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Research Article

A Toxicological Assessment of a Single-cell Protein from *Methylovorus menthalis* J25 (Psomi™)

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ABSTRACT

Objectives: Protein is an essential macronutrient required for metabolic and physiological functions. As the global population expands, identifying sustainable protein sources is increasingly important for meeting rising demands in animal feed and human food markets. *Methylovorus menthalis* J25 (Psomi™) is a single-cell protein derived from *Methylovorus menthalis* that has been reported as a sustainable protein source. This study evaluated the toxicity of J25 through a battery of toxicological studies conducted in accordance with international guidelines.

Materials and Methods: These studies evaluated the potential mutagenicity (Ames test, *in vitro* and *in vivo* mammalian chromosomal aberration assays), genetic toxicity (*in vitro* mammalian cell gene mutation) and acute, sub-acute and sub-chronic oral toxicity of J25 in Sprague Dawley rats.

Results: There was no evidence of genetic toxicity or mutagenicity in the *in vitro* studies. An acute oral LD_{50} of >5000 mg/kg Body Weight (bw) was established. There were no mortalities or any treatment-related adverse effects in the 14-day repeat dose oral toxicity study, wherein rats received low, mid, or high doses of J25 (2500, 3750, or 5000 mg/kg bw/day, n=5 rats/sex/group). These findings were repeated in the subchronic repeat-dose 90-day oral toxicity study at the same daily doses of J25 (n=10 animals/sex/group).

Conclusion: Therefore, the No-Observed-Adverse-Effect-Level (NOAEL) was established to be ≥5000 mg/kg bw. The results of these safety studies support the safe use of DeNova's protein-rich biomass ingredient (J25) for use as a novel, sustainable protein source in animal feed, companion animal supplements and human foods.

Keywords: *Methylovorus menthalis* J25; J25; Protein; Single-cell protein; Preclinical; Rodent; Safety assessment; Safety assessment; Toxicology assessment

INTRODUCTION

Protein is a critical dietary macronutrient that is required for various metabolic and physiologic functions, including the regulation of appetite, food intake, body weight, and body composition. The predominant dietary sources for proteins of animal origin are meat, fish, eggs, milk and milk products, while cereal grains, leguminous vegetables, and nuts are the main dietary sources of plant proteins [1]. In recent years, there has been an increasingly high demand for novel and sustainable protein sources to replace animal-based sources, with the most exploited non-meat protein sources including algae, insects and fungi [2]. Fermentation of single-cell organisms, including bacteria, yeasts, fungi and microalgae, grown in bioreactors to create biomass is becoming a mainstream method for manufacturing animal-free protein. Proteins manufactured in this manner are known as Single-Cell Proteins (SCPs) [3]. SCPs are established as having high biomass yields while also utilizing less energy and water, thus serving as a novel, promising and sustainable alternative protein sourcing strategy with several sources already approved for human and animal use [4]. For example, cultured proteins have been shown to be safe alternatives to fishmeal protein in numerous aquaculture species, and a cultured protein derived from *Methylococcus capsulatus* (FeedKind Pet®) has been shown to be safe as a protein source in dog feed [5].

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Naturally-occurring bacteria from the *Methylophilaceae* family are of particular interest for nutritional applications due to their ability to convert the carbon and energy from singlecarbon (C1) substrates (e.g., methanol) into protein-rich biomass [6]. Within the *Methylovorus* genus, there have been three species described including M. *glucosotrophus*, M. *mays*, and M. *menthalis* [7]. M. *menthalis* was first isolated by Russian scientists from the roots of corn mint, where it is naturally occurring in the rhizosphere of the plant [8]. The present study evaluated a SCP meal produced from the naturally-occurring microorganism, *Methylovorus menthalis* (strain J25, also known as Psomi™), isolated from a soil sample in New Brunswick, Canada (PCT/IB2022/054504) [6]. Under optimized continuous aerobic fermentation, J25 demonstrated rapid growth and accumulation of protein-rich biomass when grown on non-food C1 methanol. M. *menthalis* J25 is heat-killed and does not contain any viable cells. On a dry basis, J25 consists of 75%-85% crude protein with the remainder as lipids, ash, and carbohydrates. SCP from J25 has already been investigated in aquaculture. In one study, SCP meal from J25 was included in Atlantic salmon pre-smolt feeds at up to 30% over a 12-week supplementation period and shown to have no effects on most production performance metrics (e.g., fork length, condition factor, survival, haematocrit, red blood cell counts, viscerosomatic index, feed conversion ratio, protein efficiency ratio). Furthermore, the apparent feed intake was sufficient to support adequate growth and nutrient utilization, resulting in no effects on fish health as assessed by blood plasma biochemistry, and intestinal histopathology [6]. Similarly, there were little to no impacts on growth performance, nutrient utilization, fish health, and fillet quality in a second study, wherein J25 replaced up to 30% of traditional protein ingredients in diets of seawater phase Atlantic salmon (0.4 kg–2 kg) over a 19-week period [9]. While there have been no studies investigating the safety of J25 in humans or other mammals, Palberg et al. reviewed Methylobacteria and considered the genus to be ubiquitous in nature and non-pathogenic to humans or wildlife. Methylvorous species have primarily been isolated from plants, grassland soil, and water and waste-water samples, with no documented involvement of J25 with adverse health effects in humans [10,11].

Given the need for renewable sources of protein in animal feed and human food markets, J25 represents a viable source of protein by utilizing naturally occurring bacteria that are heat-killed and condensed and dried into a powder. However, in order to justify the use of J25 as an animal feed ingredient or dietary ingredient for use in humans, its safety profile must be thoroughly characterized. Regulatory bodies, such as the US FDA, Health Canada and the CFIA, require that new ingredients be either an approved food additive or GRAS for its intended use; that a New Dietary Ingredient (NDI) be notified; or a Natural Health Product, Novel Food, or novel feed ingredient be registered with the appropriate agency. These premarket gates ensure that novel food and feed ingredients have been assessed to minimize safety risks to humans and animals.

While it can be suggested that the general toxicity of J25 would be low based on its composition (composed predominantly of protein); data from existing feeding studies; and a general lack of reports of toxicity, it is necessary to comprehensively characterize and publish the safety of this novel ingredient for "General Recognition as Safe" requirement in animal and human foods [6,9]. As such, the aim of this study was to evaluate the safety of heat-killed M. *menthalis* J25 biomass (Psomi™) consumption through a battery of toxicological studies conducted in accordance with international guidelines, including mutagenicity (bacterial reverse mutation and *in vitro* mammalian chromosomal aberration), genotoxicity (*in vitro* mammalian cell gene mutation) and acute, sub-acute and sub-chronic oral toxicity studies. Collectively, the results demonstrate a lack of genotoxicity and toxicity in *in vivo* studies, with results supporting a No-Observed-Adverse-Effect-Level (NOAEL) of the highest dose (5000 mg/kg bw/day) tested in all studies. Therefore, the results support the safe profile of this novel SCP meal derived from M. *menthalis* J25 (Psomi™).

MATERIALS AND METHODS

Test article

Methylovorus menthalis J25 biomass (Psomi™) was provided by DeNova, Inc. (Dartmouth, Canada). The lot number for J25 used in these studies was T431 E D2P 01. The certificate of analysis reported a protein assay of 84% w/w by Kjeldahl (N*6.2) and 56% protein by BCA, while total amino acid content was 63.14% (w/w). Based on the results of pre-formulation trials conducted as part of an analytical validation study, ultrapure Type I water was selected as the vehicle for preparation of J25 formulations. All formulations were prepared fresh prior to administration, and dose confirmation analyses were performed using the Bicinchoninic Acid (BCA) protein assay to verify formulation concentrations. All experimental work was carried out at Anthem Biosciences Pvt. Ltd., Department of Preclinical Research, Bommasandra, Bangalore.

In vitro mutagenicity and genetic toxicity studies

Ames test

This study was performed in accordance with OECD Test Guideline No. 471 (2020) and complied with the OECD Principles of Good Laboratory Practice (GLP) (No. 1, 1997). All reagents and culture media were prepared in strict accordance with the guideline requirements, with no modifications to their composition [12]. The bacterial tester strains were selected in line with OECD 471 and comprised *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 (Molecular Toxicology, Inc.).

The main mutagenicity study was carried out at concentrations of 5000.0 µg, 1580.0 µg, 500.0 µg, 158.0 µg and 50.0 µg of J25/plate. Testing was performed using both the pre-incubation method, consisting of a 30-minute incubation at 35°C prior to plating, and the standard plate incorporation method (no pre-incubation period), with and without a metabolic bioactivation system (rat liver S9 microsomal fraction). All assays were carried out in triplicate, and vehicle, positive, and sterility controls were tested concurrently. Following incubation for 48–72 hours at 37 ± 1°C, bacterial revertant colonies were counted manually. Mutagenic activity was evaluated using the mutagenicity factor, calculated as the ratio of revertant colonies in the test or control group to those in the vehicle control. For strains TA98, TA1535, and TA1537, a minimum three-fold increase in revertant colonies relative to the vehicle control was considered indicative of mutagenicity. For strains TA100 and TA102, a two-fold increase was considered a positive response. In addition, the test item was deemed mutagenic if a reproducible, dose-dependent increase in revertant colony numbers was observed in one or more tester strains.

In vitro mammalian cell gene mutation test

This study was performed in accordance with OECD guidelines

(No. 490, 2016) and complied with OECD GLP (No. 1, 1997) [13]. Cultured mouse lymphoma L5178Y $TK^{+/-}$ 3.7.2C cells were used at a density of 6×10^6 cells per culture, with two replicate cultures per treatment condition. Cells were exposed to J25 at concentrations of 625 µg/mL, 312.50 µg/mL, 156.25 µg/mL, and 78.13 µg/mL, vehicle control (10% v/v autoclaved ultrapure water), or positive controls for approximately 4 hours, in the presence or absence of an exogenous metabolic activation system (S9).

Following treatment, cultures underwent a 2-day expression period before being plated for mutant selection. For non-selective growth, cells were plated in the absence of Trifluorothymidine (TFT) at approximately 1.6 cells per well (two plates per culture). For TFT-selection, cells were plated in the presence of TFT (3 µg/mL) at approximately 2,000 cells per well (four plates per culture). Mutant enumeration was performed after an 11-day incubation period.

Chromosome aberration

The *in vitro* mammalian chromosomal aberration study was conducted in accordance with OECD guidelines (No. 473, 2016) and in compliance with the OECD Principles of GLP (No. 1, 1997). Chinese hamster (*Cricetulus griseus*) ovarian CHO-K1 cells were used (11 passages). The CHO-K1 cells used were originally obtained from ATCC (lot number 61262645, catalog number CCL-61TM). The cells were subsequently cultured in the laboratory and stored in a liquid nitrogen tank. J25 biomass was tested for induction of chromosomal aberrations at 156 µg/mL, 313 µg/mL and 625 µg/mL. The CHO-K1 cells were exposed to J25 for a short term (~4 hours) in the absence (experiment 1) or presence (experiment 2) of S9, and long term (~24 hours) in the absence of S9 (experiment 3). Positive controls included Mitomycin c at 0.4 µg/mL for experiments 1 and 3 and cyclophosphamide monohydrate at 5 µg/ml for experiment 2, while the vehicle control was 10% v/v Autoclaved Ultrapure Water. All experiments were conducted in duplicate cultures. The cells were incubated at 37 ± 1 °C and 5 ± 1 % CO_2 in a CO_2 incubator. Approximately 2 hours before harvesting, 50 µL of Colcemid™ solution (9 µg/mL) was added to all cultures, the cells were then harvested and washed and trypsinized. The cells were then incubated until they detached, and trypsinized cells were added to labelled tubes containing culture medium. Cells were collected by centrifugation and mixed with 2 mL of hypotonic solution (0.8% Tri sodium citrate dihydrate) and incubated for 20 minutes at 37 ± 1 °C. Post incubation, a fixative solution was added and the cells were collected by centrifugation. Total cell count was then performed to determine the cytotoxicity and 300 stained metaphases were evaluated for occurrence of chromosomal aberrations for each concentration except for Mitomycin c where it was 50.

The *in vivo* study was conducted in accordance with OECD guidelines (No. 475, 2014) and in compliance with the OECD Principles of Good Laboratory Practice (GLP) (No. 1, 1997) [14]. Male (n=35) and female (n=35) Sprague Dawley (CD (SD) IGS) rats (7-9 weeks old at start of treatment) were sourced from Hyalasco Biotechnology (India) and randomly assigned to 5 groups: Vehicle containing ultrapure water (n=5 rats/sex), positive control group receiving cyclophosphamide (40 mg/kg i.p., n=5 rats/sex), or J25 at 500 mg/kg b.w/day (250 mg/kg b.w b.i.d.), 1000 mg/kg b.w/day (500 mg/kg b.w/day b.i.d.), or 2000 mg/kg b.w./day (1000 mg/kg b.w b.i.d., n=5 rats/sex). All treatment groups received their respective test item formulations via oral gavage at a dose volume of 10 mL/kg body weight. Throughout the study, rats were monitored for clinical signs, morbidity and mortality, and changes in body weight. Prior to sacrifice, all animals were administered the metaphase-arresting agent colchicine (4 mg/kg, intraperitoneally) approximately 2 hours to 5 hours before euthanasia. After sacrifice by carbon dioxide (CO_2) inhalation, bone marrow samples were collected from both femurs of all animals. The femora were removed, the epiphyses were cut off and the marrow was processed for histological examination. A minimum of two slides per rat were stained with 10% (v/v) Giemsa solution and examined for chromosomal and chromatid aberrations. For each animal, at least 200 evaluable metaphase cells were scored for structural abnormalities. Cytotoxicity was assessed by determining the mitotic index, calculated from the evaluation of 1,000 cells per animal.

In vivo animal studies

Animal Ethics

All studies involving animals were reviewed and approved by The Institutional Animal Ethics Committee (IAEC). Approval protocol numbers for each of the studies are as follows: IAEC protocol numbers ABD/IAEC/PR/310-23-24 (*in vivo* mammalian chromosome aberration test); ABD/IAEC/PR/294-23-24 (maximum-tolerated dose study); ABD/IAEC/PR/296-23-24 (14-day study); and ABD/IAEC/PR/332-24-25 (90-day study). The care of animals complied with the regulations of the Committee for the Control and Supervision of Experiments on Animals (CCSEA) guidelines for laboratory animal's facility published in the gazette of India, 1998 and Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Animals, housing conditions and diet

Male and/or female Sprague Dawley rats [CD(SD)IGS] were obtained from Hylasco Biotechnology (India) Pvt. Ltd., Telangana, and acclimated prior to randomization. Before study initiation, all animals were examined by a veterinarian to confirm their health status, ensuring that only healthy rats were enrolled. Animals were housed under standard laboratory conditions in an environmentally controlled, air-conditioned room with adequate fresh air circulation (10–15 air changes per hour). Across the three *in vivo* studies, room temperature and relative humidity were within ranges of 20.0°C–24.7°C and 44%–70%, respectively, under a 12-hour light/dark cycle. Rats were housed in standard polycarbonate cages with clean, autoclaved corncob bedding. Drinking water and gamma-irradiated feed (Altromin Spezialfutter GmbH & Co. KG) were provided ad libitum.

Acute oral toxicity study

This study was conducted in accordance with OECD Test Guideline 423 (Acute Oral Toxicity – acute toxic class method, 2001) and in compliance with OECD Principles of GLP (No. 1, 1997) [15]. Female Sprague Dawley rats (n=3), aged 8-9 weeks old at the time of dosing, were fasted overnight prior to treatment. Animals were dosed by oral gavage using a stepwise procedure, beginning with administration of J25 at 5000 mg/kg (1250 mg/kg bw q.i.d., n=1), followed by a confirmatory step in two additional animals at the same dose level of 5000 mg/kg (1250 mg/kg bw q.i.d., n=2). All doses were administered at a volume of 10 mL/kg body weight. The presence or absence of treatment-related mortality at each step determined progression to subsequent dosing. Following administration, animals were observed for clinical signs at 20–30 minutes, 1 hour (±10 minutes), 2 hours (±10 minutes), 3 hours (±10 minutes), and 4 hours (±10 minutes) post-dose on Day 1, and once daily thereafter for a total of 14 days. Animals were monitored twice

daily throughout the study period for morbidity and mortality, and body weights were recorded at regular intervals. On Day 15, all surviving animals were euthanized by carbon dioxide inhalation and subjected to a gross necropsy examination.

Repeat-dose 14-day oral toxicity study

This study was conducted in compliance with the OECD Principles of GLP (No. 1, 1997). A total of 40 rats, comprising both males and females (20 animals per sex), aged 5 weeks–6 weeks at the time of dosing, were randomly allocated to one of four treatment groups. Animals received either the vehicle control (ultrapure water) or J25 at low (2500 mg/kg body weight [625 mg/kg bw, q.i.d.]), mid (3750 mg/kg body weight [937.5 mg/kg bw, q.i.d.]), or high (5000 mg/kg body weight [1250 mg/kg bw, q.i.d.]) dose levels, with five rats per sex per group. The test item was administered four times daily (q.i.d.) by oral gavage for 14 consecutive days at a dose volume of 10 mL/kg body weight. All animals were monitored daily for clinical signs, twice daily for morbidity and mortality, and subjected to a detailed clinical examination once weekly. Body weights and feed consumption were recorded at weekly intervals. At the end of the treatment period (Day 15), animals were fasted, weighed and blood samples were collected from the retro-orbital plexus under mild isoflurane anesthesia for hematology, coagulation and clinical chemistry assessments. Animals were subsequently euthanized by carbon dioxide inhalation and underwent a comprehensive gross necropsy. This examination included evaluation of the external body surface, all natural orifices, and the cranial, thoracic, and abdominal cavities and their contents. Specified organs were collected and preserved for histopathological evaluation following hematoxylin and eosin staining. As no treatment-related lesions were observed in the high-dose group, histopathological examination was limited to tissues collected from the vehicle control and high-dose groups and was not extended to the low- or mid-dose groups.

Repeat-dose 90-day oral toxicity study

This study was conducted in accordance with OECD Test Guideline 408 (adopted 25 June 2018) and in compliance with the OECD Principles of GLP (No. 1, 1997) [16]. A total of 80 Sprague Dawley rats (40 males and 40 females), aged 5 weeks–6 weeks at the initiation of treatment, were randomly assigned to one of four treatment groups. Animals received either the vehicle control (ultrapure water) or J25 at low (2500 mg/kg body weight [625 mg/kg bw, q.i.d.]), mid (3750 mg/kg body weight [937.5 mg/kg bw, q.i.d.]), or high (5000 mg/kg body weight [1250 mg/kg bw, q.i.d.]) dose levels, with 10 rats per sex per group. The vehicle and test item formulations were administered by oral gavage at a dose volume of 10.0 mL/kg body weight, four times daily (q.i.d.) at intervals of 4 hours–5 hours, for 90 consecutive days. All animals were monitored throughout the study for signs of toxicity, including mortality and morbidity, clinical observations, body weight changes, and feed consumption. Additional evaluations included ophthalmological examinations, cage rotation, and neurological assessments incorporating a functional observation battery, all conducted during Week 13. Clinical pathology assessments comprised urinalysis (Week 13) and hematology, clinical chemistry, coagulation, and hormonal analyses performed on Day 91. Terminal vaginal cytology was evaluated on Day 90. Serum concentrations of triiodothyronine (T3), thyroxine (T4), and Thyroid-Stimulating Hormone (TSH) were quantified using ELISA kits, with standard samples analyzed in duplicate. All surviving animals were euthanized by carbon dioxide inhalation on Day 91 and subjected to a comprehensive gross necropsy, including examination of the external body surface, all natural orifices, and the cranial, thoracic and abdominal cavities and their contents. Selected organs were weighed and processed for histopathological examination following hematoxylin and eosin staining. An overview of the experimental timeline is presented in Figure 1.

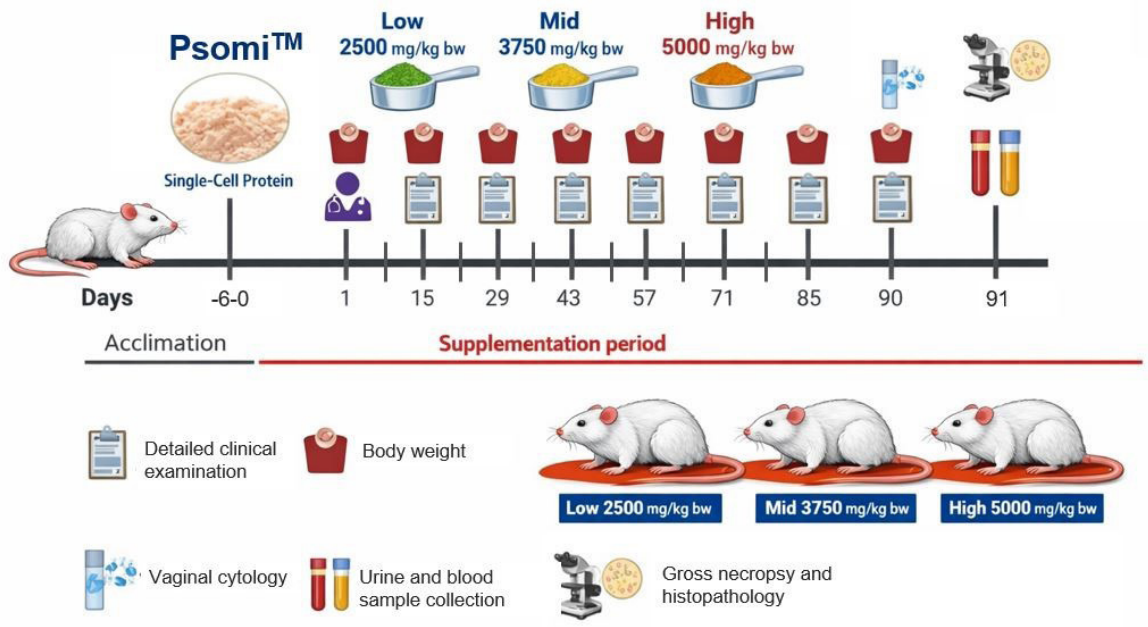

Figure 1: Timeline and experimental procedures for the subchronic 90-day oral toxicity study assessing the safety of J25. Animals were assessed by a veterinarian on day 1, and only healthy animals were included in the study. Detailed clinical examinations and body weights were measured on Days 1, 8, 15, 22, 29, 36, 43, 50, 57, 64, 71, 78, 85, 90 for all animals throughout the experimental period. An ophthalmological examination, and a neurological examination/functional observation battery of tests were also conducted in the 13th week of the study. Urinalysis was completed during the 13th week of the study using metabolic cages.

Statistics

Statistical analyses were conducted using Graph Pad Prism (version 5.03; Graph Pad Software). Data were analyzed using either an unpaired, two-tailed Student's t-test or one-way Analysis Of Variance (ANOVA) followed by Dunnett's post hoc test, as appropriate for the experimental design and group comparisons. In the chromosome aberration assays, Fisher's exact test was applied to compare the total number of aberrant cells in each treatment group (*in vitro*) and mean percentage aberrant cells and mean percent aberration per metaphase (*in vivo*) with the vehicle control. Linear regression analysis was performed for parameters showing statistically significant differences across treatment groups relative to the vehicle control. For hormonal analyses (T3, T4, and TSH), the mean of duplicate absorbance readings for each standard was plotted against concentration using nonlinear regression with sigmoidal curve fitting in GraphPad Prism (version 5.03). Concentrations of unknown samples were determined by interpolation from the standard curves and adjusted according to the appropriate dilution factors. Male and female data were analyzed separately. Outliers were identified using Grubbs' test. All statistical analyses were performed at a 95% confidence level, with significance set at $P < 0.05$.

RESULTS

In vitro studies

Ames test

The mutagenic potential of J25 was evaluated by assessing its ability to induce reverse mutations in *Salmonella typhimurium* strains in both the absence and presence of metabolic activation (S9). An initial range-finding study was conducted to identify appropriate concentrations for the main assay. Results from the preliminary study indicated that the highest tested concentration of J25 (5000.0 µg/plate) did not produce cytotoxic effects using either the plate incorporation or pre-incubation methods, with or without S9 activation (data not shown). Consequently, 5000.0 µg/plate was selected as the top concentration for the main study. In the main experiment, the mean number of histidine-positive (His^+) revertant colonies in J25-treated groups was comparable to that observed in the vehicle control (ultrapure water) across all tester strains using both the plate incorporation and pre-incubation methods, irrespective of metabolic activation as shown in Tables 1 and 2. In contrast, the positive control substances elicited clear mutagenic responses, as evidenced by statistically significant increases in His^+ revertant colonies in all strains under both test conditions, thereby confirming the sensitivity and validity of the assay system. Overall, the results demonstrated that J25 did not induce mutagenic activity at any of the concentrations tested (5000.0 µg/plate, 1580.0 µg/plate, 500.0 µg/plate, 158.0 µg/plate, 50.0 µg/plate, 15.8 µg/plate, 5.0 µg/plate, and 1.6 µg/plate).

Table 1: Results of the main bacterial reverse mutation test conducted using the pre-incubation method with J25.

Group and test item concentration (µg/ plate)	Revertant colonies per plate (mean ± standard deviation, n=3, CFU/plate)														
	TA98			TA100			TA102			TA1535			TA1537		
Basal control (-S9)	32.7	±	2.5	169	±	9.5	286.3	±	3.1	18	±	2	20.3	±	4.6
Vehicle control (DMSO) (-S9)	35.3	±	3.5	147.7	±	7.1	258	±	2	20	±	3.6	19	±	1
Vehicle control (sterile ultra-pure water) (-S9)	29.3	±	4.2	182	±	6	293.3	±	6.4	18.3	±	1.5	20	±	1
Positive mutagen*	598.7	±	10.1	748	±	28	608.7	±	16.8	820	±	17.1	284.7	±	8.4
J25 at 50 (-S9)	22.7	±	1.5	175.3	±	4.7	242.7	±	23.7	17	±	1	19.3	±	0.6
J25 at 158 (-S9)	26	±	4.4	163.7	±	6.7	271.7	±	5.1	17.3	±	3.8	20.7	±	2.5
J25 at 500 (-S9)	25.3	±	4.7	157	±	4.4	249	±	3	17	±	3.6	19.7	±	2.5
J25 at 1,580 (-S9)	28	±	4.6	148.7	±	1.5	298.7	±	6.4	17.3	±	2.1	17.7	±	2.1
J25 at 5,000 (-S9)	30	±	4	175.7	±	5.1	268.7	±	9	16.3	±	1.5	15	±	4.4
Basal control (+S9)	38.3	±	4	175	±	6.1	334.7	±	6.1	26	±	4.4	17.7	±	4
Vehicle control (DMSO) (+S9)	39	±	2.6	151.7	±	4.7	306	±	5.3	22	±	1.7	17.3	±	1.5
Vehicle control (sterile ultra-pure water) (+S9)	37.3	±	1.2	143.3	±	6.7	341.7	±	7.2	24.3	±	2.9	19	±	2.6
Positive mutagen*	758.7	±	9	1797.3	±	16.2	670.7	±	10.1	360	±	28.8	234.7	±	9
J25 at 50 (+S9)	32.3	±	2.5	119	±	9.5	361.3	±	8.6	24.3	±	4.2	20.3	±	1.5
J25 at 158 (+S9)	42.7	±	2.1	164.7	±	5	342	±	6.2	24.7	±	3.2	18	±	2
J25 at 500 (+S9)	42.3	±	4	162.7	±	2.5	329	±	9	22.7	±	2.1	18.3	±	4.2
J25 at 1,580 (+S9)	41.3	±	3.2	149	±	8.2	308	±	6.6	25	±	3.6	17.3	±	4.9
J25 at 5,000 (+S9)	39.7	±	4	157	±	9.6	290.7	±	9.1	22.3	±	1.5	17	±	2

Note: *Positive mutagens were as follows: 2-nitroflourene for TA98 without S9 and 2-aminoanthracene for TA98 with S9; sodium azide for TA100/TA1535 without S9 and 2-aminoanthracene for TA100/TA1535 with S9; cumene hydroperoxide for TA102 without S9 and 2-aminoanthracene for TA102 with S9; and ICR191 for TA1537 without S9 and 2-aminoanthracene for TA1537 with S9.

Table 2: Results of the main bacterial reverse mutation test conducted using the plate incorporation method with J25.

Group and test item concentration (µg/ plate)	Revertant colonies per plate (mean ± standard deviation, n=3, CFU/plate)														
	TA98			TA100			TA102			TA1535			TA1537		
Basal control (-S9)	29.3	±	3.2	174.3	±	8.6	289.3	±	9	18	±	1.7	20.3	±	0.6
Vehicle control (DMSO) (-S9)	28.7	±	4	135	±	2.6	300	±	2	18	±	2.6	19.3	±	3.5.
Vehicle control (sterile ultra-pure water) (-S9)	32	±	2	158	±	6.2	275.3	±	6.4	18.3	±	1.5	19.3	±	2.5
Positive mutagen*	385.3	±	16.2	762	±	53.3	648	±	20	625.3	±	24.1	402.7	±	24.1
J25 at 50 (-S9)	24.7	±	2.5	162.3	±	5.7	244	±	8	18.7	±	1.5	20	±	3.6
J25 at 158 (-S9)	33	±	1	166	±	2.6	275	±	6	18.3	±	2.5	18.3	±	4.7
J25 at 500 (-S9)	28	±	2.6	158.7	±	5.1	242.3	±	4.2	16	±	2	19.7	±	1.5
J25 at 1,580 (-S9)	29	±	2.6	148.7	±	2.5	265	±	1	20.7	±	3.2	18	±	2.6
J25 at 5,000 (-S9)	26.7	±	4	142.3	±	1.2	264.7	±	8.4	20.3	±	2.1	19.7	±	2.1
Basal control (+S9)	43	±	1	154	±	3.6	346.3	±	4.7	29	±	3	21.7	±	3.1
Vehicle control (DMSO) (+S9)	35	±	2.6	170.3	±	4.9	344	±	4	21.3	±	4.2	19.3	±	4
Vehicle control (sterile ultra-pure water) (+S9)	40	±	2	163.3	±	6.7	334.7	±	7.6	24.7	±	2.5	22	±	4.6
Positive mutagen*	764.7	±	20.8	1534	±	44.1	776.3	±	29.7	252	±	4	132.7	±	6.4
J25 at 50 (+S9)	40	±	4.6	152.7	±	5	295	±	5.6	19.3	±	4.2	20	±	1
J25 at 158 (+S9)	38.3	±	4.5	153.7	±	4.5	348.3	±	9.5	23	±	1.7	20.7	±	2.1
J25 at 500 (+S9)	40.7	±	3.2	146.7	±	9.9	319.3	±	8.3	21	±	2	20	±	2.6
J25 at 1,580 (+S9)	41.3	±	3.5	162	±	5.2	334.7	±	5	22	±	1	21	±	3.5
J25 at 5,000 (+S9)	38.7	±	3.5	174.3	±	8.5	293	±	6.1	25.7	±	2.1	16.7	±	1.5

Note: *Positive mutagens were as follows: 2-nitroflourene for TA98 without S9 and 2-aminoanthracene for TA98 with S9; sodium azide for TA100/TA1535 without S9 and 2-aminoanthracene for TA100/TA1535 with S9; cumene hydroperoxide for TA102 without S9 and 2-aminoanthracene for TA102 with S9; and ICR191 for TA1537 without S9 and 2-aminoanthracene for TA1537 with S9.

In vitro Mammalian Cell Gene Mutation test

The mutagenic potential of J25 was further evaluated using the L5178Y $TK^{+/-}$ mouse lymphoma cell assay by determining its ability to induce forward gene mutations and/or structural chromosomal damage at the Thymidine Kinase (TK) locus (TK+/-TK-/-). An initial cytotoxicity experiment was conducted using concentrations of 625, 312.50 µg/mL, 156.25 µg/mL, 78.13 µg/mL, 39.06 µg/mL and 19.53 µg/mL J25 along with ultrapure water as the vehicle control and a media control in the absence (4h treatment) and presence (4h treatment) of the metabolic activation system. There was a dose-dependent decrease in suspension growth and relative total growth in cultures exposed with J25 at the tested concentrations in absence (~4h) and presence (~4h) of the metabolic activation system (data not shown). However, there was no cytotoxicity, which is classified by OECD guideline 490 as 10% - 20% relative total growth. Based on these results, concentrations of 625 µg/mL, 312.50 µg/mL, 156.25 µg/mL, and 78.13 µg/mL were selected for the main mutagenicity study.

An absolute increase in TK mutants was not observed in any of the tested concentrations of *Methylovorus menthalis* J25 in the absence or presence of S9 as shown in Table 3 and Table 4. Furthermore, there was no statistically significant increase in the frequency of mutants at any of the tested concentrations of *Methylovorus menthalis* J25. All the values of treated groups were similar to the vehicle control values and the Mutant frequency values were in the acceptable range of 126 × 10^{-6} provided by Global Evaluation Factor (GEF) as described in OECD test guideline 490. Therefore, the results demonstrate that at concentrations up to 5000 µg/mL, J25 is not mutagenic as it did not increase the mutation frequency above the vehicle control when tested in mouse L5178Y TK+/- 3.7.2C cells, in the presence or absence of metabolic activation (S9).

In vitro and *in vivo* Mammalian Bone Marrow Chromosomal Aberration test

The *in vitro* mammalian bone marrow chromosomal aberration study was conducted to evaluate the ability of J25 to induce structural chromosomal aberrations in CHO-K1 cells *in vitro*. The assessment was done in both the presence and absence of an exogenous metabolic activation system (S9). In a dose-range finding study, *Methylovorus menthalis* J25 was found to be non-cytotoxic at all tested concentrations (from 19.53 µg/mL to 625 µg/mL) in comparison to the respective vehicle control (10% v/v autoclaved ultrapure water) in the absence (4h and 24 h) or presence (4 h) of S9 (data not shown). Therefore, in the main study, *Methylovorus menthalis* J25 was tested for induction of chromosomal aberrations at 156 µg/mL, 313 µg/mL and 625 µg/mL. The observed percentage of cells with structural chromosome aberrations for the cells exposed to the *Methylovorus menthalis* J25 for 4 hours at 156 µg/mL, 313 µg/mL and 625 µg/mL was 1.33%, 1.00% and 1.00%

in absence of S9, and in the presence of metabolic activation the observed percentage of cells with structural chromosome aberrations was 1.33%, 1.00% and 1.67%, respectively (Table 5). In long-term exposure for 24 hours, in the absence of S9 at 156 µg/mL, 313 µg/mL and 625 µg/mL, the observed percentage of cells with structural chromosome aberrations was 1.00%, 1.33% and 1.67%, respectively as shown in Table 5. The observed number of aberrant cells in the *Methylovorus menthalis* J25 exposed cultures was not significantly different from those exposed to respective vehicle controls. However, the positive controls significantly induced structural aberrations relative to the vehicle control, confirming the validity of the experimental design. Based on the results, J25 does not induce structural chromosomal aberrations in CHO-K1 cells *in vitro* and is considered nongenotoxic for inducing clastogenicity.

The *in vivo* mammalian bone marrow chromosomal aberration study was conducted to evaluate the chromosomal aberration potential of J25 in the bone marrow of rats treated by oral gavage. There was no cytotoxicity observed in the dose range finding study, as there were no significant differences in the mitotic index in the *Methylovorus menthalis* J25-treated groups (at 500, 1000, 2000 mg/kg bw/day, n=2 males and 2 females/group) compared to the vehicle control group, nor was there any mortality or morbidity (data not shown). Thus, the highest dose of 2000 mg/kg b.w/day (1000 mg/kg b.w. b.i.d.) was selected as the highest dose for the main study.

In the main chromosomal aberration study, all animals were assessed to be normal and adverse clinical signs were not observed prior to their scheduled sacrifice. Furthermore, there was no statistically significant increase in the incidence of structural chromosome aberrations in *Methylovorus menthalis* J25-treated groups relative to the respective vehicle control groups either in male or female rats after dose administration as shown in Table 6. On the other hand, cyclophosphamide monohydrate, the positive control, induced a statistically significant increase in the incidence of structural chromosome aberrations in both male and female rats relative to the vehicle control, confirming the validity of the study design. Therefore, the results indicate that J25 does not result in chromosomal aberration or toxicity to the bone marrow of Sprague Dawley rats at doses up to 2000 mg/kg bw (1000 mg/kg b.w. b.i.d.) and is therefore considered as having no significant genotoxic potential.

Table 3: Mutagenicity Study of J25 in the absence of S9 (4h).

Treatment/ Group	Rep	Colony Sizing													
		Plate 1		Plate 2		Plate 3		Plate 4		Sum	Sum	Sum	CEM	MF (× 10^{-06})	MI
		Small	Large	Small	Large	Small	Large	Small	Large	'S'	'L'	'S+L'			
Vehicle control (10% water)	A	11	4	10	4	9	2	12	3	42	13	55	7.50×10^{-05}	65.79	1.02
	B	11	3	10	0	12	1	13	2	46	6	52	7.50×10^{-05}	63.03	0.98
Media Control (F0P Medium)	A	10	4	9	2	11	2	8	3	38	11	49	7.00×10^{-05}	63.06	0.98
	B	11	1	11	3	10	3	10	3	42	10	52	7.50×10^{-05}	63.03	0.98
4-NQO (0.1 µg/mL)	A	42	8	50	7	47	8	51	10	190	33	223	4.35×10^{-04}	639.71*	9.93
	B	50	7	50	10	46	6	47	8	193	31	224	4.35×10^{-04}	659.09*	10.23
J25 78.13 µg/mL	A	10	1	11	3	12	3	11	2	44	9	53	7.50×10^{-05}	70.09	1.09
	B	13	3	12	3	11	2	13	0	49	8	57	8.00×10^{-05}	72.07	1.12
J25 156.25 µg/mL	A	13	2	13	3	10	2	9	3	45	10	55	7.50×10^{-05}	74.26	1.15
	B	13	0	9	2	11	1	12	4	45	7	52	7.50×10^{-05}	74.26	1.15
J25 312.50 µg/mL	A	9	1	12	2	14	2	10	4	45	9	54	7.50×10^{-05}	76.53	1.19
	B	12	1	10	0	10	2	9	3	41	6	47	6.50×10^{-05}	64.36	1
J25 625 µg/mL	A	11	1	13	2	13	3	12	3	49	9	58	8.00×10^{-05}	81.63	1.27
	B	10	2	11	1	10	1	12	2	43	6	49	7.00×10^{-05}	76.09	1.18

Abbreviations: Colony Sizing and Mutation Frequency. CEM = Cloning Efficiency in Mutant Plates; MF = Mutant Frequency per clonable cells (× 10-06 cells); MI = Mutation Index; * = Significantly different from control, Statistical analysis by One-way ANOVA followed by Dunnett's post-hoc test at P <0.05

Table 4: Mutagenicity Study of J25 in the presence of S9 (4h).

Treatment/ Group	Rep	Colony Sizing													
		Plate 1		Plate 2		Plate 3		Plate 4		Sum	Sum	Sum	CEM	MF (× 10^{-06})	MI
		Small	Large	Small	Large	Small	Large	Small	Large	'S'	'L'	'S+L'			
Vehicle control (10% water)	A	12	4	13	2	13	3	12	3	50	12	62	8.50×10^{-05}	76.58	1.01
	B	12	2	11	4	11	3	13	2	47	11	58	8.00×10^{-05}	74.77	0.99
Media Control (F0P Medium)	A	10	3	10	2	11	3	12	0	43	8	51	7.00×10^{-05}	61.40	0.81
	B	9	4	10	2	11	3	8	4	38	13	51	7.00×10^{-05}	63.06	0.83

Benzo[a]pyrene (2.5 µg/mL)	A	53	10	47	11	52	9	54	10	206	40	246	5.10×10^{-04}	836.07*	11.05
	B	50	11	52	9	49	10	48	9	199	39	238	4.85×10^{-04}	866.07*	11.44
J25 78.13 µg/mL	A	9	2	10	3	9	4	11	2	39	11	50	7.00×10^{-05}	69.31	0.92
	B	10	0	11	2	10	1	8	3	39	6	45	6.50×10^{-05}	66.33	0.88
J25 156.25 µg/mL	A	9	2	10	1	8	4	12	2	39	9	48	6.50×10^{-05}	83.33	1.10
	B	11	1	10	2	9	2	9	4	39	9	48	6.50×10^{-05}	86.67	1.15
J25 312.50 µg/mL	A	7	1	8	2	9	1	10	2	34	6	40	5.50×10^{-05}	83.33	1.10
	B	9	2	9	0	8	2	10	4	36	8	44	6.00×10^{-05}	88.24	1.17
J25 625 µg/mL	A	9	1	8	1	10	0	8	1	35	3	38	5.50×10^{-05}	90.16	1.19
	B	9	1	10	1	8	1	9	1	36	4	40	5.50×10^{-05}	90.16	1.19

Abbreviations: Colony Sizing and Mutation Frequency. CEM = Cloning Efficiency in Mutant Plates; MF = Mutant Frequency per clonable cells (× 10-06 cells); MI = Mutation Index; * = Significantly different from control, Statistical analysis by One-way ANOVA followed by Dunnett's post-hoc test at P <0.05.

Table 5: Cytogenetic Evaluation of CHO-K1 Cells Treated with *Methylovorus menthalis* J25.

Exposure	Metabolic Activation	Cell Exposure Time (hours)	Cytotoxicity[a] (%)	Cells Scored	Normal Cells	Aberrant Cells	% Aberrant cells
Vehicle Control[b]	Absence	4	0	300	299	1	0.33
MMC, 0.4 µg/mL			21.2	50	25	25	50*
Methylovorus menthalis J25			-	-	-	-	-
156 µg/mL			0	300	296	4	1.33
313 µg/mL			0	300	297	3	1
625 µg/mL			0	300	297	3	1
Vehicle Control[b]	Presence	4	0	300	298	2	0.67
CP, 5 µg/mL			29.44	300	277	23	7.67*
Methylovorus menthalis J25			-	-	-	-	-
156 µg/mL			0	300	296	4	1.33
313 µg/mL			0	300	297	3	1
625 µg/mL			0	300	295	5	1.67
Vehicle Control[b]	Absence	24	0	300	297	3	1
MMC, 0.4 µg/mL			20.87	50	26	24	48*
Methylovorus menthalis J25			-	-	-	-	-
156 µg/mL			0	300	297	3	1
313 µg/mL			0	300	296	4	1.33
625 µg/mL			0	300	295	5	1.67

Note: [a]Cytotoxicity (%) determined by comparing the relative increase in cell count of treated cultures to vehicle cultures.
[b]Autoclaved ultrapure water (10% v/v) was used as vehicle. MMC: Mitomycin C and CP: Cyclophosphamide monohydrate.
* Statistically significant compared to respective vehicle control at P<0.05, by Fisher's exact test.

Table 6: Evaluation of chromosomal aberrations in male and female rats exposed to J25.

Gender	Dose & Group	Mitotic Index	Reduction in Mitotic Index (%)	No. of cells examined	No. of Aberrations					Incidental Observations				No. of Aberrant Cells including gaps	No. of Aberrant Cells excluding gaps	%Aberrant cells
					ctb	cte	csb	cse	Others	ctg	csg	P	EN gaps			
Males	G1 & 0	6.20	0	1000	4	0	1	0	0	3	0	0	0	8	5	0.50
	G2 -cyclophosphamide & 20.0 mg/kg b.w	5.86	5.48	500	31	3	6	1	0	4	1	0	0	46	41*	8.20
	G3 - M. *menthalis* J25 & 500 [250 mg/kg b.w (b.i.d)]	6.08	2.58	1000	2	0	0	0	0	3	0	0	0	5	2	0.20
	G4 - M. *menthalis* J25 & 1000[500 mg/kg b.w (b.i.d)]	5.76	7.10	1000	3	0	1	0	0	2	0	0	0	6	4	0.40
	G5 - M. *menthalis* J25 & 2000 [1000 mg/kg b.w (b.i.d)]	5.68	8.39	1000	2	0	1	0	0	3	0	0	0	6	3	0.30

Females	G1 & 0	6.46	0.00	1000	3	0	0	0	0	3	0	0	0	6	3	0.30
	G2 -cyclophosphamide & 20.0 mg/kg b.w	5.90	8.67	500	26	2	8	2	0	4	0	0	0	42	38	7.60*
	G3 - M. *menthalis* J25 & 500 [250 mg/kg b.w (b.i.d)]	5.76	10.84	1000	4	0	1	0	0	3	0	0	0	8	5	0.50
	G4 - M. *menthalis* J25 & 1000[500 mg/kg b.w (b.i.d)]	5.96	7.74	1000	3	0	0	0	0	4	2	0	0	9	3	0.30
	G5 - M. *menthalis* J25 & 2000 [1000 mg/kg b.w (b.i.d)]	5.78	10.53	1000	3	0	1	0	0	4	1	0	0	9	4	0.40

Abbreviations: ctb-Chromatid break; cte-Chromatid exchange, csb-Chromosome break; cse-Chromosome exchange; Incidental observations (gaps/numerical) type of aberrations such as chromatid gap (ctg), chromosome gap (csg), Polyploidy (P); Endoreduplication (EN). MI - Mitotic Index; The % Aberrant cells were calculated excluding the gaps. *Statistically significant compared to the vehicle control at p <0.05 (Fisher's exact test)

In vivo studies

Acute toxicity: Maximum tolerated dose study

A maximum tolerated dose study was performed to evaluate the acute systemic toxicity potential of J25 following a single administration. In step I, a dose of 5000 mg/kg body weight/day [1250 mg/kg b.w (q.i.d.)] was administered to an animal and there was no mortality/morbidity observed and no adverse clinical signs during the post-dose observation. Therefore, an additional set of two animals (Step II) was administered with the same dose, wherein the same results were observed after monitoring for 14 consecutive days' post-administration. There were also no adverse effects observed on body weight and body weight gain, nor were there any treatment related gross pathological abnormalities (data not shown) on day 15. Based on these results, it was concluded that the LD_{50} of *Methylovorus menthalis* J25 (Psomi™) is greater than 5000 mg/kg body weight/day [1250 mg/kg b.w. (q.i.d.)] under the conditions of this study.

Repeated dose toxicity: 14-day study

Subsequently, a 14-day repeated-dose study was conducted to evaluate the systemic toxicity of J25 following daily oral administration for 14 consecutive days. No adverse clinical signs of toxicity, morbidity, or mortality were observed in any treatment group throughout the study period. In addition, no treatment-related effects on body weight, body weight gain, or feed consumption were noted in either sex when compared with the respective vehicle control groups (data not shown). Although a statistically significant reduction in body weight was observed in mid-dose animals, and decreases in percent body weight change were noted in mid-dose and high-dose males relative to vehicle controls, these findings were not dose-dependent and were confined to males only. Consequently, these changes were considered as not treatment-related.

Similarly, no treatment-related adverse effects were observed in hematology, coagulation or clinical chemistry parameters observed in any treated group of either sex when compared with the respective vehicle control group. In addition, no treatment-related gross pathological findings were identified at necropsy. Although statistically significant differences were noted in certain parameters, including white blood cell count, neutrophils, lymphocytes, eosinophils, monocytes, hemoglobin, hematocrit, mean cell volume, platelets, cholesterol, total bilirubin, calcium, glucose, and sodium—in J25 treated animals, these changes were considered non-adverse as shown in Table 7. The observed differences lacked dose dependency, were limited to a single sex, and were not supported by corresponding changes in related parameters. Furthermore, all values remained within normal physiological ranges. All other hematology and clinical chemistry parameters showed no statistically significant differences between treatment groups and were therefore considered incidental (data not shown).

Table 7: Selected hematology, coagulation and clinical chemistry parameters (mean ± standard deviation) following 14-days of administration of J25 (n=5/group).

Gender	Group & Dose (mg/kg b.w./day)		WBC (10^3 cells/µL)	Neut × 10^3 cells/µL	Lymph × 10^3 cells/µL	Eos × 10^3 cells/µL	HGB (g/dL)	HCT (%)	MCV (fL)	Mono × 10^3 cells/µL	Platelet (10^3 cells/µL)	Cholesterol (mg/dL)	Total bilirubin	Ca (mg/dL)	Glucose (mg/dL)	Na+ (mmol/L)
Males	G1 & 0 (q.i.d)	Mean	7.47	1.01	6.02	0.05	14.56	45.60	64.62	0.26	1011.2	71.80	0.08	9.58	111.20	139.58
		SD (±)	2.32	0.42	2.21	0.03	0.55	1.13	1.19	0.08	458.41	6.53	0.04	0.25	10.18	1.55
	G2 & 2500 [625 mg/kg b.w (q.i.d.)]	Mean	12.94 *(↑)	1.27	11.25 *(↑)	0.09 *(↑)	14.56	45.46	63.60	0.20	1346.20	65.00	0.06	9.98 *(↑)	104.40	139.56
		SD (±)	3.00	0.25	3.10	0.03	0.65	2.20	2.56	0.05	114.45	21.00	0.05	0.22	14.33	0.41
	G3 & 3750 [937.5 mg/kg b.w (q.i.d.)]	Mean	9.75	0.25 *(↑)	6.95	0.10 *(↑)	13.94	43.56	64.78	0.29	1242.00	50.00 *(↓)	0.00 *(↓)	9.48	98.2	139.60
		SD (±)	1.89	0.87	2.08	0.01	0.55	1.47	1.61	0.09	403.83	5.70	0	0.13	7.19	0.95
	G4 & 5000 [1250 mg/kg b.w (q.i.d.)]	Mean	7.40	1.41	5.63	0.07	14.44	44.60	63.38	0.21	1108.80	53.40	0.02	9.62	105.60	138.82
		SD (±)	1.41	0.65	1.6	0.02	0.54	1.32	1.85	0.07	175.24	5.73	0.04	0.28	11.67	2.12

Females	G1 & 0 (q.i.d)	Mean	4.92	0.82	3.89	0.07	14.14	41.18	60.76	0.09	912.80	67.20	0	9.60	101.80	139.68
		SD (±)	0.52	0.21	0.66	0.01	0.27	2.05	1.3	0.02	207.51	13.66	0	0.17	8.70	0.51
	G2 & 2500 [625 mg/kg b.w (q.i.d.)]	Mean	6.80	2.32	4.06	0.07	13.82	41.48	63.60 *(↑)	0.25 *(↑)	1321.20 *(↑)	69.80	0.02	9.54	125.80 *(↑)	137.82 *(↓)
		SD (±)	2.27	1.62	0.64	0.05	0.41	1.35	1.01	0.10	299.38	5.85	0.04	0.11	21.88	1.05
	G3 & 3750 [937.5 mg/kg b.w (q.i.d.)]	Mean	5.12	1.73	3.07	0.12	14.76 *(↑)	44.08 *(↑)	63.14 *(↑)	0.15	976.40	63.60	0	9.14 *(↓)	110.60	138.70
		SD (±)	2.37	1.62	0.93	0.07	0.34	1.52	1.54	0.08	165.85	14.94	0	0.15	2.97	0.69
	G4 & 5000 [1250 mg/kg b.w (q.i.d.)]	Mean	6.20	1.64	4.19	0.08	14.42	43.52	62.62	0.22	1343. 60*(↑)	61.80	0	9.28	101.40	139.90
		SD (±)	2.42	1.45	1.12	0.03	0.33	1.14	0.70	0.12	169.88	18.55	0	0.38	3.36	0.51

Abbreviations: SD (±) - Standard Deviation; *Statistically significant ($p<0.05$) difference with respect to the control group (G1); ↑ - Increase; ↓ - Decrease.

With respect to the assessment of organs, there were no treatment-related adverse effects observed on the absolute and relative organ weights of all treatment groups of both sexes compared to the respective vehicle control group. The only statistically different value (relative organ weight of the brain in mid-dose males) was not considered to be test item-related as the change was not dose-dependent and there were no associated microscopic findings. Furthermore, upon microscopic examination, no test item-related adverse histopathological findings were observed in any of the specified organs of the high dose group compared to the control group. The few histopathology findings observed in the study were sporadic and lacked consistency, hence, they were considered incidental or background to the Sprague Dawley rats used in the study. The observations included: Mononuclear cell infiltration in the liver of 1 male in the vehicle group and 1 female of the high dose J25 group; and necrosis, interstitial, unilateral mononuclear cells observations in the kidneys of 1 male in the vehicle group and 1 male in the high dose J25 group. In light of these findings, the test item *Methylovorus menthalis* J25 (Psomi™) when administered by oral gavage to Sprague Dawley rats of both the sexes daily for 14 consecutive days was found to be tolerable up to 5000 mg/kg body weight/day [1250 mg/kg b.w. (q.i.d.)] under the experimental conditions and doses employed.

Subchronic repeated dose toxicity: 90-day study

A 90-day repeated-dose toxicity study was conducted to evaluate the safety profile of J25 following daily oral gavage administration for 90 consecutive days.

Mortality/morbidity, clinical signs, vaginal cytology and ophthalmological and neurological examination: There was no mortality/morbidity in vehicle control and J25 test item treatment groups. Similarly, there were no treatment-related adverse clinical signs, no treatment-related ophthalmological abnormalities, nor were there any treatment-related adverse effects on the stages of the estrus cycle (data not shown). Furthermore, no treatment-related adverse effects were observed on the functional observation battery of tests performed in the animals of the high dose group of both sexes compared to the respective vehicle control group (data not shown). Hence, the examination was not extended to low or mid-dose groups.

Body weight and feed consumption: There were no treatment-related adverse effects on body weight, body weight gain, or feed consumption in females treated with all doses of J25 compared to vehicle during the experimental period. Statistically significant decreases in body weight and body weight gain were observed in males of all dose levels from week 3 onwards which correlated with reduced feed consumption (data now shown), and hence, was considered as treatment related as shown in Table 8.

Table 8: Body weight and body weight gain of J25-fed males over a 90-day supplementation period (n=10/group).

	Group & Dose (mg/kg b.w./day)	-	Day 1	Day 8	Day 15	Day 22	Day 29	Day 36	Day 43	Day 50	Day 57	Day 64	Day 71	Day 78	Day 85	Day 90
Body weight (g)	G1 & 0 (q.i.d)	Mean	185.95	257.00	321.99	380.55	423.61	460.25	492.94	520.11	546.65	555.95	575.21	585.48	609.71	611.52
		SD (±)	14.49	22.44	30.46	34.57	36.48	43.16	44.30	48.15	53.65	54.51	63.05	65.42	69.37	69.74
	G2 &2500 [625mg/kg b.w (q.i.d.)]	Mean	186.19	238.44	296.6	344. 89*(↓)	383. 06*(↓)	410. 08*(↓)	427. 38*(↓)	450. 99*(↓)	476. 44*(↓)	490. 07*(↓)	509. 63*(↓)	518. 88*(↓)	535. 65*(↓)	536. 87*(↓)
		SD (±)	14.76	15.61	28.35	34.07	38.59	40.95	36.24	43.50	43.72	46.32	49.07	51.36	53.91	54.10
	G3 & 3750 [937.5 mg/kg b.w (q.i.d.)]	Mean	185.49	243.15	303.61	347.98	386.75	415. 27*(↓)	435. 61*(↓)	458. 09*(↓)	485. 29*(↓)	499. 4*(↓)	524.25	533.13	549. 44*(↓)	550. 92*(↓)
		SD (±)	14.35	19.84	23.80	26.64	28.85	33.82	36.64	42.32	41.57	41.36	42.04	40.21	37.84	37.83
	G4 &5000 [1250 g/kg b.w (q.i.d.)]	Mean	187.04	242.28	304.34	344. 61*(↓)	381. 04*(↓)	403. 82*(↓)	423. 09*(↓)	447. 85*(↓)	476. 12*(↓)	492. 41*(↓)	514. 12*(↓)	524. 26*(↓)	535. 21*(↓)	536. 69*(↓)
		SD (±)	15.34	21.82	26.60	28.56	35.73	38.39	40.91	45.24	46.54	47.23	48.08	48.09	51.63	51.83

	Group & Dose (mg/kg b.w./day)	-	Day 1-8	Day 1-15	Day 1-22	Day 1-29	Day 1-36	Day 1-43	Day 1-50	Day 1-57	Day 1-64	Day 1-71	Day-1-78	Day1-85	Day1-90	-
Body weight gain (%)	G1 & 0 (q.i.d)	Mean	38.15	73.02	104.58	127.81	147.49	165.17	179.95	194.03	199.05	209.28	214.75	227.84	228.79	-
		SD (±)	3.77	6.19	8.36	9.20	12.84	14.23	15.70	19.30	19.94	24.02	24.91	27.87	27.81	-
	G2 &2500 [625 mg/kg b.w (q.i.d.)]	Mean	28.32*(↓)	59.29*(↓)	85.40*(↓)	105.98*(↓)	120.40*(↓)	126.87*(↓)	139.15*(↓)	156.28*(↓)	163.56*(↓)	174.10*(↓)	179.06*(↓)	188.10*(↓)	188.74*(↓)	-
		SD (±)	6.33	8.95	13.75	17.00	16.46	14.92	15.54	19.44	20.46	22.28	23.42	25.15	25.18	-
	G3 & 3750 [937.5 mg/kg b.w (q.i.d.)]	Mean	31.10*(↓)	63.81*(↓)	87.90*(↓)	108.88*(↓)	124.16*(↓)	135.15*(↓)	147.40*(↓)	162.15*(↓)	169.73*(↓)	183.35*(↓)	188.24*(↓)	197.11*(↓)	197.92*(↓)	-
		SD (±)	4.04	7.04	11.07	12.41	13.07	14.81	19.78	19.68	19.94	21.42	21.69	21.36	21.59	-
	G4 & 5000 [1250 mg/kg b.w (q.i.d.)]	Mean	29.66*(↓)	62.76*(↓)	84.49*(↓)	104.02*(↓)	116.35*(↓)	126.64*(↓)	139.74*(↓)	154.93*(↓)	163.59*(↓)	175.20*(↓)	180.74*(↓)	186.59*(↓)	187.37*(↓)	-
		SD (±)	7.40	6.23	9.32	13.85	17.44	18.40	18.61	19.29	18.64	18.52	20.28	21.53	21.45	-

Abbreviations: SD (±) - Standard Deviation; *Statistically significant ($p<0.05$) difference with respect to the control group (G1); ↑ - Increase; ↓ - Decrease.

However, the change was found to be non-dose dependent; the decrease was less than 13% compared to the vehicle control group; the change was observed in a single sex; and the reduced body weight gain compared to day 1 body weight was associated with no correlated changes in other evaluated parameters. Therefore, the change was considered as non-adverse. Similarly, a statistically significant decrease in feed consumption was observed in males of all dose levels from week 2 onwards which correlated with the reduced body weight and body weight gain; hence, the effects were considered as treatment related. However, the change was less than 20% compared to the vehicle control group; observed in a single sex; and was associated with no correlated changes in other evaluated parameters. Hence, the change was considered non-adverse. Therefore, there were no toxicologically significant treatment-related adverse effects on body weights, body weight gains or feed consumption in all treatment groups of both sexes during the study period.

Clinical pathology

Several parameters showed statistically significant differences in J25-treated animals compared with controls. However, these findings were considered incidental, as they lacked dose dependency, remained within historical control ranges, were minimal in magnitude, occurred in only one sex, and were not supported by corresponding changes in other evaluated parameters, including histopathological findings. All other clinical chemistry parameters assessed were not significantly different between treatment groups (data not shown).

A statistically significant decrease in T3 levels was observed in females at all dose levels compared with the respective vehicle control group. Furthermore, a statistically significant increase in T4 levels was observed in low and high dose group females compared with the respective vehicle control group. A statistically significant increase in TSH levels was also observed in mid and high dose females. However, the observed changes were found to be either non-dose dependent, observed in a single sex, or the values were within the historical control range. In addition, there were no correlated changes in thyroid organ weight or on microscopic examination of the thyroid. Hence, the observed changes were not considered as treatment related.

As indicated, several of these findings represented non-adverse changes because they were accompanied by no adverse histopathological changes or changes in absolute or relative organ weights. Although there were statistically significant differences in absolute organ weights in the liver, pituitary gland and testes in J25 treated males (high dose), in addition to reduced fasting body weights of J25-treated males (low, mid and high doses), the changes were considered as secondary to the decreased body weight gain in males observed at the end of the treatment period. Furthermore, the changes were observed in a single sex and there were no associated microscopic findings. Hence, the changes were determined to be non-adverse as shown in Table 9 and Table 10.

No external or internal gross pathological changes were observed in any of the treatment groups during gross necropsy examination. In terms of histopathological examination, mineralization was observed in the kidneys of the high dose (5000 mg/kg b.w./day) females that was determined to be a test item related non-adverse histopathological finding. However, these findings were not observed in males belonging to the same group. Mineralization was observed in six females out of ten dosed at 5000 mg/kg b.w. per day. The observed mineralization was multifocal, unilateral or bilateral in distribution at minimal to mild severity. Most of the mineralized areas were located near the corticomedullary junction. The observed mineralization was not associated with any other pathological findings like degeneration, necrosis or hyperplasia in kidneys. Furthermore, there were no associated correlative changes in kidney function parameters like creatinine, blood urea nitrogen, or urinalysis and kidney weights. Therefore, the observed mineralization in kidneys was considered as a non-adverse change. Single isolated findings of alveolar hemorrhage and chronic inflammation were observed in the lungs; unilateral tubular degeneration was observed in the testes; and cellular debris were observed in the lumen of the epididymides. However, these changes were not considered to be attributed to the test item as they lacked consistency. All other histopathology findings observed

in the study animals were sporadic in nature and also observed in the concurrent vehicle control animals; hence, the changes were considered incidental or background to the Sprague Dawley rats used in the study (data not shown).

In conclusion, the NOAEL of *Methylovorus menthalis* J25 was determined to be 5000 mg/kg ([1250 mg/kg b.w (q.i.d.)]) b.w./day under the tested dose levels and experimental conditions employed.

Table 9: Selected urinalysis and clinical chemistry parameters (mean ± standard deviation) following 90-days administration of J25 (n=10/group).

Gender	Group & Dose (mg/kg b.w./day)		Urine volume (mL)	Urine pH	Cholesterol (mg/dL)	HDL (mg/dL)	Total protein (g/dL)	Total bilirubin (mg/dL)	Ca (mg/dL)	Globulin (calc, g/dL)	Albumin (g/dL)	Alkaline phosphatase (U/L)	Cl- (mmol/L)
Males	G1 & 0 (q.i.d)	Mean	9.50	7.90	61.20	55.80	6.66	0.13	9.86	5.48	1.18	88.50	114.64
		SD (±)	2.72	0.66	13.36	10.37	0.28	0.05	0.21	0.23	0.06	11.98	1.11
	G2 & 2500 [625 mg/kg b.w (q.i.d.)]	Mean	9.20	7.45	46. 40*(↓)	43. 10*(↓)	6.91	0.07 *(↓)	9.45 *(↓)	5.70	1.21	93.80	115.03
		SD (±)	2.57	0.60	6.82	5.45	0.22	0.05	0.29	0.27	0.11	19.56	1.23
	G3 & 3750 [937.5 mg/kg b.w (q.i.d.)]	Mean	12.50 *(↑)	7.15 *(↓)	58.50	53.90	6.99*(↑)	0.13	9.42 *(↓)	5.84 *(↑)	1.15	98.60	115.16
		SD (±)	3.41	0.24	12.12	9.98	0.24	0.05	0.21	0.22	0.10	17.05	1.66
	G4 & 5000 [1250 mg/kg b.w (q.i.d.)]	Mean	10.20 *(↑)	6.70 *(↓)	48.50	45.40	7.09 *(↑)	0.14	9.47 *(↓)	5.98 *(↑)	1.11	87.10	115.46
		SD (±)	3.29	0.35	14.49	11.11	0.32	0.05	0.31	0.33	0.10	10.19	1.23
Females	G1 & 0 (q.i.d)	Mean	7.70	7.20	66.40	56.70	7.12	0.14	9.77	5.55	1.57	46.50	116.46
		SD (±)	3.16	0.48	9.89	6.65	0.53	0.05	0.32	0.39	0.18	13.42	1.08
	G2 & 2500 [625 mg/kg b.w (q.i.d.)]	Mean	7.20	7.30	66.00	57.40	7.12	0.13	9.49	5.71	1.41	56.70	115.30
		SD (±)	2.62	0.48	12.20	8.90	0..36	0.05	0.26	0.30	0.14	11.78	1.35
	G3 & 3750 [937.5 mg/kg b.w (q.i.d.)]	Mean	7.60	7.05	63.50	56.40	7.12	0.13	9.44	5.74	1.38*(↓)	69.80 *(↑)	115.03 *(↓)
		SD (±)	1.84	0.16	16.53	11.32	0.29	0.07	0.57	0.28	0.06	20.08	1.42
	G4 & 5000 [1250 mg/kg b.w (q.i.d.)]	Mean	8.40	7.15	71.80	65.80	8.00 *(↑)	0..16	9.42	6.49 *(↑)	1.5	52.50	114. 74*(↓)
		SD (±)	3.27	0.34	17.20	12.70	0.48	0.05	0.33	0.31	0.21	12.40	1.06

Abbreviations: SD (±) - Standard Deviation; *Statistically significant ($p<0.05$) difference with respect to the control group (G1); ↑ - Increase; ↓ - Decrease.

Table 10: Selected hematology and hormonal parameters, fasting body weights and absolute and relative organ weights (mean ± standard deviation) following 90-days administration of J25 (n=10/group).

Gender	Group & Dose (mg/kg b.w./day)		White blood cells (10^3 cells/µL)	Lymphocytes (10^3 cells/µL)	Neutrophiles (10^3 cells/µL)	Prothrombin time (sec)	Platelet (10^3 cells/µL)	T3 (ng/mL)	T4 (ng/mL)	TSH (ng/mL)	Liver (absolute, g)	Pituitary (absolute, g)	Fasted body weight (%)	Testes/ ovaries (relative, %)
Males	G1 & 0 (q.i.d)	Mean	10.35	7.13	2.51	25.16	924.70	2.259	96.490	6.969	18.60825	0.01578	580.47	0.61755
		SD (±)	2.76	2.16	1.03	1.48	88.18	0.475	24.546	1.618	2.58027	0.00368	63.56	0.14844
	G2 & 2500 [625 mg/kg b.w (q.i.d.)]	Mean	9.55	6.83	2.13	25.51	988.60	1.713	99.900	5.589	16.23361	0.01619	507.87 *(↓)	0.74661
		SD (±)	1.80	1.43	1.00	1.48	141.70	0.335	30.057	1.786	1.80343	0.00223	47.02	0.05468
	G3 & 3750 [937.5 mg/kg b.w (q.i.d.)]	Mean	12.50	8.65	3.18	23.80	1025.00	2.075	90.984	6.380	16.46502	0.01527	515.77 *(↓)	0.67498
		SD (±)	3.12	2.06	1.29	1.75	138.66	0.581	22.166	2.209	2.49746	0.00175	40.10	0.14905
	G4 & 5000 [1250 mg/kg b.w (q.i.d.)]	Mean	14.03 *(↑)	9.81 *(↑)	3.42	23.37 *(↓)	1003	1.924	104.938	6.912	15.29523 *(↓)	0.01272 *(↓)	499.58 *(↓)	0.76190 *(↑)
		SD (±)	3.32	2.29	2.05	1.12	136.28	0.510	23.764	1.207	2.19427	0.00171	49.75	0.10904

Females	G1 & 0 (q.i.d)	Mean	7.06	5.21	1.31	22.73	798.40	6.087	55.201	6.413	9.63275	0.01620	301.56	0.06136
		SD (±)	2.11	1.47	0.49	1.37	113.40	2.885	16.006	1.313	0.94653	0.00371	16.44	0.00693
	G2 & 2500 [625 mg/kg b.w (q.i.d.)]	Mean	8.45	6.46	1.46	24.22	898.60	2.915 *(↓)	76.282 *(↑)	7.070	9.26528	0.01579	291.85	0.07093
		SD (±)	2.27	1.86	0.55	1.55	99.95	0.438	10.541	3.534	1.31735	0.00252	26.61	0.01678
	G3 & 3750 [937.5 mg/kg b.w (q.i.d.)]	Mean	8.69	6.11	2.09	24.23	937.10 *(↑)	3.629 *(↓)	60.744	9.588 *(↑)	9.21437	0.0185	291.06	0.06418
		SD (±)	1.63	0.99	1.40	1.26	65.22	0.742	11.947	3.216	1.31334	0.00356	26.68	0.01062
	G4 & 5000 [1250 mg/kg b.w (q.i.d.)]	Mean	10.41 *(↑)	7.17	2.58 *(↑)	22.00	862.60	4.127 *(↓)	78.814 *(↑)	10.664 *(↑)	9.63736	0.01877	304.34	0.05900
		SD (±)	3.60	2.97	1.51	1.42	79.96	1.657	6.700	2.869	1.28498	0.00449	34.74	0.00929

Abbreviations: SD (±) - Standard Deviation; *Statistically significant ($p<0.05$) difference with respect to the control group (G1); ↑ - Increase; ↓ - Decrease.

DISCUSSION

A comprehensive battery of toxicological studies conducted on *Methylovorus menthalis* J25 (supplied by DeNova Inc.) in accordance with relevant OECD guidelines demonstrated no evidence of *in vitro* or *in vivo* toxicity. Collectively, the results of the genetic toxicology assessments, including the bacterial reverse mutation assay, *in vitro* and *in vivo* mammalian chromosomal aberration assays, and the mammalian cell gene mutation assay, indicated that J25 is non-mutagenic at all tested dose levels. In the acute oral toxicity study, J25 exhibited an LD_{50} >5000 mg/kg body weight (1250 mg/kg bw, q.i.d). Furthermore, no mortality or treatment-related adverse effects were observed across evaluated parameters in the 14-day repeated-dose oral toxicity study following daily administration of low (2500 mg/kg bw [625 mg/kg bw q.i.d.]), mid (3750 mg/kg bw [937.5 mg/kg bw q.i.d.]), or high (5000 mg/kg bw [1250 mg/kg bw q.i.d.]) doses of J25. Consistent findings were observed in the subchronic 90-day repeated-dose oral toxicity study, in which daily administration of J25 at doses up to 5000 mg/kg bw/day (1250 mg/kg bw q.i.d.) resulted in no treatment-related adverse effects. Accordingly, the highest dose tested was identified as the No-Observed-Adverse-Effect Level (NOAEL). These findings are summarized in Figure 2.

Figure 2: Schematic summary of the experimental findings. Results from a battery of genetic toxicity tests demonstrate that J25 is non-mutagenic. In an acute toxicity experiment, an LD_{50} of >5000 mg/kg bw was derived for J25. These findings extended to a repeat dose 14-day study, wherein a NOAEL of 5000 mg/kg bw/day was determined for J25. Lastly, in the 90-day sub chronic oral toxicity study, there were no adverse or toxicologically significant findings at the highest dose tested. Therefore, the established NOAEL for J25 was 5000 mg/kg bw/day. This figure was created using BioRender https://BioRender.com.

The battery of four genetic toxicology studies provides consistent evidence that J25 is non-mutagenic. In the bacterial reverse mutation test (Ames test), J25 did not result in an increased number of revertants in 5 strains of *Salmonella typhimurium* at concentrations up to 5000 µg/plate. Similar results were observed in the *in vitro* mammalian cell gene mutation test and *in vitro* mammalian bone marrow chromosomal aberration assay, wherein the highest tested concentrations of J25 demonstrated a lack of mutagenic and clastogenic effects. These findings extended to an *in vivo* genetic toxicity assay, as demonstrated by the results from the *in vivo* chromosomal aberration assay suggesting a lack of toxicity to the bone marrow of Sprague Dawley rats (2000 mg/kg bw (1000 mg/kg b.w. b.i.d.). These results are in line with the expected genetic toxicity profile of a fermented protein-rich biomass product, as genetic toxicity studies for other fermented proteins consistently show that these ingredients are not genotoxic when produced using safe fermentation methods [17–19]. Collectively, the battery of genetic toxicity studies demonstrated that J25 is non-mutagenic.

Although these studies are important for assessing the genetic toxicity potential of J25, the *in vivo* bone marrow chromosomal aberration test only assessed the acute effects of administration of J25. Since J25 is intended to be added to animal feed and human foods that would be ingested chronically, *in vivo* repeat-dose studies are required to better characterize the toxicology profile of J25. Therefore, a maximum-tolerated dose study was first conducted to assess the acute toxicity of J25. Administration of J25 by oral gavage up to 5000 mg/kg/b.w. in female Sprague Dawley rats showed no adverse effects on body weight, body weight gain, or on gross necropsy examination. Therefore, the LD_{50} of J25 was determined to be >5000 mg/kg b.w. These data suggest a generally safe profile and low acute toxicity for J25, as the highest tested dose as per OECD guidelines was shown not to be toxic.

Subsequently, the toxicity of repeated doses of J25 were investigated in a 14-day study dose-range finding study. Rats administered J25 up to 5000 mg/kg bw/day showed no toxicologically significant or treatment-related adverse effects relative to the vehicle on all assessed parameters, which included: Clinical signs, body weight and weight gain, feed intake, hematology, coagulation or clinical chemistry parameters, organ weights, gross pathological findings, and microscopic examination of tissues. Thus, the subchronic toxicity of J25 was investigated in a subsequent repeat-dose 90-day study.

In the 90-day repeat dose oral toxicity study, there was no mortality reported in rats that received J25 at doses up to 5000 mg/kg bw/day. Even though there were reports of reduced body weights and feed intake in J25-treated rats, similar findings were reported in a comparable 90-day toxicity study involving oral administration of a fungal (*Neurospora crassa*) protein-rich biomass (55%-60% protein) at concentrations of 0, 1,000 mg/kg, 2,500 mg/kg, and 5,000 mg/kg body weight/day. While there was a significant decrease in food consumption among both male and female animals in the higher dose groups as compared to the controls, the authors also noted that these effects were likely due to prolonged satiety in the animals, particularly in the 5000 mg/kg/day group [18]. It has been well-established that protein consumption increases appetite-reducing hormones (GLP-1, PYY, CCK) and reduces hunger-inducing ghrelin [20]. Thus, a higher protein intake can induce a natural reduction in feed intake and appetite due to increased satiety. Nonetheless, the changes in body weight and feed intake were all found to be non-dose dependent; the decreases were minor relative to the vehicle control group; the change was observed in a single sex; and there were no correlated changes in other evaluated parameters. Thus, the changes were considered as non-adverse.

While there were certain parameters in the clinical chemistry analyses that were significantly different in J25-treated animals relative to the vehicle control; all changes were considered non-adverse and non-toxicologically significant. The differences were considered to be non-toxicologically significant as the differences in values occurred in only one sex; the changes were non-dose dependent; and/or the values remained within a normal, healthy physiological range. Furthermore, several of these findings were considered as non-adverse changes because they were accompanied by no histopathological changes or changes in organ weights. While there were a few reported histopathology findings, they were considered incidental or background to the Sprague Dawley rats used in the study since they lacked consistency and were sporadic [21]. With respect to urinalysis parameters of J25-treated rats, a statistically significant decrease in urine pH of mid and high dose males and statistically significant increase in urine volume of mid-dose and high-dose males was observed relative to the vehicle control group. However, these findings were not unexpected as intake of a high protein diet often increases urination. The kidneys work harder when challenged with a high protein load to filter and expel nitrogenous waste products (urea) that are created during protein metabolism [22]. Therefore, this process can result in a well-characterized and understood urea-induced osmotic diuresis, which requires more water to flush out waste. Nonetheless, there were no correlated changes observed in the other parameters that were evaluated, and the observed change was therefore not considered to be treatment related.

Based on the results, it was concluded that were no treatment-related adverse effects on all assessed parameters, including the external and internal gross pathological assessment, organ weights, or on urinalysis, hormone, hematology, and coagulation in any of the animals at all tested dose levels of J25 compared to the vehicle control groups. Therefore, the highest dose tested (5000 mg/kg bw/day) was determined to be the NOAEL for J25. While there are no available toxicological assessments of comparable protein-rich products from *Methylovorus* species, these results are in accordance with findings from other 90-day toxicity studies conducted on protein-rich biomasses. For example, Choi et al. assessed the safety a protein-rich powder derived from *Xanthobacter* sp. SoF1 in a 90-day study in rats, and also reported the NOAEL as the highest dose tested (1500 mg/kg bw/day) [23]. The authors described *Xanthobacter* sp. SoF1 (SoF1) as an autotrophic hydrogen-oxidizing bacteria that produces protein-rich biomass and has potential to be an alternative protein source. This heat-inactivated biomass of SoF consisted of 68% protein (using Kjeldahl factor [Nx5.83]). Similarly, a protein-rich powder derived from heat treatment of the whole-cell biomass of polyhydroxybutyrate-deficient *Cupriavidus necator* was assessed in a 90-day repeated-dose oral toxicity study in rats, and a NOAEL of 3000 mg/kg bw/day, the highest dose tested, was determined [19]. Lozy et al. also assessed the toxicity of a fungal (*Neurospora crassa*) protein-rich biomass (55-60% protein) at concentrations of 0, 1,000 mg/kg, 2,500 mg/kg, and 5,000 mg/kg body weight/day over a 90-day administration period [18]. In this study, the NOAEL for the dried *N. crassa* biomass ingredient was determined to be 5,000 mg/kg body weight/day, the highest

dose tested, which is comparable to the value derived in the present study for J25. Fermented protein-rich biomass products have also been shown to be safe in other species, including beagle dogs. In a study by Longshaw et al., beagles were fed diets containing up to 8% cultured protein (FeedKind Pet®, 74.68% crude protein derived from obligate methanotroph bacteria *Methylococcus capsulatus* Bath—alongside *Aneurinibacillus danicus*, *Brevibacillus agri*, and *Cupriavidus cauae*) for 6 months, then fed control diets for a further two months [4]. The diets were well tolerated with no reduction in feed intake and were readily digested, providing all of the essential nutrients required. The results showed that FeedKind Pet® is safe as a protein source for dogs and can be included at up to 8% of the total diet with no harmful side effects. Collectively, the studies consistently demonstrate that fermented protein rich biomass products have a strong safety profile, as they were consistently shown to be safe at the highest doses tested in numerous subchronic studies [6].

CONCLUSION

Based on the findings described above, the results demonstrate that J25 was safe and well tolerated under the conditions of these studies, as no treatment related adverse effects of toxicological significance were observed in the *in vitro* genetic toxicity assays or in rats administered J25 at any dose level. Accordingly, the NOAEL for J25 was determined to be ≥ 5000 mg/kg body weight in both male and female Sprague Dawley rats following daily oral gavage administration for 90 consecutive days under the experimental conditions employed.

As the animal feed and human food markets search for sustainable protein sources and animal-based meat alternatives, *Methylovorus* presents as a viable option for a protein source for the feed and food industries. Not only is DeNova's protein biomass product protein-rich (approximately 75%-85%) but it is also highly digestible. Protein and essential amino acid digestibilities of J25 were high (86%–96%) at 20% inclusion in a study in freshwater phase pelleted diets. Additionally, when increased to 26 % at the expense of plant proteins in pelleted diets, high dietary protein and essential amino acid digestibilities were maintained (average 94%). On the other hand, plant sources of protein, such as legumes, are lower in protein concentration and can be less digestible. Therefore, protein from *Methylovorus* presents as a viable and sustainable protein source. Collectively, the results of the comprehensive battery of safety studies support the safe use of DeNova's protein-rich biomass ingredient (Psomi™) for use in animal feed, companion animal foods and supplements, and human foods (e.g., dietary supplements and conventional foods, including alternative meat products).

AUTHOR CONTRIBUTIONS

All experiments, data curation and formal statistical analysis were performed at Anthem Biosciences Pvt. Ltd., Department of Preclinical Research, Bommasandra, Bangalore. S.S. – investigation, supervision and review of data, writing of original draft; A.C. – investigation, study design, supervision; N.G. – investigation, supervision, resources; C.H. – conceptualization, investigation, study design, supervision and review of data. All authors participated in revision of the manuscript and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The following authors (SS, AC, NG, CH) are employed by KGK Science, Inc. This study received funding from DeNova Inc. The funder commissioned KGK Science Inc. to design and oversee the conduct of the studies, and to prepare the manuscript. Experimental work and analysis of results were completed by the Department of Preclinical Research at Anthem Biosciences Pvt. Ltd. The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; or in the writing of the manuscript. The funder made the decision to publish the results. We declare that Roberto Armenta is an employee of DeNova; however, any reference to commercial companies, product names and/or organism identifiers is for accuracy and does not represent endorsement by the authors.

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