a dietary ingredient with a daily intake of at least 10^9 live bacteria. The tests described in this paper have shown that the bacterium does not give rise to any genotoxic effects. The rat study showed that intake of up to $3.3–5 \times 10^{11}$ live *A. soehngenii* cells/kg body weight/day did not result in any adverse events. Translated to human use the observed NOAEL provides a safe dosage for humans of at least 1.2×10^{13}, which exceeds the recommended dosage in food more than 100-fold.

In conclusion, the results of the toxicity studies, combined with the observations from the clinical studies, support the safety of *A. soehngenii* CH106 for oral intake as a food ingredient or food supplement.

CONFLICT OF INTEREST

The authors did not report any conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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