



Subchronic feeding, allergenicity, and genotoxicity safety evaluations of single strain bacterial protein

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ABSTRACT

Microbial proteins are potentially important alternatives to animal protein. A safety assessment was conducted on a *Clostridium* protein which can serve as a high-quality protein source in human food. A battery of toxicity studies was conducted comprising a 14-day dose-range finding dietary study in rats, 90-day dietary study in rats and in vitro genotoxicity studies. The allergenic potential was investigated by bioinformatics analysis. In the 90-day feeding study, rats were fed diets containing 0, 5.0, 7.5, and 10% *Clostridium* protein. The *Clostridium* protein-containing diets were well-tolerated and no adverse effects on the health or growth were observed. Significant reductions in neutrophil counts were observed in all female rats compared to controls, which were slightly outside of reference ranges. These effects were not deemed to be adverse due to the absence of comparable findings in male rats and high physiological variability of measured values within groups. A No-Observed-Adverse-Effect-Level (NOAEL) of at least 10% *Clostridium* protein, the highest dose tested and corresponding to 5,558 and 6,671 mg/kg body weight/day for male and female rats, respectively, was established. No evidence of genotoxicity was observed and the allergenic potential was low. These results support the use of *Clostridium* protein as a food ingredient.

1. Introduction

Global population growth, combined with improvements in living standards, are primary contributors to the increasing demand for animal protein and pressure on agricultural systems over the past decade (Health Council of the Netherlands, 2011; Boland et al., 2013; Sans and Combris, 2015; Chapman et al., 2020; National Academy of Sciences, Engineering and Medicine, 2020). Novel proteins, i.e., those not forming a traditional part of the human diet, such as insect protein, plant-derived proteins, cultured meat, and microbial proteins can play an important role in helping meet the protein requirements of the growing population (Boland et al., 2013; Hadi and Brightwell, 2021). Of these novel alternatives, microbial proteins, obtained by cultivation of microalgae, fungi, and bacteria, have several beneficial properties, including rapid growth rates, the ability to utilize a wide range of substrates and relatively high protein levels (Linder, 2019). From a nutritional perspective, they can

provide a complete source of protein, delivering all essential amino acids, as well as contributing to vitamin and minerals intakes (Matassa et al., 2016; Linder, 2019).

Although the potential benefits of single cell proteins (SCPs) as novel protein sources are well-documented, there are still relatively few products available commercially. Furthermore, the microbial proteins currently available are primarily derived from microalgae or fungal sources with bacterial proteins remaining under-utilized largely due to challenges associated with their commercial production (Matassa et al., 2016; Linder, 2019; Sillman et al., 2020).

On account of their novel source, microbial proteins are generally subject to regulatory approvals worldwide prior to being placed on the market for use as protein sources in human food. The mycoprotein marketed under the brand name Quorn™ is a rare example of an SCP which has undergone a full safety assessment and is available commercially. Produced from the filamentous fungi *Fusarium venenatum*, the mycoprotein was successfully notified to the United States

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Abbreviations

AOAC	Association of Official Analytical Chemists	LUC	large unstained cells
ALP	alkaline phosphatase	LYMPH	lymphocytes
ALT	alanine aminotransferase	MCH	mean corpuscular hemoglobin
AST	aspartate aminotransferase	MCHC	mean corpuscular hemoglobin concentration
BASO	basophils	MCV	mean corpuscular volume
CBPI	Cytokinesis-Block Proliferation Index	MMC-C	mitomycin C
CP	<i>Clostridium</i> protein	MONO	monocytes
DIN	German Institute for Standardization	NEUT	neutrophils
DRF	dose range-finding	NIR	Near Infra-Red
EOS	eosinophils	NOAEL(s)	No-Observed-Adverse-Effect-Level(s)
EU	European Union	OECD	Organization for Economic Co-operation and Development
FARRP	Food Allergy Research and Resource Program	PTL	platelet
FDA	Food and Drug Administration	RBC	Red Blood Cells
FOB	functional observational batter	RDW	red blood cell distribution width
GLP	good laboratory practices	RETIC	reticulocytes
GRAS	Generally Recognized As Safe	SCFA	short-chain fatty acid
HCT	hematocrit	SCP	single cell protein
HBSS	Hanks' Balanced Salt Solution	-S9	without metabolic activation
HDL	high-density lipoprotein	+S9	with metabolic activation
HGB	hemoglobin	S9	metabolic activation system
ILSI	International Life Sciences Institute	TSH	thyroid stimulating hormone
IOM	Institute of Medicine	U.S.	United States
ISO	International Organization for Standardization	WGS	Whole Genome Sequence
LDL	low density lipoprotein	WBC	white blood cells
		WHO	World Health Organization

(U.S.) Food and Drug Administration (FDA) as Generally Recognized As Safe (GRAS) for use as a protein source in human food in 2002 (GRN No. 91; [U.S. FDA, 2002](#)). Another *Fusarium*-derived protein was also notified as GRAS to the U.S. FDA in 2021 (GRN No. 904; [U.S. FDA, 2021](#)) which is compositionally similar to Quorn™. To date however, no bacterial protein has undergone a safety assessment for use in human foods in either the U.S. or European Union (EU).

This research describes the safety evaluation of a novel bacterial protein produced by *Clostridium tyrobutyricum* fermentation using an economical food-grade sugar feedstock. To this end, the *Clostridium* protein was subjected to a standard battery of toxicological tests, consisting of a 90-day dietary feeding study in rats, a bacterial reverse mutation test and an in vitro mammalian micronucleus assay. These studies were conducted in accordance with current toxicological guidelines to establish the safety of novel food ingredients, including protein sources.

2. Materials and methods

2.1. Test item and experimental diets

Clostridium protein is the dried killed cells obtained from *Clostridium tyrobutyricum* fermentation using a corn-derived sugar feedstock. The test item was an off-white powder (min. 80% protein; see [Table 1](#) for the product specifications) manufactured by Superbrewed Food (Delaware, United States). The microbial source is an asporogenous strain generated by natural evolution from a wild-type isolate first isolated in Delaware (United States). The strain was fully characterized using whole genome sequence (WGS) analysis and is deposited in the CABI international culture collection under reference number SD20/06. The separated cells after anaerobic fermentation are heat-treated to reduce nucleic acid levels to not more than 4% by weight of *Clostridium* protein.

Nutrient analyses were conducted on *Clostridium* protein using Association of Official Analytical Chemists (AOAC) and International Organization for Standardization (ISO) methods: crude protein (AOAC 990.03), crude fat (AOAC 2003.05), ash (AOAC 942.05), moisture

Table 1

Specification for *Clostridium* protein intended as a cultured protein in food.

Parameter	Specification (g/100 g)
Crude protein	≥80
Crude fat	≤3
Ash	≤6
Moisture	≤10
Ammonia nitrogen	≤1
Nucleic acids (RNA + DNA)	≤4
Heavy Metals	
Lead	≤0.3
Cadmium	≤0.1
Arsenic	≤0.1
Mercury	≤0.5
Microbiology	
Aerobic plate count	≤100,000
Yeast and mold	≤100
<i>E. coli</i> 0157-H7	Absent
<i>Salmonella</i>	Absent

(AOAC 930.15) and neutral detergent fiber (ISO 16472:2006 and AOAC 2002:04). The amino acid profile was analyzed using AOAC 994.12 method and the in vitro digestibility measured with Megazymes Protein Digestibility Kit (Wicklow, Ireland), using pepsin, trypsin and chymotrypsin. Vitamin and mineral profiles were measured using AOAC methods: riboflavin (AOAC 970.65), vitamin B6 (J. AOAC 88, 30–37) and vitamin B12 (AOAC 952.20), calcium, phosphorus, sodium and iron (AOAC 984.27). The typical nutrient profile of *Clostridium* protein is presented in [Table 2](#).

Experimental diets for use in the 14-day dose range finding and 90-day repeated dose oral toxicity studies, respectively, were manufactured by SSNIFF Spezialdiäten GmbH (Soest, Germany) and analyzed for nutrient composition by Fumoprep Kft. (Budapest, Hungary). The dietary treatments containing graded levels of *Clostridium* protein were prepared by partial replacement of casein in the basal (control) diet. The mineral profile of *Clostridium* protein was taken into account in

Table 2Nutrient composition of *Clostridium* protein used in the diets.

Nutrient	Amount
Proximate (g/100 g)	
Crude protein	83.5
Crude fat	1.51
Ash	2.61
Neutral detergent fiber	0.65
Carbohydrate	6.38
Nucleic acids	2.68
Moisture	2.85
Amino acids (g/100 g)	
Lysine	8.57
Methionine	2.49
Threonine	3.87
Histidine	1.22
Leucine	6.15
Isoleucine	5.82
Aspartic acid	12.2
Glutamic acid	10.1
Serine	3.57
Glycine	3.85
Arginine	3.86
Alanine	5.82
Tyrosine	3.16
Valine	4.76
Phenylalanine	3.51
Proline	2.49
Cysteine	0.81
Tryptophan	0.78
Vitamins (mg/kg)	
Riboflavin	1.07
Vitamin B6	0.82
Vitamin B12	0.014
Minerals (g/kg)	
Sodium	2.28
Calcium	0.11
Phosphorus	4.27
Iron	0.12

formulation of the diets in order to minimize nutritional differences among experimental groups (see Table 3 for the diets used in the 90-day study in rats; a mineral mixture and adjusted mineral mixture were used in different amounts for the formulation of each experimental diet).

Measurement of moisture (Method A), crude ash (Method M), crude protein (Method C), total fat (Method C), crude fiber (Method H), and amino acids (Method I) in the experimental diets was conducted using the relevant procedures described in Commission Regulation (EC) No 152/2009 (European Commission, 2009). The mineral contents were determined using German Institute for Standardization (DIN) EN method 15621:2017–10, except for the selenium content which was analyzed using DIN EN method 17053:2018–03. Test item content and homogeneity in the experimental diets were analyzed using a validated near infra-red (NIR) method (data not shown).

2.2. Good laboratory practice and regulatory guidelines

The toxicity studies were conducted by Charles River Laboratories Den Bosch BV (The Netherlands). All institutional and national guidelines for the care and use of laboratory animals were followed. The studies were reviewed and agreed by the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. within the framework of the project license approved by the Central Authority for Scientific Procedures on Animals (CCD) as required by the Dutch Act on Animal Experimentation.

With the exception of the 14-day range finding study and diet

Table 3Dietary composition of rats fed *Clostridium* protein for 90 days.

	Dietary level			
	0% CP (Control)	5% CP	7.5% CP	10% CP
Nutritional Value (as analyzed)				
Moisture (%)	11.5	11.4	11.5	11.5
Crude ash (%)	3.6	3.7	3.7	3.9
Crude protein (Nx6,25) (%)	17.1	17.4	17.3	17.5
Total fat (%)	4.9	5.2	5.1	5.0
Crude fiber (%)	2.2	2.6	3.1	3.3
Composition (as formulated)				
Casein (%)	19.60	15.05	12.78	10.51
<i>Clostridium</i> protein ^a (%)	–	5.00	7.50	10.00
Corn starch (%)	39.23	38.94	38.80	38.65
Maltodextrin (%)	13.20	13.20	13.20	13.20
Sucrose (%)	10.00	10.00	10.00	10.00
Cellulose powder (%)	6.00	6.00	6.00	6.00
Soybean oil (%)	5.00	5.00	5.00	5.00
Inulin/Pectin 1:1 (%)	1.00	1.00	1.00	1.00
Vitamin mix (%)	1.00	1.00	1.00	1.00
Mineral mix ^b (%)	4.00	2.00	1.00	–
Mineral mix (Adjusted) ^c (%)	–	2.00	3.00	4.00
Minerals and Trace Elements (as analyzed)				
Sodium (%)	0.18	0.18	0.17	0.18
Calcium (%)	0.59	0.59	0.62	0.63
Phosphorus (%)	0.42	0.41	0.42	0.42
Iron (mg/kg)	73.0	77.5	72.6	72.3
Manganese (mg/kg)	45.4	40.7	40.9	45.0
Zinc (mg/kg)	60.3	59.6	61.5	62.9
Copper (mg/kg)	11.4	12.8	12.5	14.4
Selenium (mg/kg)	0.37	0.39	0.52	0.65

Abbreviations: CP, *Clostridium* protein.

^a Calculated based on 83.5% crude protein content.

^b One kilogram of feed contains: calcium carbonate, 295 g; calcium phosphate dibasic, 133 g; potassium phosphate dibasic, 250 g; tri-potassium citrate hydrate, 70 g; potassium sulfate, 42 g; sodium chloride, 111 g; magnesium oxide, 39 g; iron (III) citrate hydrate, 8.65 g; manganese chloride, 2.4 g; zinc carbonate, 2.43 g; copper (II) oxide, 400 mg; potassium iodide, 10 mg; sodium fluoride, 60 mg; cobalt sulfate pentahydrate, 60 mg; sodium molybdate dihydrate, 20 mg; chromium potassium sulfate dodecahydrate, 10 mg; lithium chloride, 10 mg; sucrose (ad 100), 45.95 g.

^c One kilogram of feed contains: calcium carbonate, 305 g; calcium phosphate dibasic, 125 g; potassium phosphate dibasic, 246 g; tri-potassium citrate hydrate, 74 g; potassium sulfate, 48 g; sodium chloride, 100 g; magnesium oxide, 39 g; iron (III) citrate hydrate, 6.8 g; manganese chloride, 1.64 g; zinc carbonate, 2.34 g; copper (II) oxide, 410 mg; potassium iodide, 10 mg; sodium fluoride, 60 mg; cobalt sulfate pentahydrate, 60 mg; sodium molybdate dihydrate, 10 mg; chromium potassium sulfate dodecahydrate, 10 mg; lithium chloride, 10 mg; sucrose (ad 100), 51.65 g.

analysis,¹ studies were carried out according to the Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (OECD, 1998) and EC Commission Directive 2004/10/EC of 11 February 2004 (European Commission, 2004). The 90-day study was conducted using test methods described in OECD Guideline Test No. 408 (OECD, 2018) and FDA Redbook Chapter IV.C.4. a (U.S. FDA, 2000a). The bacterial reverse mutation assay was conducted using methods described in the OECD Guideline Test No. 471 (OECD, 1997 and 2020) and FDA Redbook Chapter IV.C.1a (U.S. FDA, 2000b). The in vitro mammalian cell micronucleus test was conducted according to OECD Guideline Test No. 487 (OECD, 2016).

The allergenic potential of *Clostridium* protein was evaluated by in silico methods using the criteria described by Codex Alimentarius Commission on foods derived from biotechnology (Codex Alimentarius Commission, 2009; Goodman et al., 2008).

¹ Dietary analysis necessitated the use of non-validated modeling software; however, the process was fully documented and verified.

2.3. Repeated-dose toxicity

2.3.1. 14-Day range finding study in rats

In dose-range finding (DRF) study not conducted in accordance with GLP, 5 male Sprague Dawley rats per group (6–8 weeks of age; 185–232 g) were administered *Clostridium* protein in the diet at concentrations of 0, 2.5, 5.0, 7.5, or 10.0% for 14 days. Feed pellets containing the required dietary concentrations of *Clostridium* protein were produced by SSNIFF Spezialdiäten GmbH (Soest, Germany), wherein casein was substituted with the test item at the appropriate concentration. The control group of animals received standard AIN rodent feed pellets.

Animals were monitored for mortality and clinical signs of toxicity, body weight and body weight gains, feed intake, locomotor activity, and subjected to standard hematology, coagulation, clinical chemistry, including uric acid, urinalysis evaluations, as well as measurement of organ weights (brain, adrenal glands, heart, kidneys, liver, spleen and thymus), gross pathology, and histopathology of left hind paw and kidneys. Unless specifically treated, microbial proteins can contain high levels of nucleic acids (Ritala et al., 2017), which when consumed, can result in elevated plasma uric acid levels leading to joint issues/gout and kidney stones. Hind paw histology evaluations represent a marker for adverse joint effects.

2.3.2. 90-Day dietary feeding study in rats

In the 90-day feeding study, male and female Crl:CD Sprague Dawley rats (10 per sex per group; at least 6 weeks of age and 139–255 g) were administered diets containing 0, 5.0, 7.5, or 10.0% *Clostridium* protein. Feed pellets containing the required dietary concentrations of *Clostridium* protein were produced by SSNIFF Spezialdiäten GmbH (Soest, Germany), wherein casein was substituted with the test item at the appropriate concentration. The control group of animals received a standard AIN rodent feed pellets. The nutrient compositions of the diets fed in the study are provided in Table 3.

Each animal was identified using a subcutaneously implanted electronic identification chip that was implanted shortly after arrival at the Test Facility. All animals were allowed to acclimate to the facility for at least 5 days prior to study initiation. Up to 5 animals of the same sex and same dose group were housed together in polycarbonate cages (Makrolon type IV, height 18 cm or Makrolon type 2000P, height 21.5 cm) containing sterilized wooden fibers as bedding material (Lignocel S 8–15, JRS - J. Rettenmaier & Söhne GmbH + CO. KG, Rosenberg, Germany) and equipped with water bottles containing municipal tap water. Target animal room environmental conditions consisted of a temperature range of 18–24 °C, 40–70% humidity, 12 h light and 12 h dark, and 10 or more air exchanges per hour.

During the study, animals were assessed for mortality (twice daily), clinical signs of toxicity (at least once daily), arena observations (weekly), body weights and feed consumption (weekly), water intake (visual inspection throughout study period), ophthalmic examinations (week 13 for control and highest-dose groups), functional observational battery (FOB; week 13: hearing ability, pupillary reflex and static righting reflex; fore- and hind-limb grip strength; locomotor activity), and estrous stage (day of scheduled necropsy for females). At interim periods (Day 30 and Day 50) and at the end of the study, standard hematology, blood coagulation, clinical chemistry, uric acid, and urinalysis parameters were measured in all dose groups.

Study animals surviving until scheduled euthanasia were deeply anesthetized using isoflurane and subsequently exsanguinated and subjected to a full *post-mortem* examination, including evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities. Absolute and relative organ weights (brain, heart, kidneys, stomach, large and small intestines, liver, lung, reproductive organs, thyroid, lymph nodes, spleen, pancreas, amongst other glands and tissues) were collected, weighed, and subjected to macro- and microscopic histopathological evaluations by a board-certified

toxicological pathologist with training and experience in laboratory animal pathology.

All statistical tests were conducted at the 5% significance level with all pairwise comparisons conducted using two sided tests and reported at the 1% and 5% levels, unless otherwise noted. Analyses were conducted as compared to control group for body weights, body weight gains, hematology, coagulation, clinical chemistry, FOB quantitative values, and absolute and relative organ weights using parametric/non-parametric methods. Levene's test was used to assess the homogeneity of group variances. The groups were compared using an overall one-way ANOVA F-test if Levene's test was not significant or the Kruskal-Wallis test if it was significant. If the overall F-test or Kruskal-Wallis test was found to be significant, then pairwise comparisons were conducted using Dunnett's or Dunn's test, respectively. A Fisher's exact test was used to conduct pairwise group comparisons of interest in relation to FOB qualitative incidence results.

2.4. Genotoxicity tests

2.4.1. Bacterial reverse mutation (Ames) test

The potential of *Clostridium* protein and/or its metabolites to induce reverse mutations at the histidine locus in *Salmonella typhimurium* (*S. typhimurium*) strains TA98, TA100, TA1535, and TA1537, and at the tryptophan locus of *Escherichia coli* (*E. coli*) strain WP_{2uvrA}-pkM101 was assessed in a bacterial reverse mutation assay in the presence or absence of an exogenous mammalian metabolic activation system (S9). Rat S9 homogenate was obtained from Trinova Biochem GmbH (Giessen, Germany) and prepared from male Sprague Dawley rats that had been intraperitoneally induced with Aroclor 1254 (500 mg/kg body weight).

This assay was conducted consistent with OECD Guideline No. 471 (bacterial reverse mutation test, adopted 21 July 1997, corrected 26 June 2020) using the “treat and wash” modification. This adjustment is necessary when testing substances such as proteins, containing, or capable of releasing, amino acids such as tryptophan and histidine, which can interfere with the assay results (i.e., false positives). Using this method, actively growing bacteria are exposed to the test item in suspension, centrifuged, washed free of the test item, and plated out in top agar as per the standard method. In the first experiment, *Clostridium* protein, dissolved in water, was evaluated in triplicate at concentrations of 0, 52, 164, 512, 800, 1,600, 5,000 µg/plate, with 5% S9 or without. In the second experiment, concentrations of 275, 492, 878, 1,568, 2,800, and 5,000 µg/mL were evaluated with 10% S9 or without. The negative control samples consisted of water, while the positive control substances without S9 consisted of sodium azide (for TA1535), 2-nitrofluorene (for TA1537 and TA98), methylmethanesulfonate (for TA100), 4-nitroquinoline N-oxide (for WP_{2uvrA}-pkM101), and 2-aminoanthracene for all strains with S9.

The incubation mixtures were incubated in a shaking bath for 90 min ± 5 min by 70 rpm at 37 °C. After incubation, all bacterial suspensions were washed with approximately 9 mL of washing solution (Oxoid No. 2 nutrient broth in phosphate buffered saline (1:7 v/v)) followed by a centrifugation step for 30 min at 1,750 rpm. After washing, the bacterial pellet was briefly vortexed. Top agar in top agar tubes was molten and heated to 45 °C. To each resuspended pellet one molten top agar was added. The ingredients were mixed on a Vortex and the content of the mixed tube poured onto a selective agar plate. After solidification of the top agar, the plates were inverted and incubated in the dark at 37.0 ± 1.0 °C for 48 ± 4 h.

The revertant colonies were counted automatically with a Sorcerer Colony Counter, whereas plates with sufficient test item precipitate to interfere with automated colony counting were counted manually. Evidence of test item precipitate on the plates and the condition of the bacterial background lawn was evaluated when considered necessary, macroscopically and/or microscopically by using a dissecting microscope.

2.4.2. *In vitro* mammalian cell micronucleus test

The *in vitro* cell micronucleus test used peripheral human lymphocytes collected from healthy adult, non-smoking, volunteers (aged 18–35 years). In every assay, appropriate vehicle and positive controls were included, which consisted of the following: water as negative control; without S9 activation: mitomycin C (MMC-C) as direct-acting clastogen and colchicine as direct acting aneugen; with metabolic activation: cyclophosphamide was used as an indirect acting clastogen. Vehicle for positive controls was Hanks' Balanced Salt Solution (HBSS) without calcium and magnesium.

Rat S9 was obtained from Trinova Biochem GmbH (Giessen, Germany) and was prepared from male Sprague Dawley rats that had been dosed orally with a suspension of phenobarbital (80 mg/kg body weight) and β -naphthoflavone (100 mg/kg body weight).

2.4.2.1. Dose range-finding assay. Lymphocytes were cultured for 48 ± 2 h and thereafter exposed to 7.8, 16, 31, 63, 125 and 250 μ g test item/mL culture medium for 24 h in the absence of S9-mix. The cytotoxicity of the test item in the lymphocyte cultures was determined using the Cytokinesis-Block Proliferation Index and precipitation of test substance monitored to indicate concentrations to be used in the full assays.

2.4.2.2. First cytogenic assay. Based on the results of the range-finding assay, lymphocytes were cultured for 48 ± 2 h and thereafter exposed in duplicate to concentrations of 0, 16, 31, and 63 μ g/mL of test item for 3 h in the absence and presence of S9-mix. After 3 h exposure, the cells were separated from the exposure medium by centrifugation (5 min, 365 g). The supernatant was removed, and the cells were rinsed once with 5 mL HBSS. After a second centrifugation step, HBSS was removed, and cells were re-suspended in 5 mL culture medium with cytochalasin B (5 μ g/mL) and incubated for another 24 h (1.5 times normal cell cycle).

2.4.2.3. Second cytogenic assay. Lymphocytes were cultured for 48 ± 2 h and thereafter exposed in duplicate to concentrations of 7.8, 16, and 31 μ g/mL of the test item with cytochalasin B (5 μ g/mL) for 24 h in the absence of S9-mix. Appropriate vehicle and positive controls were included in the second cytogenetic assay.

A minimum of 500 (with a maximum deviation of 5%) cells per culture were counted, scoring cells with one, two or more nuclei (multinucleated cells). The cytostasis/cytotoxicity was determined by calculating the Cytokinesis-Block Proliferation Index (CBPI). The highest dose level examined for micronuclei was 31 μ g/mL due to precipitation in the culture medium. The lowest dose level 7.8 μ g/mL had little or no cytotoxicity (approximately the same as solvent control). At least 1,000 (with a maximum deviation of 5%) binucleated cells per culture were examined by light microscopy for micronuclei.

2.5. Protein analysis for the evaluation of allergenic potential

The Food Allergy Research and Resource Program (FARRP) at the University of Nebraska-Lincoln assessed the risks of an allergenic response by humans from consumption of *Clostridium* protein using *in silico* analysis. The proteins were predicted from the genome of the species using the GLIMMER2 software at Johns Hopkins University. The predicted protein sequences were compared to the [AllergenOnline.org](https://allergenonline.org) version 20 using the Holland Computing Center server at the University of Nebraska. The software used was FASTA version 36.3.8 in batch mode using the criteria outlined in the [Codex Alimentarius Commission](https://www.codexalimentarius.org), 2009 guideline ([Codex Alimentarius Commission](https://www.codexalimentarius.org), 2009). [AllergenOnline.org](https://allergenonline.org) version 20 is a searchable public database containing 2171 allergens and putative allergen sequences which allows for the identification of proteins that may present a potential risk of allergenic cross-reactivity. The database sequences represented 873 protein-taxonomic proteins from 392 allergenic sources. The recommended criteria for cross-reactivity were that proteins with >35%

amino acid sequence identity over 80 or more amino acids could represent proteins that might lead to IgE cross-reactivity and potential allergic reactions for those with significant pre-existing allergies. The sequences of *Clostridium* proteins and the individual sequence matched allergens were also compared to the NCBI Protein database (2020) using BLASTP version 2.9.0+ (2019) to consider the relevance of the alignment. The *E* score threshold used was 10 and the calculated *E* scores were recorded to understand the relevance of the matches. The sequences identified to meet these criteria were compiled to show the best FASTA match to an allergen in [AllergenOnline.org](https://allergenonline.org) 20 including the sequence identity, alignment length, and *E* score as well as results from the BLASTP to NCBI proteins.

3. Results

3.1. Characterization of the clostridium source

The strain has been unambiguously identified as *C. tyrobutyricum* using WGS analysis and bioinformatics was used to confirm the absence of any genetic element sequences that code for virulence factors or protein toxins. WGS analysis also showed that the strain has not acquired any antimicrobial resistance.

Nutrient analyses demonstrate that *Clostridium* protein comprises 83% protein and 2.7% nucleic acids (see [Table 2](#)). The result of an *in vitro* digestibility assay using pepsin, trypsin and chymotrypsin indicate that 99.1% of the protein fraction was digested under the experimental conditions. The B vitamin content was analyzed to be 1.07 mg/kg riboflavin, 0.82 mg/kg vitamin B6 and 0.014 mg/kg vitamin B12, and mineral content to be 1.28 g/kg sodium, 0.11 g/kg calcium, 4.27 g/kg phosphorus and 0.12 g/kg iron. The mineral profile of *Clostridium* protein was taken into account to minimize nutritional differences between the dietary treatments.

3.2. 14-Day range finding study in rats

The dietary concentrations of 2.5, 5.0, 7.5, or 10.0% *Clostridium* protein corresponded to mean intakes of 2,099, 4,153, 6,372, and 8,457 mg *Clostridium* protein/kg body weight/day, respectively. There was no mortality or clinical signs of toxicity, and no test item-related differences in body weights or body weight gains, food consumption, or coagulation parameters, locomotor activity, gross pathology, organ weights, clinical chemistry, or histopathology. All recorded findings were within the range of background pathology encountered in rats of the age and strain tested. With regards to hematology, statistically significant ($p \leq 0.05$) reductions in white blood cell, lymphocyte, monocyte, eosinophil, basophil, and large unstained cells (LUC) counts were measured in high-dose male rats, as well as increased mean corpuscular volume (MCV). Basophils were also decreased in males in Groups 2 and 3. Other statistically significant findings were without a dose-response relationship and were of a magnitude that were not considered toxicologically significant. All hematology values measured were within historical control ranges for the age and strain of rat. No statistically significant test item-related effects on uric acid levels or other urinalysis parameters were reported.

Based on a lack of toxicologically significant adverse effects reported in the dose range-finding study, concentrations of 5, 7.5, and 10% *Clostridium* protein were used in the 90-day study.

3.3. 90-Day dietary feeding study

The dietary concentrations of 5, 7.5 or 10% *Clostridium* protein corresponded to mean intakes of 2,749, 4,080, and 5,558 mg *Clostridium* protein/kg body weight/day in male rats, and 3,158, 5,000, and 6,671 mg *Clostridium* protein/kg body weight/day in female rats, respectively (referred to as Groups 2, 3, and 4). Over the course of the study, there was no test item-related mortality, clinical signs of toxicity, or

statistically significant effects on food intake (see Fig. 1), body weights (see Fig. 2), ophthalmic evaluations, FOB results, or gross macroscopic or microscopic pathology evaluations (data provided as supplementary information). The only organ effects noted were slight, but statistically significantly decreased relative (to body) liver weights observed in Group 4 females and Groups 2 and 3 males, compared to the control group (see Table 4). However, the decreases were very slight in magnitude, not dose-dependent (in males), were associated with high physiological variability within groups, and were not associated with any microscopic findings in the livers. As such, it was concluded that the decreased relative liver weights was not a test item-related effect. All other microscopic findings were not considered to be related to the test item, as findings were sporadic, without a dose-response pattern, or consistent with incidence in aging rats.

The effect of graded dietary levels of *Clostridium* protein on hematology and serum chemistry parameters is shown in Tables 5 and 6, respectively. With regards to hematology (See Table 5), statistically significant effects observed at the end of the study in female rats consisted of decreased neutrophils (Groups 2, 3, and 4), monocyte counts and percentage (Groups 3 and 4), total bilirubin (Groups 3 and 4), and thyroid stimulating hormone (TSH) (Group 4). In male rats, the only hematology value that was statistically significantly different from control values was reduced high-density lipoprotein (HDL) cholesterol in Groups 3 and 4.

No adverse effects on blood coagulation parameters were noted in any of the groups, compared to control animals. As well, no test item-related effects on uric acid levels or urinalysis parameters were measured in groups at the end of the study, although female rats in groups 1 and 2 had statistically significantly decreased serum uric acid concentrations vs. control.

In reviewing the interim hematology measurements (Day 30 and 50; data provided as supplemental information) no statistically significant differences in monocyte or neutrophil counts or percentages were measured at Day 30 in any of the male or female dose groups, while a significant reduction in neutrophils was measured in all dosed males at Day 54 without any significant differences observed in any female groups. At this same time point (Day 54), female rats (Group 3 and 4) had significantly reduced monocytes, while male rats did not. At the end of the study, no significant differences were observed in males for these endpoints. Total bilirubin was decreased in group 3 and 4 females at Day 54, which persisted to the end of the study.

Inclusion of *Clostridium* protein at graded levels in the diet of the rats had no statistically significant effect on any serum chemistry parameters at the interim periods (Day 30 and 50; data provided as supplemental information) or at the end of the study.

3.4. Bacterial reverse mutation test

At concentrations of up to 5,000 µg/plate, *Clostridium* protein did not elicit an increase in the number of revertants in tester strains TA98, TA100, TA1535, and TA1537 with or without S9-mix, compared to the negative control samples in either of the 2 experiments conducted. A slight precipitate was observed at the highest concentration in Experiment 2 only, although bacterial background lawn was normal in all cases and there was no evidence of cytotoxicity.

The results of the bacterial mutation assay are presented in Table 7. With tester strain WP₂uvrA-pkM101, a dose-dependent increase in revertants was observed in the presence of S9 in Experiment 1, which reached a 2.1-fold increase over the control sample at the highest concentration (28 in the solvent control and 58 at 5,000 µg/plate). No increase was observed without S9. To confirm the results, the test was repeated in tester strain WP₂uvrA-pkM101 with S9 and no increase in revertants was observed. In Experiment 2, no increase in revertant counts compared to the negative control was observed in tester strain WP₂uvrA-pkM101. As 2 tests confirmed that WP₂uvrA-pkM101 did not elicit an increase in revertants, it was concluded that the results from the first test were not biologically relevant.

The negative control and strain-specific positive control values were within the laboratory historical control data ranges, indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

3.5. In vitro mammalian cell micronucleus test

In the DRF evaluation (7.8, 16, 31, 63, 125 and 250 µg test item/mL culture medium for 24 h without S9), the test item precipitated at concentrations of ≥31 µg/mL. The cytostasis % at the concentrations tested ranged from 6 to 15%. The pH and osmolality of a concentration of 16 µg/mL were 7.55 and 291 mOsm/kg respectively (compared to 7.52 and 300 mOsm/kg in the solvent control).

The results of the two main cytogenic assays are presented in Table 8. Based on the results of the DRF, the 1st cytogenic assay was conducted with concentration of 0, 16, 31, and 63 µg/mL (3-h exposure with and without S9). Precipitation of the test item was observed at the highest concentration in both tests and only minimal (≤5%) cytostasis was measured at any concentration. There was no statistically significant increase in the number of micronuclei counted at any concentration as compared to the solvent control samples.

In the second cytogenic assay conducted at concentrations of 0, 8, 16, and 31 µg/mL (24-h exposure time without S9), test item precipitation was observed at the highest concentration. No statistically significant increase in the number of micronuclei counted at any concentration as compared to the solvent control samples.

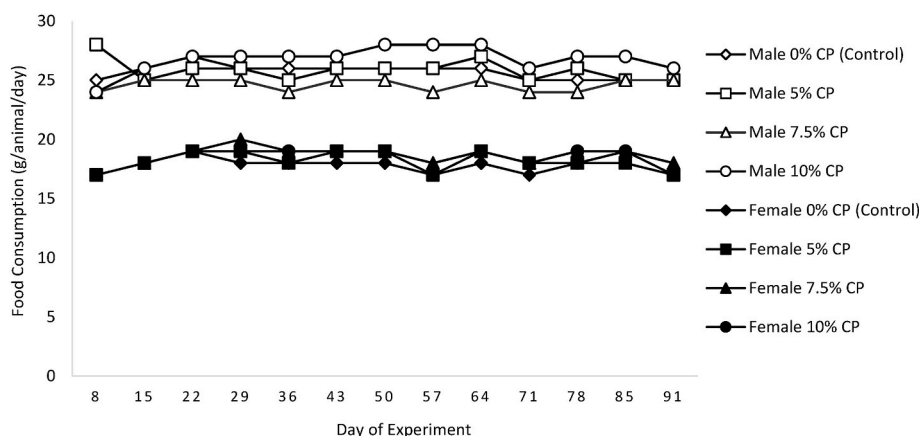


Fig. 1. Food consumption of female and male rats fed experimental diets for 90 days.

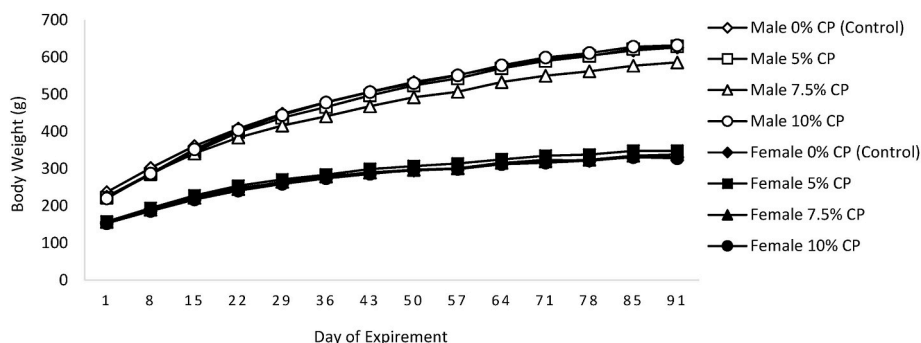


Fig. 2. Body weights of female and male rats fed experimental diets for 90 days.

Table 4

Organ weight/body weight ratio of male and female rats fed experimental diets for 90 days.

	Dietary level (mean $\pm$ standard deviation)							
	Males				Females			
	0% CP (Control)	5% CP	7.5% CP	10% CP	0% CP (Control)	5% CP	7.5% CP	10% CP
Body Weight (g)	601 $\pm$ 58	601 $\pm$ 57	560 $\pm$ 67	605 $\pm$ 61	310 $\pm$ 25	327 $\pm$ 32	312 $\pm$ 19	312 $\pm$ 30
Organ/Body Weight Ratios (%)								
Brain	0.37 $\pm$ 0.04	0.38 $\pm$ 0.04	0.40 $\pm$ 0.05	0.37 $\pm$ 0.04	0.65 $\pm$ 0.06	0.63 $\pm$ 0.04	0.66 $\pm$ 0.05	0.65 $\pm$ 0.06
Pituitary	0.002 $\pm$ 0.0002	0.002 $\pm$ 0.0004	0.003 $\pm$ 0.0004	0.002 $\pm$ 0.0004	0.01 $\pm$ 0.001	0.01 $\pm$ 0.001	0.01 $\pm$ 0.001	0.01 $\pm$ 0.001
Heart	0.29 $\pm$ 0.15	0.29 $\pm$ 0.02	0.30 $\pm$ 0.02	0.29 $\pm$ 0.02	0.38 $\pm$ 0.04	0.36 $\pm$ 0.03	0.37 $\pm$ 0.05	0.36 $\pm$ 0.04
Liver	2.29 $\pm$ 0.14	2.15 ^a $\pm$ 0.14	2.12 ^{**} $\pm$ 0.12	2.20 $\pm$ 0.06	2.79 $\pm$ 0.16	2.71 $\pm$ 0.19	2.66 $\pm$ 0.15	2.61 ^a $\pm$ 0.13
Thyroids	0.003 $\pm$ 0.001	0.004 $\pm$ 0.001	0.004 $\pm$ 0.001	0.003 $\pm$ 0.001	0.01 $\pm$ 0.001	0.01 $\pm$ 0.001	0.01 $\pm$ 0.001	0.005 $\pm$ 0.001
Thymus	0.70 $\pm$ 0.13	0.07 $\pm$ 0.01	0.06 $\pm$ 0.02	0.06 $\pm$ 0.01	0.11 $\pm$ 0.03	0.10 $\pm$ 0.02	0.10 $\pm$ 0.01	0.10 $\pm$ 0.02
Kidneys	0.61 $\pm$ 0.04	0.64 $\pm$ 0.05	0.63 $\pm$ 0.05	0.63 $\pm$ 0.05	0.69 $\pm$ 0.03	0.70 $\pm$ 0.06	0.70 $\pm$ 0.06	0.69 $\pm$ 0.05
Adrenals	0.01 $\pm$ 0.001	0.01 $\pm$ 0.001	0.01 $\pm$ 0.002	0.01 $\pm$ 0.001	0.02 $\pm$ 0.002	0.02 $\pm$ 0.003	0.02 $\pm$ 0.002	0.02 $\pm$ 0.002
Spleen	0.15 $\pm$ 0.02	0.15 $\pm$ 0.02	0.15 $\pm$ 0.02	0.16 $\pm$ 0.02	0.16 $\pm$ 0.01	0.17 $\pm$ 0.02	0.17 $\pm$ 0.02	0.16 $\pm$ 0.01
Testes	0.68 $\pm$ 0.04	0.66 $\pm$ 0.11	0.71 $\pm$ 0.06	0.66 $\pm$ 0.08	–	–	–	–
Prostate gland	0.26 $\pm$ 0.03	0.25 $\pm$ 0.04	0.26 $\pm$ 0.03	0.25 $\pm$ 0.05	–	–	–	–
Epididymides	0.23 $\pm$ 0.02	0.23 $\pm$ 0.04	0.25 $\pm$ 0.02	0.23 $\pm$ 0.03	–	–	–	–
Seminal vesicles	0.37 $\pm$ 0.07	0.34 $\pm$ 0.06	0.38 $\pm$ 0.06	0.38 $\pm$ 0.04	–	–	–	–
Ovaries	–	–	–	–	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01	0.05 $\pm$ 0.01
Uterus	–	–	–	–	0.30 $\pm$ 0.14	0.22 $\pm$ 0.12	0.38 $\pm$ 0.15	0.20 $\pm$ 0.04

Abbreviations: –, not applicable; CP, *Clostridium* protein.

^a Dunnett-test based on pooled variance significant at 5% level.

The positive control chemicals, mitomycin C, colchicine and cyclophosphamide all produced a statistically significant increase in the number of binucleated cells with micronuclei. In addition, the number of binucleated cells with micronuclei found in the positive control cultures was within the 95% control limits of the distribution of the historical positive control database. It was therefore concluded that the test conditions were adequate, and that the metabolic activation system (S9-mix) functioned properly.

The results support that *Clostridium* protein is not clastogenic or aneugenic in human lymphocytes under the conditions of the experimental.

3.6. Protein analysis for the evaluation of allergenic potential

Results of the predicted protein sequences of *Clostridium tyrobutyricum* evaluation against the [AllergenOnline.org](https://allergenonline.org/) 20 database of known and putative allergens found 23 alignments that were 80 residues or longer with a sequence identity of $\geq 35\%$. The sequences of the matched proteins listed as allergens or putative allergens were further evaluated by BLASTP with the NCBI Protein database. Based on the Codex guideline, each match above the threshold could represent a potential cross reactive protein for consumers with allergies to the sequence matched protein. However, matches noted for *Clostridium* protein were much higher in identity to other microbial proteins from sources that are not known to be allergenic than to proteins of other allergenic sources. Furthermore, comparison of the *Clostridium* proteins to the NCBI Protein

database demonstrated that *Clostridium* protein sequences are not unique and are common in bacterial species. In addition, the sequences of the allergens and information in [AllergenOnline.org](https://allergenonline.org/) for identifying allergens demonstrated that these are not potent and highly unique sequences.

The results of the bioinformatics search support that *Clostridium* protein does not represent a risk of allergic cross-reactivity for consumers.

4. Discussion

The International Life Sciences Institute (ILSI) has developed a 2-tiered, weight of evidence strategy for the safety assessment of protein products produced through biotechnology (Delaney et al., 2008; Hammond et al., 2013). The objective of using a tiered approach for the evaluation of the totality of evidence is to allow certain types of data to be allocated more weight than others, leading to greater confidence in the overall safety determination. The individual components of the evaluation are separated into Tier 1 and Tier 2, respectively to optimize the robustness of the process. Tier 1 is a potential hazard identification and includes an assessment of (1) the biological function or mode of action and intended application of the protein; (2) comparison of the amino acid sequence of the protein to other known proteins, particular those known to be toxic or allergenic and those considered to be anti-nutrients. Where Tier 1 is not sufficient to allow a reasonable certainty of no harm, additional safety testing, such as in vivo toxicity

Table 5

Hematology analysis of male and female rats fed experimental diet for 90 days.

Parameter measured	Dietary level (mean $\pm$ standard deviation)									
	Male					Female				
	0% CP (Control)	5% CP	7.5% CP	10% CP	Historical data	0% CP (Control)	5% CP	7.5% CP	10% CP	Historical data
WBC ($\times 10^9/L$)	9.98 $\pm$ 2.44	8.80 $\pm$ 1.20	9.63 $\pm$ 1.73	10.2 $\pm$ 1.30	11.8 $\pm$ 2.35	6.17 $\pm$ 1.36	4.89 $\pm$ 1.56	3.54** $\pm$ 0.93	4.92 $\pm$ 1.77	6.90 $\pm$ 1.78
NEUT ($\times 10^9/L$)	1.13 $\pm$ 0.29	0.85 $\pm$ 0.10	1.16 $\pm$ 0.89	0.88 $\pm$ 0.17	1.50 $\pm$ 0.52	0.89 $\pm$ 0.46	0.55 $\pm$ 0.42	0.46* $\pm$ 0.19	0.43** $\pm$ 0.11	0.70 $\pm$ 0.16
LYMPH ($\times 10^9/L$)	8.42 $\pm$ 2.13	7.54 $\pm$ 1.12	8.02 $\pm$ 1.08	8.96 $\pm$ 1.34	9.60 $\pm$ 2.36	4.93 $\pm$ 1.02	4.08 $\pm$ 1.37	2.90** $\pm$ 0.84	4.28 $\pm$ 1.70	5.30 $\pm$ 1.52
MONO ($\times 10^9/L$)	0.20 $\pm$ 0.08	0.20 $\pm$ 0.10	0.20 $\pm$ 0.07	0.17 $\pm$ 0.04	0.20 $\pm$ 0.13	0.17 $\pm$ 0.08	0.10* $\pm$ 0.06	0.06** $\pm$ 0.03	0.09 $\pm$ 0.04	0.20 $\pm$ 0.07
EOS ($\times 10^9/L$)	0.15 $\pm$ 0.03	0.14 $\pm$ 0.04	0.17 $\pm$ 0.06	0.16 $\pm$ 0.03	0.10 $\pm$ 0.09	0.10 $\pm$ 0.04	0.11 $\pm$ 0.05	0.09 $\pm$ 0.04	0.08 $\pm$ 0.03	0.10 $\pm$ 0.06
BASO ($\times 10^9/L$)	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.03 $\pm$ 0.01	0.00 $\pm$ 0.03	0.01 $\pm$ 0.01	0.01 $\pm$ 0.01	0.004 $\pm$ 0.01	0.01 $\pm$ 0.01	0.00 $\pm$ 0.00
LUC ($\times 10^9/L$)	0.06 $\pm$ 0.02	0.05 $\pm$ 0.03	0.06 $\pm$ 0.03	0.06 $\pm$ 0.03	–	0.06 $\pm$ 0.04	0.05 $\pm$ 0.02	0.03 $\pm$ 0.01	0.04 $\pm$ 0.02	–
NEUT (%)	11.5 $\pm$ 2.15	9.74 $\pm$ 1.40	11.4 $\pm$ 5.86	8.76 $\pm$ 2.38	13.0 $\pm$ 4.37	14.1 $\pm$ 4.60	11.2 $\pm$ 6.85	13.1 $\pm$ 5.16	9.76 $\pm$ 4.03	11.7 $\pm$ 4.78
LYMPH (%)	84.2 $\pm$ 2.30	85.6 $\pm$ 2.54	84.0 $\pm$ 5.76	87.3 $\pm$ 2.80	83.2 $\pm$ 5.17	80.3 $\pm$ 5.14	83.5 $\pm$ 7.18	81.7 $\pm$ 5.20	85.8 $\pm$ 4.04	84.2 $\pm$ 5.19
MONO (%)	2.03 $\pm$ 0.72	2.27 $\pm$ 1.20	2.07 $\pm$ 0.65	1.64 $\pm$ 0.38	2.20 $\pm$ 1.07	2.78 $\pm$ 1.03	1.98 $\pm$ 0.83	1.78* $\pm$ 0.79	1.85* $\pm$ 0.67	2.30 $\pm$ 0.80
EOS (%)	1.52 $\pm$ 0.33	1.58 $\pm$ 0.42	1.70 $\pm$ 0.43	1.54 $\pm$ 0.31	1.10 $\pm$ 0.64	1.63 $\pm$ 0.63	2.30 $\pm$ 0.99	2.53* $\pm$ 0.90	1.64 $\pm$ 0.63	1.50 $\pm$ 0.69
BASO (%)	0.22 $\pm$ 0.06	0.20 $\pm$ 0.05	0.22 $\pm$ 0.04	0.26 $\pm$ 0.05	0.20 $\pm$ 0.20	0.20 $\pm$ 0.12	0.14 $\pm$ 0.05	0.11 $\pm$ 0.07	0.12 $\pm$ 0.04	0.20 $\pm$ 0.15
LUC (%)	0.56 $\pm$ 0.13	0.58 $\pm$ 0.31	0.62 $\pm$ 0.34	0.55 $\pm$ 0.23	0.80 $\pm$ 0.50	1.03 $\pm$ 0.56	0.88 $\pm$ 0.22	0.70 $\pm$ 0.27	0.80 $\pm$ 0.28	0.90 $\pm$ 0.44
RBC ($\times 10^{12}/L$)	8.54 $\pm$ 0.19	8.45 $\pm$ 0.36	8.41 $\pm$ 0.38	8.32 $\pm$ 0.49	8.85 $\pm$ 0.45	7.92 $\pm$ 0.54	7.64 $\pm$ 0.26	7.70 $\pm$ 0.34	7.77 $\pm$ 0.31	8.03 $\pm$ 0.35
RETIC ($\times 10^9/L$)	192 $\pm$ 28.0	176 $\pm$ 23.0	170 $\pm$ 26.0	191 $\pm$ 25.8	200 $\pm$ 37.3	167 $\pm$ 62.8	146 $\pm$ 34.0	154 $\pm$ 27.9	129 $\pm$ 28.9	174 $\pm$ 33.9
RDW (%)	13.0 $\pm$ 0.40	13.0 $\pm$ 0.52	12.8 $\pm$ 0.37	13.4 $\pm$ 0.66	12.9 $\pm$ 0.71	12.2 $\pm$ 0.60	12.1 $\pm$ 0.36	12.1 $\pm$ 0.29	11.7 $\pm$ 0.27	11.7 $\pm$ 0.46
HGB (g/L)	152 $\pm$ 4.00	149 $\pm$ 4.50	149 $\pm$ 3.90	146 $\pm$ 5.60	15.6 $\pm$ 0.74	141 $\pm$ 6.60	137 $\pm$ 4.40	138 $\pm$ 4.50	138 $\pm$ 5.20	15.0 $\pm$ 0.63
HCT (L/L)	0.46 $\pm$ 0.01	0.45 $\pm$ 0.01	0.46 $\pm$ 0.01	0.45 $\pm$ 0.02	0.46 $\pm$ 0.02	0.42 $\pm$ 0.02	0.42 $\pm$ 0.02	0.42 $\pm$ 0.01	0.42 $\pm$ 0.02	0.43 $\pm$ 0.02
MCV (fL)	53.9 $\pm$ 1.36	53.6 $\pm$ 1.90	54.2 $\pm$ 1.41	53.8 $\pm$ 1.55	51.6 $\pm$ 2.03	53.5 $\pm$ 2.87	54.3 $\pm$ 1.26	54.2 $\pm$ 1.27	54.1 $\pm$ 1.32	53.3 $\pm$ 1.97
MCH (pg)	17.7 $\pm$ 0.42	17.6 $\pm$ 0.68	17.7 $\pm$ 0.63	17.6 $\pm$ 0.63	17.7 $\pm$ 0.69	17.9 $\pm$ 1.07	18.0 $\pm$ 0.39	17.9 $\pm$ 0.55	17.8 $\pm$ 0.45	18.5 $\pm$ 0.52
MCHC (g/L)	329 $\pm$ 4.10	328 $\pm$ 3.60	327 $\pm$ 5.80	328 $\pm$ 4.50	344 $\pm$ 8.77	334 $\pm$ 4.70	331 $\pm$ 4.90	331 $\pm$ 6.70	330 $\pm$ 4.50	349 $\pm$ 9.78
PLT ($\times 10^9/L$)	950 $\pm$ 156.5	927 $\pm$ 64.4	963 $\pm$ 128.9	991 $\pm$ 131.3	920 $\pm$ 155.5	825 $\pm$ 70.6	804 $\pm$ 114.2	834 $\pm$ 62.7	827 $\pm$ 91.2	895 $\pm$ 164.1

Abbreviations: BASO, basophils; CP, *Clostridium* protein; EOS, eosinophils; HCT, hematocrit; HGB, hemoglobin; LUC, large unstained cells; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; NEUT, neutrophils; PTL, platelet; RBC, red blood cells; RDW, red blood cell distribution width; RETIC, reticulocytes; WBC, white blood cells.

*p $\leq$ 0.05 determine with Anova & Dunnett.

**p $\leq$ 0.01 determine with Anova & Dunnett.

studies are conducted under Tier 2.

While *Clostridium* protein does not contain any transgenic proteins, the approach can broadly be applied to novel microbial proteins. *C. tyrobutyricum* is well-documented in the published literature on account of its ability to produce the short-chain fatty acid (SCFA) butyrate (Liu et al., 2006; Michel-Savin et al., 1990). The species is commonly found in milk and cheese, and is responsible for the defect known as “late-blowing” in semi-hard cheeses, where gas production results in holds, fissures and bursting of the cheese (EFSA, 2005; Ghoddusi and Sherburn, 2010; Brändle et al., 2016). There are no reports in the published literature associating *C. tyrobutyricum* with toxin production or pathogenicity in humans or animals. The findings of the genome-wide analysis conducted on the *Clostridium* strain are consistent with the published literature, confirming the absence of any genes encoding for toxins commonly associated with *Clostridium* species or any known virulence factors. Although these data indicate that *Clostridium* protein

will not be associated with any safety concerns when used as an ingredient in human food, no studies were identified in the published literature in which *C. tyrobutyricum*, or proteins derived from *Clostridium* per se, were fed to humans or animals.

On this basis, a battery of toxicity studies were undertaken on account of the novelty of bacterial proteins, and particularly *Clostridium* protein as a human food ingredient. Genotoxicity studies are not normally conducted on novel proteins but considering the absence of any significant history of use of *Clostridia* or products derived thereof, as food or feed ingredients, evaluation of the genotoxic potential was considered pertinent to the safety evaluation. Consistent with the WGS and bioinformatics analysis, the results of the in vitro genotoxicity tests demonstrate that *Clostridium* protein is non-genotoxic.

The methodology used for the toxicity assessment of *Clostridium* protein is consistent with the general principles laid down by the FDA in the Redbook Chapter III (U.S. FDA, 2000c) and EFSA’s Guidance on

Table 6

Serum chemistry analysis of male and female rats fed experimental diets for 90 days.

Parameter measured	Dietary level (mean ± standard deviation)									
	Male					Female				
	0% CP (Control)	5% CP	7.5% CP	10% CP	Historical data	0% CP (Control)	5% CP	7.5% CP	10% CP	Historical data
ALT (U/L)	47.1 ± 13.4	40.9 ± 2.3	43.2 ± 5.7	42.4 ± 3.6	38.1 ± 7.74	35.7 ± 5.5	41.5 ± 17.5	33.6 ± 4.1	40.8 ± 7.0	39.6 ± 16.7
AST (U/L)	99.3 ± 32.8	99.0 ± 9.7	105 ± 15.1	101 ± 15.1	86.2 ± 14.1	84.5 ± 19.3	101 ± 48.7	85.5 ± 9.0	107 ± 36.7	88.5 ± 27.4
ALP (U/L)	138 ± 57.0	102 ± 28.2	92.2 ± 21.7	108 ± 30.0	112 ± 27.1	60.0 ± 29.2	63.8 ± 49.6	49.4 ± 12.5	42.6 ± 10.9	56 ± 19.0
Total Protein (g/L)	64.7 ± 1.70	63.7 ± 1.66	63.1 ± 1.32	63.7 ± 1.56	64.7 ± 2.60	79.5 ± 4.16	74.6 ± 4.03	75.8 ± 5.29	75.3 ± 4.46	71.6 ± 4.04
Albumin (g/L)	37.7 ± 1.13	37.2 ± 1.43	36.3 ± 1.30	37.1 ± 1.07	31.6 ± 1.46	50.9 ± 2.99	47.1 ± 2.93	48.2 ± 4.39	47.2 ± 3.83	37.8 ± 2.63
Total Bilirubin (umol/L)	2.69 ± 0.30	2.72 ± 0.19	2.55 ± 0.30	2.87 ± 0.30	2.4 ± 0.33	3.70 ± 0.55	3.25 ± 0.72	2.44*** ± 0.83	2.87* ^a ± 0.54	2.9 ± 0.48
Urea (mmol/L)	4.21 ± 0.84	3.99 ± 0.42	3.93 ± 0.40	4.11 ± 0.77	4.7 ± 0.86	5.57 ± 0.56	5.15 ± 0.52	5.58 ± 0.83	5.18 ± 0.67	5.9 ± 1.30
Creatine (umol/L)	28.6 ± 5.2	28.3 ± 1.5	27.3 ± 3.0	28.4 ± 3.2	38.1 ± 2.51	39.4 ± 3.4	38.2 ± 3.2	38.8 ± 4.2	41.3 ± 4.6	45.2 ± 3.53
Glucose (mmol/L)	9.38 ± 1.08	9.27 ± 0.86	8.83 ± 1.25	9.93 ± 1.05	8.52 ± 1.37	10.2 ± 1.16	10.8 ± 1.23	11.0 ± 1.22	10.2 ± 1.50	8.25 ± 1.10
Cholesterol (mmol/L)	1.86 ± 0.22	1.64 ± 0.28	1.49*** ± 0.30	1.61 ± 0.18	1.53 ± 0.40	1.81 ± 0.31	1.97 ± 0.37	1.78 ± 0.22	1.70 ± 0.33	2.09 ± 0.50
Triglycerides (mmol/L)	0.76 ± 0.24	0.70 ± 0.37	0.66 ± 0.35	0.66 ± 0.19	0.64 ± 0.23	0.59 ± 0.23	0.49 ± 0.07	0.48 ± 0.12	0.44 ± 0.11	0.50 ± 0.15
HDL (mmol/L)	1.26 ± 0.12	1.10 ± 0.18	1.00*** ± 0.21	1.08** ± 0.13	–	1.30 ± 0.20	1.38 ± 0.23	1.31 ± 0.15	1.20 ± 0.23	–
LDL (mmol/L)	0.34 ± 0.04	0.35 ± 0.06	0.30 ± 0.04	0.32 ± 0.04	–	0.35 ± 0.06	0.38 ± 0.08	0.35 ± 0.06	0.34 ± 0.05	–
Sodium (mmol/L)	144 ± 0.7	143 ± 0.8	144 ± 0.7	143 ± 1.0	142 ± 1.89	142 ± 1.3	142 ± 0.8	142 ± 0.8	143 ± 1.2	142 ± 1.90
Potassium (mmol/L)	3.92 ± 0.14	3.94 ± 0.18	4.03 ± 0.16	4.02 ± 0.26	3.99 ± 0.21	3.68 ± 0.22	3.59 ± 0.17	3.69 ± 0.15	3.72 ± 0.22	3.59 ± 0.34
Chloride (mmol/L)	109 ± 0.8	109 ± 1.1	109 ± 0.9	108 ± 1.4	104 ± 1.4	108 ± 2.1	107 ± 1.2	107 ± 1.5	108 ± 1.6	103 ± 2.1
Calcium (mmol/L)	2.62 ± 0.04	2.60 ± 0.07	2.68 ± 0.14	2.71 ± 0.13	2.59 ± 0.11	2.73 ± 0.09	2.67 ± 0.10	2.77 ± 0.17	2.74 ± 0.12	2.67 ± 0.12
Phosphorus (mmol/L)	1.99 ± 0.14	1.90 ± 0.11	2.02 ± 0.07	2.03 ± 0.09	–	1.53 ± 0.27	1.53 ± 0.26	1.49 ± 0.26	1.64 ± 0.39	–
Triiodothyronine	0.48 ± 0.06	0.60* ^a ± 0.11	0.57 ± 0.14	0.49 ± 0.10	–	0.70 ± 0.12	0.73 ± 0.11	0.69 ± 0.10	0.71 ± 0.13	–
Thyroxine	47.2 ± 5.36	46.9 ± 6.16	48.7 ± 4.75	51.4 ± 7.63	–	27.8 ± 8.21	29.6 ± 7.43	28.3 ± 5.25	25.5 ± 6.18	–
THS	0.60 ± 0.49	0.58 ± 0.24	0.53 ± 0.37	0.60 ± 0.39	–	0.22 ± 0.16	0.35 ± 0.64	0.15 ± 0.07	0.07* ^b ± 0.03	–

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CP, *Clostridium* protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; THS, thyroid stimulating hormone.

* $p \leq 0.05$.

** $p \leq 0.01$.

^a Anova & Dunnett.

^b Kruskal-Wallis & Dunn.

Novel Foods (EFSA, 2021). However, it is generally recognized that the standard battery of testing in animals has limitations when applied to macronutrients on the basis that there are practical limitations in deriving No-Observed-Adverse-Effect-Levels (NOAELs) or tolerability limits. The concentration of the test item generally cannot be incorporated into the diet at sufficiently high levels to derive the conventional 100-fold safety factor allowing for intra- and inter-species variation without resulting in nutritional imbalances which can lead to secondary consequences such as adverse physiological effects (EFSA, 2021). Consequently, the findings of the toxicity study must be considered in conjunction with the characterization of the *Clostridium* source and the nutritional composition of the product to demonstrate the safety under the proposed conditions of use. Thus, the overall assessment is a weight of evidence approach to determine the safety of *Clostridium* protein as an alternative source of protein in human food.

The quality of protein according to the Institute of Medicine (IOM) is principally defined by digestibility and amino acid composition (IOM,

2005). In terms of nutritional value, *Clostridium* protein is a source of all essential amino acids (Table 2) and meets or exceeds the requirements laid down by the World Health Organization (WHO) for protein source on a g/100 g protein basis for all individual amino acids. Under the conditions of a commercial in vitro assay using pepsin, trypsin and chymotrypsin, 99.1% of the protein fraction was digested. Furthermore, in the 90-day dietary feeding study, rats fed diets containing *Clostridium* protein as a partial replacement for casein displayed equivalent growth with no reduction in food consumption relative to the controls. The comparable growth performance of the results among all dietary treatment groups is consistent with *Clostridium* protein providing a digestible and high-quality protein source for rats. The experimental diets used in the animal studies were nutritionally balanced by adjusting for the compositional profile of *Clostridium* protein. Consequently, there were minimal differences in protein, ash, fat or mineral profiles between the dietary treatments (generally <0.2% variation between parameters). The only exception was crude fiber which was observed to increase from

Table 7Mutagenic response of *Clostridium* protein in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

Concentration (μg/plate)	Revertant colonies per plate (mean ± standard deviation)									
	<i>S. typhimurium</i> TA98		<i>S. typhimurium</i> TA100		<i>S. typhimurium</i> TA1535		<i>S. typhimurium</i> TA1537		<i>E. coli</i> WP2uvrA (pkM101)	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Experiment 1										
Negative control	6 ± 2	9 ± 5	91 ± 9	84 ± 10	5 ± 3	4 ± 3	4 ± 1	6 ± 3	14 ± 4	28 ± 9
52	6 ± 4	6 ± 2	72 ± 15	86 ± 8	6 ± 1	5 ± 2	2 ± 2	9 ± 5	22 ± 14	38 ± 12
164	7 ± 3	7 ± 3	70 ± 3	72 ± 12	8 ± 2	8 ± 1	3 ± 2	6 ± 6	20 ± 7	38 ± 12
512	7 ± 4	10 ± 5	74 ± 3	80 ± 12	4 ± 1	11 ± 2	4 ± 1	6 ± 2	22 ± 2	48 ± 7
1,600	7 ± 2	14 ± 1	70 ± 6	99 ± 19	9 ± 2	6 ± 2	4 ± 1	15 ± 6	13 ± 5	53 ± 5
5,000	10 ± 2 ⁿ NP	10 ± 3 ⁿ NP	58 ± 6 ⁿ NP	79 ± 12 ⁿ NP	7 ± 4 ⁿ NP	8 ± 2 ⁿ NP	6 ± 1 ⁿ NP	6 ± 3 ⁿ NP	9 ± 5 ⁿ NP	58 ± 7 ⁿ NP
Positive control	1172 ± 81	706 ± 97	200 ± 21 ^s	1216 ± 106	121 ± 12	237 ± 148	44 ± 6	258 ± 44	627 ± 100	154 ± 47
Additional Experiment: <i>Escherichia coli</i> Reverse Mutation Assay										
Solvent control	–	–	–	–	–	–	–	–	–	40 ± 6
52	–	–	–	–	–	–	–	–	–	48 ± 2
164	–	–	–	–	–	–	–	–	–	60 ± 7
512	–	–	–	–	–	–	–	–	–	58 ± 5
1600	–	–	–	–	–	–	–	–	–	63 ± 5
5000	–	–	–	–	–	–	–	–	–	53 ± 2 ^{NP}
Positive control	–	–	–	–	–	–	–	–	–	152 ± 7
Experiment 2										
Negative control	12 ± 6	9 ± 3	75 ± 19	59 ± 2	9 ± 3	7 ± 1	2 ± 1	9 ± 2	33 ± 2	52 ± 13
275	11 ± 4	9 ± 1	73 ± 9	73 ± 15	6 ± 3	10 ± 2	2 ± 1	5 ± 2	18 ± 6	66 ± 2
492	11 ± 4	10 ± 2	78 ± 14	72 ± 10	6 ± 1	7 ± 2	4 ± 1	2 ± 1	16 ± 4	64 ± 9
878	2 ± 1	9 ± 2	79 ± 6	62 ± 8	10 ± 2	12 ± 2	3 ± 2	3 ± 0	21 ± 5	55 ± 2
1,568	8 ± 6	12 ± 4	70 ± 6	56 ± 8	9 ± 1	9 ± 2	2 ± 1	4 ± 1	18 ± 4	64 ± 10
2,800	8 ± 3 ^{NP}	12 ± 2 ^{NP}	73 ± 9 ^{NP}	60 ± 10 ^{NP}	10 ± 4 ^{NP}	9 ± 3 ^{NP}	2 ± 1 ^{NP}	3 ± 2 ^{NP}	16 ± 2 ^{NP}	74 ± 9 ^{NP}
5,000	11 ± 2 ⁿ SP	14 ± 2 ⁿ SP	78 ± 6 ⁿ SP	57 ± 9 ⁿ SP	7 ± 2 ⁿ SP	14 ± 4 ⁿ SP	3 ± 1 ⁿ SP	5 ± 4 ⁿ SP	12 ± 7 ⁿ SP	67 ± 9 ⁿ SP
Positive control	891 ± 116	283 ± 6	338 ± 157	706 ± 157	95 ± 21	175 ± 21	42 ± 9	58 ± 9	487 ± 53	133 ± 23

Abbreviations: –, not applicable; -S9, without metabolic activation; +S9, with metabolic activation.

^{NP} No precipitate.^{SP} Slight precipitate.ⁿ Normal bacterial background lawn.

Positive control = MMS 800 μg/ml.

*Mean number of revertant colonies/3 replicate plates with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain.

2.2 g/100 g (control diet) to 3.3 g/100 (10% *Clostridium* protein in the diet), but this was not associated with any apparent detrimental effects on growth or health of the animals.

In addition to providing a source of protein, *Clostridium* protein contains B vitamins as a consequence of the bacterial source, including 0.014 mg/kg of vitamin B12. One of the primary uses of *Clostridium* protein as a food ingredient is in vegan cheese-type products which are widely found on the market in the U.S. A 25 g serving of *Clostridium* protein as part of a vegan cheese product would supply around 0.3 μg vitamin B12, contributing around 14% of the Recommended Daily Allowance (RDA) for vitamin B12 for adults of 2.4 μg/day set by the IOM (1998). Considering that B vitamin intake can be a concern for individuals consuming plant-based diets, the vitamin B12 content of *Clostridium* protein adds to the potential nutritional value of the ingredient as an alternative protein source in food.

The only statistically significant effects measured in the 90-day feeding study consisted of a small number of hematology findings, primarily in females. Reduced neutrophil counts (Groups 2, 3, and 4) and monocyte percentage (Groups 3 and 4) and counts (Groups 2, 3, and 4), as well as reduced total bilirubin (Groups 3 and 4) were observed in female rats only at the end of the study.

The laboratory historical values for female Sprague Dawley rats from the performing laboratory for these parameters were $0.9 \times 10^9/L \pm 0.38$ for neutrophil count and $0.2 \times 10^9/L \pm 0.07$ for monocyte counts. The measured values in Groups 3 and 4 were slightly outside a standard deviation of the reference range for females (Table 5). These findings were not considered to be adverse, primarily due to the fact that no concurrent findings were measured in male rats at any dose and measurements were subject to high inter-group variability.

Bilirubin is produced by the breakdown of heme proteins, like hemoglobin, first to biliverdin and then into bilirubin, which is carried in the plasma to the liver, where it is conjugated to form bilirubin

diglucuronide and excreted in the urine and feces (Hull and Agarwal, 2014). Total bilirubin includes both the conjugated and unconjugated (free) forms. Measurement of bilirubin levels typically serve as a measure of liver and bile tract function, with highly elevated levels typically indicative of liver damage, hemolysis, or other disorders, which can precipitate as jaundice and ultimately neurological damage at extremely high concentrations (VanWagner and Green, 2015). However, recent data indicate associations of slightly elevated serum bilirubin being protective of cardiovascular and metabolic health in certain populations (Hinds and Stec, 2019), which may be attributed to its hormonal signaling and antioxidant properties. In contrast, Creeden et al. (2021) reviewed associations of decreased bilirubin levels and numerous adverse health conditions related to the cardiovascular, metabolic, and organ diseases. However, the exact role and mechanism in these associations are still being elucidated. The reduction of total bilirubin in female rats of Groups 3 and 4 (2.44 and 2.87 μmol/L, respectively) remained within the reference range for this strain, sex, and age of rat (2.9 μmol/L ± 0.48). The lack of any associated adverse physiological effects and absence of a concurrent reduction in bilirubin in male rats in any group, further supports that the decrease in bilirubin was an incidental finding and not being biologically significant.

Statistically significantly reduced HDL cholesterol levels in Group 3 and 4 rats, compared to controls, was not considered to be biologically relevant or an adverse effect, as the mean values in all groups were all within a standard deviation of each other, there were no concurrent changes in any of the other lipids or triglyceride levels, nor were there any effects seen at interim evaluations, or any such effects observed in female rats. As such, in the absence of any other evidence to indicate adverse health effects from the consumption of *Clostridium* protein, these findings were not considered to be biologically relevant.

Other statistically significant hematology findings were observed sporadically at interim evaluations or only in low or mid-dose groups of

Table 8

Number binucleated cells with micronuclei of human lymphocyte cultures treated with *Clostridium* protein in the cytogenetic assays.

Concentration (µg/ mL)	Mean CBPI	Cytostasis (%)	Number of binucleated cells with micronuclei ^a		
			1000	1000	2000
			A	B	A + B
3-h treatment: S9					
0	2.28	0	0	0	0
16	2.21	5	1	2	3
31	2.28	0	1	1	2
63 ^b	2.26	1	1	1	2
0.25 MMC-C	1.85	33	26	22	48****
0.05 Colch	1.41	68	41	50	91****
3-h treatment: +S9					
0	2.34	0	1	1	2
16	2.30	3	1	1	2
31	2.33	1	0	0	0
63 ^b	2.34	0	1	0	1
Cyclophosphamide	1.56	58	23	28	51****
24-h treatment: S9					
0	1.46	0	1	1	2
8	1.49	−9	0	0	0
16	1.53	−17	0	1	1
31 ^b	1.33	28	1	0	1
0.15 MMC-C	1.17	62	14	8	22****
0.05 Colch	1.12	100	1 ^c	2 ^c	3**

Abbreviations: MMC-C, mitomycin C; -S9, without metabolic activation; +S9, with metabolic activation.

** Significantly different from control group (Chi-square test), $p < 0.01$.

**** Significantly different from control group (Chi-square test), $p < 0.0001$.

^a 1000 binucleated cells were scored for the presence of micronuclei. Duplicate cultures are indicated by A and B.

^b The test item precipitated in the culture medium.

^c 71 and 43 binucleated cells were scored for the presence of micronuclei, respectively.

one sex. As these statistical findings did not persist throughout the study (i.e., not statistically significantly at the final evaluation), were not dose-dependent, and/or observed in only one sex, these were not considered to be toxicologically relevant.

An important safety consideration in the production of microbial proteins is the high nucleic acid content and the potential elicitation of gout and kidney stones due to the ingestion of purine compounds derived from RNA breakdown, which increases uric acid concentrations in the blood (Kamel and Kramer, 1979; Ritala et al., 2017). During the manufacture of *Clostridium* protein, steps are undertaken to reduce the levels of nucleic acids to less than 4% on a weight basis. Based on the results of the 90-day study in rats, especially hind gut histology, no impact on uric acid levels were observed in any of the groups of animals consuming *Clostridium* protein, which supports that the nucleic acid content would not be a safety concern following long-term consumption of this ingredient by humans.

The protein analysis supports that *Clostridium* protein does not pose a realistic risk of food allergy to consumers. These findings are consistent with the low oral allergenic risk generally associated with microbial species and in contrast to other novel proteins gaining increasing interest as substitutes for traditional food sources, such as insect protein and plant-derived protein. In this respect, *Clostridium* protein offers advantages over for example, insect protein for which cross-reactivity with shellfish (Leung et al., 1996; EFSA, 2015; Barre et al., 2018) could present an added allergy risk.

The utility of *Clostridium* protein as a nutrient source in the diet is further supported by data in the published literature on other bacterial proteins. Although, the number of studies conducted in animals using bacterial proteins per se is limited, a number of studies are available in chickens, minks, and pigs in which animals were fed proteins from a mixture of bacteria fermented on natural gas, ammonia and oxygen

(Øverland et al., 2004; Hellwing et al., 2005, 2006 and 2007). In these studies, bacterial protein partially replaced other commonly used protein sources in the diet of the animals with no detrimental effects on growth performance or blood parameters. By comparison, *Clostridium* protein is derived from a single well-characterized strain grown on a corn-derived sugar feedstock, which limits the potential for variability or carryover of unintended impurities. The nucleic acid content is also considerably lower than reported in these alternative bacterial proteins (e.g., 10 and 12% in Øverland et al., 2004 and Hellwing et al., 2007, vs. below 4% in *Clostridium* protein). From a safety perspective, therefore, *Clostridium* protein offers many advantages which will aid its commercialization as a human food ingredient.

5. Conclusion

Based on the results of the 90-day feeding study in rats, the intake of up to 10% *Clostridium* protein in the diet, corresponding to 5,558 and 6,671 mg/kg body weight/day for male and female rats, respectively, was not associated with any adverse effects on the growth or health of the animals compared to controls. Therefore, the NOAEL under the experimental conditions was at least 10% *Clostridium* protein in the diet, the highest dose tested. *Clostridium* protein was not mutagenic in the *S. typhimurium* and *E. coli* reverse mutation test up to the highest concentration tested and did not elicit an increase in the incidence of micronuclei in human lymphocytes. Bioinformatics analysis confirms that protein produced from the production strain is unlikely to represent any risk of allergenicity to consumers. Taken together, these results support that *Clostridium* protein is a safe and high-quality source of protein for human consumption.

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CRedit authorship contribution statement

Tom Jonaitis: Validation, Writing – original draft, Writing – review & editing, Visualization. **Elizabeth A. Lewis:** Conceptualization, Writing – original draft, Writing – review & editing, Visualization. **Nicky Lourens:** Investigation. **Angelique Groot:** Investigation. **Richard E. Goodman:** Formal analysis. **Daniel Mitchell:** Investigation. **Alon Karpol:** Supervision. **Bryan Tracy:** Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Tom Jonaitis and Elizabeth Lewis received consultancy fees to assist in designing, placing, and interpreting the results of the studies, as well as drafting this manuscript. Nicky Lourens and Angelique Groot were responsible for the conduct of the studies at CRL. Richard E. Goodman was hired to conduct the protein analysis study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fct.2022.112878>.

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SUPPLEMENTARY INFORMATION

Table of Content

1	Subchronic Toxicity Study in Rats	2
1.1	Body Weight and Feed Intake Results.....	2
	Table 1.1: Body weight (g) males	2
	Table 1.2: Body weight (g) females	2
	Table 1.3: Food consumption (g/animal/day) males.....	3
	Table 1.4: Food consumption (g/animal/day) females	3
	Table 1.5: Relative food consumption (g/kg body weight/day) males.....	4
	Table 1.6: Relative food consumption (g/kg body weight/day) females.....	4
1.2	Interim Hematology Evaluations (Day 30 and Day 50)	5
	Table 1.7: Hematology analysis of male rats fed treatment diets for 29 days	5
	Table 1.8: Hematology analysis of female rats fed treatment diets for 30 days	6
	Table 1.9: Hematology analysis of male rats fed treatment diets for 54 days	7
	Table 1.10: Hematology analysis of female rats fed treatment diets for 54 days	8
1.3	Coagulation Evaluation (Day 90).....	9
	Table 1.11: Coagulation analysis of male and female rats fed treatment diets for 90 days	9
1.4	Interim Biochemistry Evaluations (Day 30 and Day 50)	10
	Table 1.12: Serum chemistry analysis of male rats fed treatment diets for 54 days	10
	Table 1.13: Serum chemistry analysis of female rats fed treatment diets for 54 days.....	11
1.6	End of Study Urinalysis and Uric Acid Results	12
	Table 1.14. Urinalysis of male rats fed treatment diets for 92 days	12
	Table 1.15. Urinalysis of Female Rats fed Treatment Diets for 93 days.....	12

1 Subchronic Toxicity Study in Rats**1.1 Body Weight and Feed Intake Results****Table 1.1:** Body weight (g) males

Administration Period	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
Day 1	237 $\pm$ 15.4	222 $\pm$ 16.8	225 $\pm$ 14.6	220 $\pm$ 21.8
Day 8	302 $\pm$ 19.1	285 $\pm$ 17.9	287 $\pm$ 19.6	287 $\pm$ 23.5
Day 15	361 $\pm$ 26.7	345 $\pm$ 18.5	341 $\pm$ 29.2	352 $\pm$ 24.4
Day 22	408 $\pm$ 35.0	398 $\pm$ 22.2	384 $\pm$ 36.7	404 $\pm$ 28.4
Day 29	448 $\pm$ 40.8	437 $\pm$ 28.8	416 $\pm$ 45.1	444 $\pm$ 34.5
Day 36	479 $\pm$ 35.0	466 $\pm$ 35.0	441 $\pm$ 48.7	478 $\pm$ 39.0
Day 43	507 $\pm$ 49.0	497 $\pm$ 38.8	468 $\pm$ 53.4	506 $\pm$ 43.2
Day 50	533 $\pm$ 54.7	524 $\pm$ 42.3	492 $\pm$ 57.5	530 $\pm$ 49.7
Day 57	552 $\pm$ 57.3	543 $\pm$ 44.9	507 $\pm$ 61.5	551 $\pm$ 48.2
Day 64	575 $\pm$ 59.0	570 $\pm$ 49.5	533 $\pm$ 62.4	578 $\pm$ 53.6
Day 71	596 $\pm$ 60.1	590 $\pm$ 52.5	550 $\pm$ 65.4	599 $\pm$ 58.6
Day 78	604 $\pm$ 60.2	603 $\pm$ 53.3	562 $\pm$ 66.5	611 $\pm$ 61.6
Day 85	618 $\pm$ 61.7	622 $\pm$ 58.3	577 $\pm$ 68.1	628 $\pm$ 65.8
Day 91	626 $\pm$ 62.1	629 $\pm$ 61.0	586 $\pm$ 67.3	632 $\pm$ 65.3

Abbreviations: CP, *Clostridium* protein.**Table 1.2:** Body weight (g) females

Administration Period	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
Day 1	157 $\pm$ 8.3	158 $\pm$ 15.5	157 $\pm$ 14.5	153 $\pm$ 12.3
Day 8	192 $\pm$ 10.7	194 $\pm$ 17.6	190 $\pm$ 12.8	186 $\pm$ 15.2
Day 15	224 $\pm$ 11.9	228 $\pm$ 19.2	222 $\pm$ 11.8	217 $\pm$ 18.2
Day 22	248 $\pm$ 15.5	254 $\pm$ 23.4	245 $\pm$ 14.2	241 $\pm$ 15.7
Day 29	263 $\pm$ 16.5	271 $\pm$ 25.6	265 $\pm$ 15.8	259 $\pm$ 18.8
Day 36	281 $\pm$ 18.7	284 $\pm$ 24.1	279 $\pm$ 12.6	274 $\pm$ 22.3
Day 43	288 $\pm$ 17.5	299 $\pm$ 27.7	290 $\pm$ 17.6	286 $\pm$ 29.2
Day 50	295 $\pm$ 21.5	307 $\pm$ 30.9	297 $\pm$ 18.3	297 $\pm$ 32.2
Day 57	300 $\pm$ 19.8	314 $\pm$ 30.4	301 $\pm$ 16.4	299 $\pm$ 23.1
Day 64	315 $\pm$ 22.5	325 $\pm$ 35.9	316 $\pm$ 18.9	312 $\pm$ 26.1
Day 71	324 $\pm$ 17.2	335 $\pm$ 34.1	320 $\pm$ 18.8	316 $\pm$ 26.7
Day 78	321 $\pm$ 19.1	338 $\pm$ 32.4	324 $\pm$ 21.2	322 $\pm$ 29.4
Day 85	332 $\pm$ 24.0	348 $\pm$ 30.9	335 $\pm$ 17.7	331 $\pm$ 28.0
Day 91	331 $\pm$ 23.8	348 $\pm$ 34.0	338 $\pm$ 22.1	328 $\pm$ 32.1

Abbreviations: CP, *Clostridium* protein.

Table 1.3: Food consumption (g/animal/day) males

Administration Period	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
Days 1-8	25 $\pm$ 1.0	28 $\pm$ 6.0	24 $\pm$ 0.2	24 $\pm$ 0.3
Days 8-15	26 $\pm$ 0.9	25 $\pm$ 0.1	25 $\pm$ 0.6	26 $\pm$ 0.4
Days 15-22	27 $\pm$ 1.2	26 $\pm$ 0.2	25 $\pm$ 1.8	27 $\pm$ 0.5
Days 22-29	26 $\pm$ 1.0	26 $\pm$ 0.4	25 $\pm$ 2.1	27 $\pm$ 0.1
Days 29-36	26 $\pm$ 0.8	25 $\pm$ 1.1	24 $\pm$ 2.3	27 $\pm$ 1.0
Days 36-43	26 $\pm$ 0.8	26 $\pm$ 1.0	25 $\pm$ 1.5	27 $\pm$ 1.2
Days 43-50	26 $\pm$ 1.2	26 $\pm$ 1.6	25 $\pm$ 1.8	28 $\pm$ 1.9
Days 50-57	26 $\pm$ 0.0	26 $\pm$ 1.2	24 $\pm$ 1.6	28 $\pm$ 1.5
Days 57-64	26 $\pm$ 1.7	27 $\pm$ 0.6	25 $\pm$ 0.8	28 $\pm$ 1.2
Days 64-71	25 $\pm$ 0.6	25 $\pm$ 0.5	24 $\pm$ 1.0	26 $\pm$ 1.3
Days 71-78	25 $\pm$ 0.6	26 $\pm$ 0.7	24 $\pm$ 0.9	27 $\pm$ 0.8
Days 78-85	25 $\pm$ 0.6	25 $\pm$ 0.3	25 $\pm$ 1.5	27 $\pm$ 1.5
Days 85-91	25 $\pm$ 0.1	25 $\pm$ 0.0	25 $\pm$ 0.7	26 $\pm$ 0.0
Mean of Means	26	26	25	27

Abbreviations: CP, *Clostridium* protein.

Table 1.4: Food consumption (g/animal/day) females

Administration Period	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
Days 1-8	17 $\pm$ 0.7	17 $\pm$ 0.6	17 $\pm$ 0.3	17 $\pm$ 0.9
Days 8-15	18 $\pm$ 0.6	18 $\pm$ 0.2	18 $\pm$ 0.8	18 $\pm$ 0.4
Days 15-22	19 $\pm$ 0.7	19 $\pm$ 0.3	19 $\pm$ 0.6	19 $\pm$ 0.5
Days 22-29	18 $\pm$ 0.6	19 $\pm$ 0.1	20 $\pm$ 1.3	19 $\pm$ 0.2
Days 29-36	18 $\pm$ 0.6	18 $\pm$ 0.0	19 $\pm$ 0.1	19 $\pm$ 0.1
Days 36-43	18 $\pm$ 0.9	19 $\pm$ 0.3	19 $\pm$ 0.8	19 $\pm$ 1.1
Days 43-50	18 $\pm$ 0.3	19 $\pm$ 0.6	19 $\pm$ 1.3	19 $\pm$ 0.7
Days 50-57	17 $\pm$ 1.3	17 $\pm$ 0.3	18 $\pm$ 1.4	17 $\pm$ 0.1
Days 57-64	18 $\pm$ 0.4	19 $\pm$ 0.0	19 $\pm$ 0.2	19 $\pm$ 0.7
Days 64-71	17 $\pm$ 0.1	18 $\pm$ 1.2	18 $\pm$ 0.9	18 $\pm$ 0.8
Days 71-78	18 $\pm$ 0.1	18 $\pm$ 1.5	19 $\pm$ 1.2	19 $\pm$ 0.2
Days 78-85	19 $\pm$ 0.1	18 $\pm$ 0.8	19 $\pm$ 1.2	19 $\pm$ 0.4
Days 85-91	17 $\pm$ 0.1	17 $\pm$ 0.7	18 $\pm$ 0.1	17 $\pm$ 0.3
Mean of Means	18	18	19	18

Abbreviations: CP, *Clostridium* protein.

Table 1.5: Relative food consumption (g/kg body weight/day) males

Administration Period	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
Days 1-8	82 $\pm$ 0.5	99 $\pm$ 25.5	84 $\pm$ 1.3	85 $\pm$ 0.7
Days 8-15	71 $\pm$ 0.0	73 $\pm$ 1.9	72 $\pm$ 0.4	74 $\pm$ 0.9
Days 15-22	65 $\pm$ 0.5	65 $\pm$ 1.5	66 $\pm$ 1.9	68 $\pm$ 1.0
Days 22-29	58 $\pm$ 0.2	59 $\pm$ 0.9	59 $\pm$ 2.1	62 $\pm$ 1.5
Days 29-36	54 $\pm$ 0.1	54 $\pm$ 1.8	55 $\pm$ 2.1	57 $\pm$ 0.4
Days 36-43	51 $\pm$ 0.0	51 $\pm$ 1.1	52 $\pm$ 0.5	54 $\pm$ 0.1
Days 43-50	49 $\pm$ 0.7	50 $\pm$ 1.7	51 $\pm$ 1.2	52 $\pm$ 0.9
Days 50-57	47 $\pm$ 1.9	48 $\pm$ 1.0	47 $\pm$ 0.9	51 $\pm$ 0.2
Days 57-64	46 $\pm$ 1.1	47 $\pm$ 0.0	48 $\pm$ 0.4	48 $\pm$ 0.3
Days 64-71	43 $\pm$ 0.7	43 $\pm$ 0.2	44 $\pm$ 0.1	44 $\pm$ 0.2
Days 71-78	42 $\pm$ 0.5	43 $\pm$ 0.3	44 $\pm$ 0.4	44 $\pm$ 0.7
Days 78-85	41 $\pm$ 0.3	41 $\pm$ 0.4	42 $\pm$ 1.4	43 $\pm$ 0.5
Days 85-91	39 $\pm$ 1.0	40 $\pm$ 0.5	42 $\pm$ 0.1	41 $\pm$ 1.8
Mean of Means	53	55	54	56

Abbreviations: CP, *Clostridium* protein.**Table 1.6:** Relative food consumption (g/kg body weight/day) females

Administration Period	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
Days 1-8	88 $\pm$ 0.4	88 $\pm$ 0.2	90 $\pm$ 0.5	90 $\pm$ 2.7
Days 8-15	79 $\pm$ 0.2	79 $\pm$ 2.2	80 $\pm$ 2.1	82 $\pm$ 1.6
Days 15-22	76 $\pm$ 1.8	75 $\pm$ 2.8	79 $\pm$ 0.9	79 $\pm$ 0.9
Days 22-29	70 $\pm$ 0.2	70 $\pm$ 0.2	74 $\pm$ 1.3	75 $\pm$ 0.7
Days 29-36	65 $\pm$ 1.6	64 $\pm$ 2.2	68 $\pm$ 1.4	69 $\pm$ 0.3
Days 36-43	62 $\pm$ 1.6	63 $\pm$ 1.8	66 $\pm$ 0.7	67 $\pm$ 1.0
Days 43-50	61 $\pm$ 0.5	61 $\pm$ 2.1	64 $\pm$ 2.2	64 $\pm$ 0.3
Days 50-57	56 $\pm$ 3.5	55 $\pm$ 0.5	59 $\pm$ 1.7	58 $\pm$ 2.6
Days 57-64	59 $\pm$ 0.2	57 $\pm$ 1.3	61 $\pm$ 1.5	60 $\pm$ 0.3
Days 64-71	53 $\pm$ 0.1	54 $\pm$ 3.1	57 $\pm$ 0.7	56 $\pm$ 3.3
Days 71-78	55 $\pm$ 1.5	53 $\pm$ 3.6	58 $\pm$ 1.5	58 $\pm$ 0.1
Days 78-85	56 $\pm$ 0.2	53 $\pm$ 1.2	58 $\pm$ 1.3	56 $\pm$ 2.1
Days 85-91	52 $\pm$ 1.5	50 $\pm$ 1.5	53 $\pm$ 1.7	53 $\pm$ 1.1
Mean of Means	64	63	67	67

Abbreviations: CP, *Clostridium* protein.

1.2 Interim Hematology Evaluations (Day 30 and Day 50)

Table 1.7: Hematology analysis of male rats fed treatment diets for 29 days

	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
WBC ($\times 10^9/L$)	12.3 $\pm$ 2.74	10.8 $\pm$ 1.73	11.1 $\pm$ 1.87	13.1 $\pm$ 1.66
NEUT ($\times 10^9/L$)	1.69 $\pm$ 0.67	1.74 $\pm$ 0.87	1.56 $\pm$ 0.48	2.10 $\pm$ 0.24
LYMPH ($\times 10^9/L$)	9.97 $\pm$ 2.33	8.55 $\pm$ 1.57	9.01 $\pm$ 1.54	10.4 $\pm$ 1.64
MONO ($\times 10^9/L$)	0.23 $\pm$ 0.09	0.25 $\pm$ 0.05	0.24 $\pm$ 0.10	0.28 $\pm$ 0.08
EOS ($\times 10^9/L$)	0.20 $\pm$ 0.10	0.17 $\pm$ 0.09	0.18 $\pm$ 0.06	0.17 $\pm$ 0.03
BASO ($\times 10^9/L$)	0.04 $\pm$ 0.02	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	0.04 $\pm$ 0.01
LUC ($\times 10^9/L$)	0.13 $\pm$ 0.16	0.10 $\pm$ 0.04	0.08 $\pm$ 0.03	0.10 $\pm$ 0.03
NEUT (%)	13.9 $\pm$ 4.91	15.9 $\pm$ 7.36	14.0 $\pm$ 3.50	16.3 $\pm$ 3.08
LYMPH (%)	81.3 $\pm$ 6.25	79.0 $\pm$ 7.28	81.3 $\pm$ 3.76	79.2 $\pm$ 3.17
MONO (%)	1.86 $\pm$ 0.57	2.36 $\pm$ 0.68	2.12 $\pm$ 0.61	2.14 $\pm$ 0.67
EOS (%)	1.70 $\pm$ 1.08	1.55 $\pm$ 0.62	1.63 $\pm$ 0.55	1.30 $\pm$ 0.28
BASO (%)	0.28 $\pm$ 0.06	0.23 $\pm$ 0.07	0.24 $\pm$ 0.07	0.30 $\pm$ 0.08
LUC (%)	0.98 $\pm$ 1.00	0.90 $\pm$ 0.28	0.73 $\pm$ 0.16	0.79 $\pm$ 0.21
RBC ($\times 10^{12}/L$)	8.37 $\pm$ 0.35	7.92* $\pm$ 0.40	7.97* $\pm$ 0.33	7.60** $\pm$ 0.40
RETIC ($\times 10^9/L$)	208.2 $\pm$ 38.9	197.8 $\pm$ 30.4	170.4 $\pm$ 34.2	230.8 $\pm$ 57.8
RDW (%)	12.1 $\pm$ 0.22	12.2 $\pm$ 0.38	11.9 $\pm$ 0.15	12.4* $\pm$ 0.30
HGB (g/L)	173.7 $\pm$ 5.9	162.1** $\pm$ 5.50	165.8* $\pm$ 6.10	157.2** $\pm$ 6.20
HCT (L/L)	0.48 $\pm$ 0.01	0.46** $\pm$ 0.02	0.46 $\pm$ 0.20	0.45** $\pm$ 0.02
MCV (fL)	57.7 $\pm$ 1.60	57.6 $\pm$ 2.30	58.3 $\pm$ 1.12	58.7 $\pm$ 1.55
MCH (pg)	20.8 $\pm$ 0.48	20.5 $\pm$ 0.84	20.8 $\pm$ 0.54	20.7 $\pm$ 0.65
MCHC (g/L)	359.5 $\pm$ 5.6	356.0 $\pm$ 4.1	357.2 $\pm$ 5.6	353.0* $\pm$ 3.8
PLT ($\times 10^9/L$)	904.6 $\pm$ 195.2	880.9 $\pm$ 88.9	958.8 $\pm$ 174.4	1019.3 $\pm$ 152.6

Abbreviations: BASO, basophils; CP, *Clostridium* protein; EOS, eosinophils; HCT, hematocrit; HGB, hemoglobin; LUC, large unstained cells; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; NEUT, neutrophils; PTL, platelet; RBC, red blood cells; RDW, red blood cell distribution width; RETIC, reticulocytes; WBC, white blood cells.

* $p \leq 0.05$ determined with Anova & Dunnett.

** $p \leq 0.01$ determined with Anova & Dunnett.

Table 1.8: Hematology analysis of female rats fed treatment diets for 30 days

	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
WBC ($\times 10^9/L$)	9.49 $\pm$ 1.69	10.4 $\pm$ 3.26	9.44 $\pm$ 2.61	9.39 $\pm$ 1.62
NEUT ($\times 10^9/L$)	1.85 $\pm$ 1.73	1.32 $\pm$ 0.57	1.09 $\pm$ 0.31	0.88 $\pm$ 0.15
LYMPH ($\times 10^9/L$)	7.22 $\pm$ 1.65	8.65 $\pm$ 2.99	7.93 $\pm$ 2.30	8.12 $\pm$ 1.57
MONO ($\times 10^9/L$)	0.17 $\pm$ 0.09	0.17 $\pm$ 0.07	0.17 $\pm$ 0.08	0.17 $\pm$ 0.07
EOS ($\times 10^9/L$)	0.15 $\pm$ 0.09	0.17 $\pm$ 0.11	0.14 $\pm$ 0.06	0.13 $\pm$ 0.04
BASO ($\times 10^9/L$)	0.02 $\pm$ 0.01	0.03 $\pm$ 0.02	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01
LUC ($\times 10^9/L$)	0.07 $\pm$ 0.03	0.08 $\pm$ 0.05	0.09 $\pm$ 0.04	0.08 $\pm$ 0.03
NEUT (%)	18.5 $\pm$ 15.1	13.4 $\pm$ 7.17	12.0 $\pm$ 4.69	9.52 $\pm$ 2.22
LYMPH (%)	77.1 $\pm$ 15.5	82.2 $\pm$ 7.02	83.5 $\pm$ 4.62	86.2 $\pm$ 2.72
MONO (%)	1.79 $\pm$ 0.75	1.66 $\pm$ 0.72	1.81 $\pm$ 0.52	1.82 $\pm$ 0.83
EOS (%)	1.61 $\pm$ 0.91	1.79 $\pm$ 1.53	1.48 $\pm$ 0.63	1.45 $\pm$ 0.50
BASO (%)	0.25 $\pm$ 0.10	0.26 $\pm$ 0.10	0.22 $\pm$ 0.08	0.18 $\pm$ 0.08
LUC (%)	0.76 $\pm$ 0.26	0.74 $\pm$ 0.25	0.94 $\pm$ 0.23	0.84 $\pm$ 0.20
RBC ($\times 10^{12}/L$)	7.48 $\pm$ 0.67	7.45 $\pm$ 0.53	7.59 $\pm$ 0.24	7.55 $\pm$ 0.17
RETIC ($\times 10^9/L$)	165.8 $\pm$ 64.0	173.5 $\pm$ 37.7	162.1 $\pm$ 33.7	164.6 $\pm$ 33.5
RDW (%)	11.8 $\pm$ 0.32	11.7 $\pm$ 0.23	11.7 $\pm$ 0.16	11.5* $\pm$ 0.17
HGB (g/L)	154.4 $\pm$ 10.3	154.9 $\pm$ 12.1	155.2 $\pm$ 3.90	153.8 $\pm$ 3.90
HCT (L/L)	0.42 $\pm$ 0.03	0.43 $\pm$ 0.03	0.44 $\pm$ 0.01	0.43 $\pm$ 0.01
MCV (fL)	56.8 $\pm$ 2.01	57.7 $\pm$ 1.32	57.6 $\pm$ 1.05	57.2 $\pm$ 1.07
MCH (pg)	20.7 $\pm$ 1.00	20.8 $\pm$ 0.45	20.4 $\pm$ 0.37	20.4 $\pm$ 0.47
MCHC (g/L)	364.7 $\pm$ 8.6	360.9 $\pm$ 2.5	355.4** $\pm$ 3.5	356.1* $\pm$ 5.0
PLT ($\times 10^9/L$)	892.6 $\pm$ 138.0	905.9 $\pm$ 107.0	1008.0 $\pm$ 88.6	961.7 $\pm$ 105.4

Abbreviations: BASO, basophils; CP, *Clostridium* protein; EOS, eosinophils; HCT, hematocrit; HGB, hemoglobin; LUC, large unstained cells; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; NEUT, neutrophils; PTL, platelet; RBC, red blood cells; RDW, red blood cell distribution width; RETIC, reticulocytes; WBC, white blood cells.

* $p \leq 0.05$ determined with Kruskal-Wallis & Dunn.

** $p \leq 0.01$ determined with Kruskal-Wallis & Dunn.

Table 1.9: Hematology analysis of male rats fed treatment diets for 54 days

	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
WBC ($\times 10^9/L$)	13.6 $\pm$ 2.36	11.4 $\pm$ 2.65	12.5 $\pm$ 1.48	12.4 $\pm$ 1.49
NEUT ($\times 10^9/L$)	2.05 $\pm$ 0.30	1.12** $\pm$ 0.23	1.20** $\pm$ 0.38	1.37** $\pm$ 0.27
LYMPH ($\times 10^9/L$)	11.0 $\pm$ 2.05	9.87 $\pm$ 2.38	10.8 $\pm$ 1.19	10.5 $\pm$ 1.24
MONO ($\times 10^9/L$)	0.28 $\pm$ 0.18	0.18 $\pm$ 0.04	0.19 $\pm$ 0.09	0.26 $\pm$ 0.05
EOS ($\times 10^9/L$)	0.21 $\pm$ 0.08	0.18 $\pm$ 0.08	0.19 $\pm$ 0.06	0.16 $\pm$ 0.04
BASO ($\times 10^9/L$)	0.03 $\pm$ 0.01	0.04 $\pm$ 0.01	0.03 $\pm$ 0.01	0.04 $\pm$ 0.02
LUC ($\times 10^9/L$)	0.09 $\pm$ 0.02	0.06 $\pm$ 0.04	0.05 $\pm$ 0.02	0.08 $\pm$ 0.03
NEUT (%)	15.2 $\pm$ 1.75	9.94** $\pm$ 1.38	9.42** $\pm$ 2.40	11.0** $\pm$ 1.18
LYMPH (%)	80.3 $\pm$ 2.99	86.0** $\pm$ 2.35	86.8** $\pm$ 2.99	84.7* $\pm$ 1.72
MONO (%)	2.02 $\pm$ 1.33	1.66 $\pm$ 0.57	1.54 $\pm$ 0.71	2.10 $\pm$ 0.46
EOS (%)	1.56 $\pm$ 0.48	1.60 $\pm$ 0.63	1.46 $\pm$ 0.41	1.28 $\pm$ 0.35
BASO (%)	0.26 $\pm$ 0.09	0.30 $\pm$ 0.07	0.28 $\pm$ 0.04	0.32 $\pm$ 0.08
LUC (%)	0.64 $\pm$ 0.11	0.50 $\pm$ 0.19	0.44 $\pm$ 0.15	0.60 $\pm$ 0.19
RBC ($\times 10^{12}/L$)	9.03 $\pm$ 0.31	8.86 $\pm$ 0.66	8.82 $\pm$ 0.27	8.93 $\pm$ 0.60
RETIC ($\times 10^9/L$)	282.0 $\pm$ 41.6	224.9* $\pm$ 20.4	239.0 $\pm$ 42.2	219.5* $\pm$ 11.1
RDW (%)	13.4 $\pm$ 0.47	12.8 $\pm$ 0.44	12.9 $\pm$ 0.34	12.7 $\pm$ 0.59
HGB (g/L)	163.0 $\pm$ 3.5	160.2 $\pm$ 6.1	161.2 $\pm$ 5.4	160.6 $\pm$ 5.4
HCT (L/L)	0.49 $\pm$ 0.01	0.48 $\pm$ 0.02	0.48 $\pm$ 0.01	0.49 $\pm$ 0.02
MCV (fL)	54.4 $\pm$ 0.79	54.7 $\pm$ 2.13	54.9 $\pm$ 1.44	54.7 $\pm$ 2.12
MCH (pg)	18.1 $\pm$ 0.29	18.1 $\pm$ 0.93	18.3 $\pm$ 0.61	18.0 $\pm$ 0.82
MCHC (g/L)	333.2 $\pm$ 5.0	331.0 $\pm$ 7.0	333.2 $\pm$ 2.8	329.2 $\pm$ 4.3
PLT ($\times 10^9/L$)	1108.6 $\pm$ 189.0	936.4 $\pm$ 82.5	1006.2 $\pm$ 113.0	1095.2 $\pm$ 141.9

Abbreviations: BASO, basophils; CP, *Clostridium* protein; EOS, eosinophils; HCT, hematocrit; HGB, hemoglobin; LUC, large unstained cells; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; NEUT, neutrophils; PTL, platelet; RBC, red blood cells; RDW, red blood cell distribution width; RETIC, reticulocytes; WBC, white blood cells.

* $p \leq 0.05$ determined with Anova & Dunnett.

** $p \leq 0.01$ determined with Anova & Dunnett.

Table 1.10: Hematology analysis of female rats fed treatment diets for 54 days

	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
WBC ($\times 10^9/L$)	8.67 $\pm$ 1.29	7.69 $\pm$ 2.60	5.41 $\pm$ 1.89	5.58 ^{*b} $\pm$ 0.33
NEUT ($\times 10^9/L$)	1.34 $\pm$ 0.81	0.78 $\pm$ 0.57	0.56 $\pm$ 1.88	0.64 $\pm$ 0.13
LYMPH ($\times 10^9/L$)	6.90 $\pm$ 1.81	6.62 $\pm$ 2.19	4.64 $\pm$ 1.76	4.67 $\pm$ 0.38
MONO ($\times 10^9/L$)	0.18 $\pm$ 0.11	0.09 $\pm$ 0.04	0.06 ^{*a} $\pm$ 0.03	0.08 ^{*a} $\pm$ 0.02
EOS ($\times 10^9/L$)	0.16 $\pm$ 0.05	0.13 $\pm$ 0.07	0.11 $\pm$ 0.06	0.14 $\pm$ 0.08
BASO ($\times 10^9/L$)	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.01 $\pm$ 0.01	0.01 $\pm$ 0.01
LUC ($\times 10^9/L$)	0.07 $\pm$ 0.04	0.06 $\pm$ 0.04	0.03 $\pm$ 0.02	0.04 $\pm$ 0.01
NEUT (%)	16.6 $\pm$ 13.00	10.1 $\pm$ 5.31	11.0 $\pm$ 4.58	11.6 $\pm$ 2.57
LYMPH (%)	78.5 $\pm$ 12.8	86.3 $\pm$ 5.13	85.2 $\pm$ 4.22	83.7 $\pm$ 3.21
MONO (%)	2.06 $\pm$ 1.02	1.12 $\pm$ 0.29	1.16 $\pm$ 0.46	1.38 $\pm$ 0.44
EOS (%)	1.78 $\pm$ 0.41	1.56 $\pm$ 0.48	1.96 $\pm$ 0.78	2.52 $\pm$ 1.34
BASO (%)	0.22 $\pm$ 0.11	0.24 $\pm$ 0.09	0.14 $\pm$ 0.09	0.20 $\pm$ 0.17
LUC (%)	0.82 $\pm$ 0.36	0.72 $\pm$ 0.27	0.56 $\pm$ 0.17	0.64 $\pm$ 0.13
RBC ($\times 10^{12}/L$)	8.09 $\pm$ 0.44	8.07 $\pm$ 0.18	8.16 $\pm$ 0.23	8.39 $\pm$ 0.27
RETIC ($\times 10^9/L$)	160.4 $\pm$ 41.3	175.8 $\pm$ 30.6	177.9 $\pm$ 41.1	182.0 $\pm$ 41.4
RDW (%)	12.2 $\pm$ 0.39	12.1 $\pm$ 0.44	12.0 $\pm$ 0.26	11.7 $\pm$ 0.20
HGB (g/L)	148.4 $\pm$ 8.6	152.0 $\pm$ 2.0	148.4 $\pm$ 4.2	150.8 $\pm$ 4.1
HCT (L/L)	0.43 $\pm$ 0.03	0.44 $\pm$ 0.01	0.44 $\pm$ 0.02	0.45 $\pm$ 0.02
MCV (fL)	52.6 $\pm$ 1.02	54.3 $\pm$ 1.08	53.8 $\pm$ 0.85	53.4 $\pm$ 1.07
MCH (pg)	18.3 $\pm$ 0.49	18.8 $\pm$ 0.44	18.2 $\pm$ 0.31	17.9 $\pm$ 0.52
MCHC (g/L)	348.0 $\pm$ 4.3	347.2 $\pm$ 2.9	338.6 ^{*a} $\pm$ 7.5	336.0 ^{*a} $\pm$ 5.5
PLT ($\times 10^9/L$)	980.8 $\pm$ 80.9	1009.4 $\pm$ 79.3	997.6 $\pm$ 95.9	893.8 $\pm$ 88.0

Abbreviations: BASO, basophils; CP, *Clostridium* protein; EOS, eosinophils; HCT, hematocrit; HGB, hemoglobin, LUC, large unstained cells; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; NEUT, neutrophils; PTL, platelet; RBC, red blood cells; RDW, red blood cell distribution width; RETIC, reticulocytes; WBC, white blood cells.

^a Anova & Dunnett.

^b Kruskal-Wallis & Dunn.

* $p \leq 0.05$.

1.3 Coagulation Evaluation (Day 90)

Table 1.11: Coagulation analysis of male and female rats fed treatment diets for 90 days

	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
Male				
PT (sec)	17.1 $\pm$ 0.47	17.0 $\pm$ 0.65	17.3 $\pm$ 0.51	17.5 $\pm$ 0.43
APTT (sec)	17.9 $\pm$ 2.89	18.3 $\pm$ 2.50	17.7 $\pm$ 2.58	18.2 $\pm$ 3.17
Female				
PT (sec)	16.2 $\pm$ 0.50	15.6 ^a $\pm$ 0.48	15.8 $\pm$ 0.43	16.0 $\pm$ 0.55
APTT (sec)	17.6 $\pm$ 1.12	16.5 $\pm$ 2.03	16.8 $\pm$ 1.60	16.8 $\pm$ 0.86

Abbreviations: APTT, activated partial thromboplastin time; CP, *Clostridium* protein; PT, prothrombin time.

^a Anova & Dunnett.

* $p \leq 0.05$.

1.4 Interim Biochemistry Evaluations (Day 30 and Day 50)

Table 1.12: Serum chemistry analysis of male rats fed treatment diets for 54 days

	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
ALT (U/L)	41.6 $\pm$ 4.7	45.9 $\pm$ 2.7	53.5 ^a $\pm$ 7.2	42.7 $\pm$ 7.8
AST (U/L)	98.5 $\pm$ 8.9	109.5 $\pm$ 9.8	131.3 ^b $\pm$ 26.3	95.3 $\pm$ 12.6
ALP (U/L)	243.4 $\pm$ 131.8	211.7 $\pm$ 80.8	153.4 $\pm$ 30.4	155.1 $\pm$ 31.4
Total Protein (g/L)	70.4 $\pm$ 2.90	71.4 $\pm$ 2.42	68.5 $\pm$ 2.30	67.4 $\pm$ 2.85
Albumin (g/L)	43.0 $\pm$ 1.83	43.4 $\pm$ 0.74	41.6 $\pm$ 1.62	41.1 $\pm$ 1.57
Total Bilirubin (umol/L)	3.86 $\pm$ 0.58	3.52 $\pm$ 0.33	3.60 $\pm$ 0.89	2.88 $\pm$ 0.27
Urea (mmol/L)	4.99 $\pm$ 0.29	5.20 $\pm$ 0.75	5.26 $\pm$ 0.46	6.00 ^a $\pm$ 0.53
Creatine (umol/L)	30.1 $\pm$ 3.2	34.7 $\pm$ 2.4	33.5 $\pm$ 1.7	30.8 $\pm$ 4.0
Glucose (mmol/L)	5.84 $\pm$ 0.53	6.23 $\pm$ 0.42	5.80 $\pm$ 0.64	6.39 $\pm$ 0.36
Cholesterol (mmol/L)	1.83 $\pm$ 0.34	1.66 $\pm$ 0.37	1.48 $\pm$ 0.25	1.64 $\pm$ 0.22
Triglycerides (mmol/L)	0.58 $\pm$ 0.12	0.45 $\pm$ 0.21	0.56 $\pm$ 0.18	0.44 $\pm$ 0.17
HDL (mmol/L)	1.29 $\pm$ 0.22	1.14 $\pm$ 0.20	1.00 $\pm$ 0.23	1.13 $\pm$ 0.14
LDL (mmol/L)	0.35 $\pm$ 0.04	0.37 $\pm$ 0.12	0.31 $\pm$ 0.06	0.34 $\pm$ 0.02
Sodium (mmol/L)	144.6 $\pm$ 0.5	144.2 $\pm$ 1.3	144.8 $\pm$ 1.1	143.8 $\pm$ 0.8
Potassium (mmol/L)	4.42 $\pm$ 0.37	4.70 $\pm$ 0.47	4.47 $\pm$ 0.60	4.28 $\pm$ 0.21
Chloride (mmol/L)	105.4 $\pm$ 1.3	104.8 $\pm$ 1.6	105.6 $\pm$ 0.9	104.4 $\pm$ 1.3
Calcium (mmol/L)	2.76 $\pm$ 0.13	2.77 $\pm$ 0.13	2.69 $\pm$ 0.12	2.73 $\pm$ 0.04
Phosphorus (mmol/L)	2.30 $\pm$ 0.08	2.32 $\pm$ 0.13	2.26 $\pm$ 0.26	2.44 $\pm$ 0.05
Iron (umol/L)	37.8 $\pm$ 14.5	48.7 $\pm$ 16.7	29.6 $\pm$ 3.67	44.0 $\pm$ 14.1
Magnesium (mmol/L)	0.87 $\pm$ 0.06	0.08 $\pm$ 0.08	0.83 $\pm$ 0.10	1.07 ^{**a} $\pm$ 0.04

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CP, *Clostridium* protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

^aAnova & Dunnett.

^bKruskal-Wallis & Dunn.

* $p \leq 0.0$.

** $p \leq 0.01$.

Table 1.13: Serum chemistry analysis of female rats fed treatment diets for 54 days

	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
ALT (U/L)	34.7 $\pm$ 4.4	40.4 $\pm$ 6.8	35.2 $\pm$ 10.4	35.9 $\pm$ 6.7
AST (U/L)	90.0 $\pm$ 19.2	109.7 $\pm$ 36.1	87.4 $\pm$ 4.3	96.3 $\pm$ 10.5
ALP (U/L)	105.6 $\pm$ 45.4	95.6 $\pm$ 30.7	99.2 $\pm$ 45.8	90.6 $\pm$ 23.9
Total Protein (g/L)	81.2 $\pm$ 3.00	78.9 $\pm$ 3.94	80.5 $\pm$ 6.00	75.8 $\pm$ 6.44
Albumin (g/L)	53.3 $\pm$ 2.09	51.6 $\pm$ 4.10	52.3 $\pm$ 4.50	48.8 $\pm$ 4.79
Total Bilirubin (umol/L)	4.80 $\pm$ 0.76	3.74 $\pm$ 0.91	3.42 ^{*a} $\pm$ 0.53	2.94 ^{**a} $\pm$ 0.42
Urea (mmol/L)	5.65 $\pm$ 0.61	5.54 $\pm$ 0.91	5.86 $\pm$ 0.99	5.85 $\pm$ 1.06
Creatine (umol/L)	41.7 $\pm$ 3.1	43.1 $\pm$ 1.3	41.2 $\pm$ 4.4	44.4 $\pm$ 3.6
Glucose (mmol/L)	6.69 $\pm$ 0.614	6.50 $\pm$ 0.14	6.19 $\pm$ 0.45	6.46 $\pm$ 0.70
Cholesterol (mmol/L)	1.85 $\pm$ 0.11	1.94 $\pm$ 0.60	2.05 $\pm$ 0.59	1.99 $\pm$ 0.49
Triglycerides (mmol/L)	0.54 $\pm$ 0.09	0.54 $\pm$ 0.13	0.42 $\pm$ 0.08	0.50 $\pm$ 0.12
HDL (mmol/L)	1.32 $\pm$ 0.05	1.34 $\pm$ 0.41	1.32 $\pm$ 0.26	1.29 $\pm$ 0.25
LDL (mmol/L)	0.40 $\pm$ 0.09	0.39 $\pm$ 0.07	0.36 $\pm$ 0.11	0.40 $\pm$ 0.05
Sodium (mmol/L)	143.0 $\pm$ 1.2	142.8 $\pm$ 1.1	143.0 $\pm$ 0.7	142.8 $\pm$ 1.5
Potassium (mmol/L)	4.23 $\pm$ 0.21	4.25 $\pm$ 0.43	4.63 $\pm$ 0.40	3.82 $\pm$ 0.33
Chloride (mmol/L)	106.4 $\pm$ 1.7	105.6 $\pm$ 1.1	105.4 $\pm$ 1.8	106.2 $\pm$ 0.8
Calcium (mmol/L)	2.85 $\pm$ 0.07	2.79 $\pm$ 0.05	2.82 $\pm$ 0.09	2.66 ^{*a} $\pm$ 0.15
Phosphorus (mmol/L)	1.61 $\pm$ 0.11	1.68 $\pm$ 0.28	1.57 $\pm$ 0.41	1.31 $\pm$ 0.23
Iron (umol/L)	66.8 $\pm$ 14.1	76.3 $\pm$ 10.9	75.0 $\pm$ 17.3	64.0 $\pm$ 9.03
Magnesium (mmol/L)	1.02 $\pm$ 0.06	1.12 $\pm$ 0.05	1.07 $\pm$ 0.13	1.13 $\pm$ 0.05

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CP, *Clostridium* protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

* $p \leq 0.05$ determined with Anova & Dunnett.

** $p \leq 0.01$ determined with Anova & Dunnett.

1.6 End of Study Urinalysis and Uric Acid Results

Table 1.14. Urinalysis of male rats fed treatment diets for 92 days

	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
Volume (mL)	8.50 $\pm$ 3.84	8.50 $\pm$ 3.44	8.20 $\pm$ 5.15	11.7 $\pm$ 6.04
Specific Gravity	1.03 $\pm$ 0.01	1.03 $\pm$ 0.01	1.03 $\pm$ 0.01	1.02 $\pm$ 0.01
Urine (pH)	7.50 $\pm$ 0.41	7.60 $\pm$ 0.46	7.70 $\pm$ 0.67	7.65 $\pm$ 0.53
Uric Acid (umol/L)	39.0 $\pm$ 26.5	18.3 $\pm$ 7.72	22.2 $\pm$ 14.6	21.5 $\pm$ 7.88

Abbreviations: CP, *Clostridium* protein.

Table 1.15. Urinalysis of Female Rats fed Treatment Diets for 93 days

	Treatment level (mean $\pm$ standard deviation)			
	0% CP (Control)	5% CP	7.5% CP	10% CP
Volume (mL)	4.95 $\pm$ 2.89	6.55 $\pm$ 2.66	6.10 $\pm$ 3.06	7.25 $\pm$ 5.03
Specific Gravity	1.03 $\pm$ 0.01	1.03 $\pm$ 0.01	1.03 $\pm$ 0.01	1.03 $\pm$ 0.01
Urine (pH)	6.50 $\pm$ 0.33	6.50 $\pm$ 0.33	6.40 $\pm$ 0.32	6.55 $\pm$ 0.44
Uric Acid (umol/L)	38.1 $\pm$ 19.7	13.6** $\pm$ 8.57	16.8* $\pm$ 11.1	18.3 $\pm$ 7.17

Abbreviations: CP, *Clostridium* protein.

* $p \leq 0.05$ determined with Kruskal-Wallis & Dunn.

** $p \leq 0.01$ determined with Kruskal-Wallis & Dunn.