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A 90-day dietary study with fibrillated cellulose in Sprague-Dawley rats

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ABSTRACT

Novel forms of fibrillated cellulose offer improved attributes for use in foods. Conventional cellulose and many of its derivatives are already widely used as food additives and are authorized as safe for use in foods in many countries. However, novel forms have not yet been thoroughly investigated using standardized testing methods. This study assesses the 90-day dietary toxicity of fibrillated cellulose, as compared to a conventional cellulose, Solka Floc. Sprague Dawley rats were fed 2 %, 3 %, or 4 % fibrillated cellulose for 90 consecutive days, and parallel Solka Floc groups were used as controls. Survival, clinical observations, body weight, food consumption, ophthalmologic evaluations, hematology, serum chemistry, urinalysis, post-mortem anatomic pathology, and histopathology were monitored and performed. No adverse observations were noted in relation to the administration of fibrillated cellulose. Under the conditions of this study and based on the toxicological endpoints evaluated, the no-observed-adverse-effect level (NOAEL) for fibrillated cellulose was 2194.2 mg/kg/day (males) and 2666.6 mg/kg/day (females), corresponding to the highest dose tested (4 %) for male and female Sprague Dawley rats. These results demonstrate that fibrillated cellulose behaves similarly to conventional cellulose and raises no safety concerns when used as a food ingredient at these concentrations.

1. Introduction

Cellulose is the most abundant natural biopolymer on earth. It is present in all plants where, in conjunction with lignin and hemicelluloses, it plays an essential role in maintaining structure and providing support to cell walls. Cellulose is also present in invertebrates, algae, fungi, tunicates, and can be produced by some bacteria [1]. Cellulose and some of its derivatives have a long history of use as dietary fibers and additives in food and animal feed. Researchers have been refining cellulose fibers into single microfibrils or elementary fibrils since the 1950s using ultrasonic, mechanical, hydrolytic and oxidation treatments [2]. Today, mechanical treatments are used to break down cellulose fibers into their structural components (the microfibrils), yielding fibrillated cellulose or aggregates of fibrillated cellulose, some of which have smaller widths than previous forms of cellulose.

These novel forms of fibrillated cellulose have improved rheological characteristics and at low concentrations are useful as non-caloric stabilizers, gelling agents, thickeners, and flavour carriers in food [3]. They have demonstrated potential to improve strength and lightweighting of paper and board food packaging as both a filler and coating [4], as a protective and flexible fruit coating [5,6], and as a plastic or fiber packaging replacement [7]. As these applications of fibrillated cellulose are developed, research that characterizes their dietary safety is needed to facilitate responsible use and commercialization.

Abbreviations: % RET, percent reticulocyte; ABAS, absolute basophil; AEOS, absolute eosinophil; ALB, albumin; ALKP, alkaline phosphatase; ALT, alanine aminotransferase; ALUC, absolute large unstained cell; ANEU, absolute neutrophil; ANOVA, one-way analysis of variance; ALYM, absolute lymphocyte; AMON, absolute monocyte; ARET, absolute reticulocyte; AST, aspartate aminotransferase; BUN, urea nitrogen; CAS, Chemical Abstracts Service; CHOL, cholesterol; CREAT, creatinine; DLS, dynamic light scattering; EFSA, European Food Safety Authority; EDXS, energy-dispersive X-ray spectroscopy; FDA, U.S. Food and Drug Administration; GLOB, globulin; GLP, good laboratory practice; GLU, glucose; GRAS, generally recognized as safe; HBG, hemoglobin; HCT, hematocrit; MCH, mean corpuscular cell hemoglobin; MCHC, mean corpuscular cell hemoglobin concentration; MCV, mean corpuscular cell volume; NOAEL, no-observed-adverse-effect level; OECD, Organisation for Economic Co-operation and Development; PLT, platelet count; RBC, red blood cell count; RDW, red cell distribution width; SCOGS, Select Committee on GRAS Substances; SDH, sorbitol dehydrogenase; SEM, scanning electron microscopy; TBA, total bile acids; TBIL, total bilirubin; TEM, transmission electron microscopy; TEMPO, 2,2,6,6-tetramethyl-piperidinyloxyl; TP, total protein; TRIG, triglycerides; WBC, white blood cell count

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The objective of this study was to evaluate the dietary toxicity of fibrillated cellulose. Fibrillated celluloses are produced by freeing cellulose fibrils from a cellulose source through mechanical means, using high shear methods to delaminate the cells walls of the fibers to liberate fibrils [8,9]. Other modified forms of cellulose, produced with extra chemical steps, such as TEMPO (2,2,6,6-tetramethyl-piperidinyloxyl)-mediated oxidation of fibrillated cellulose, or acid hydrolysis to produce cellulose nanocrystals, were not the subject of this research.

To evaluate dietary toxicity, this study used standardized test guidelines promulgated by the Organisation for Economic Co-operation and Development (OECD). Regulatory agencies require the use of these validated methods to ensure study consistency and reliability. This study was conducted in accordance with OECD Test Guideline 408 to evaluate the safety of fibrillated cellulose in comparison to conventional cellulose, a widely used dietary fiber, following subchronic dietary consumption by rats. Both forms of cellulose are representative of commercial forms of cellulose fibrils intended for use in food, packaging and other applications.

Conventional cellulose and many of its derivatives have been safely used as food additives in the United States and globally for decades ([10,11,3]). From 1958 to 1969, the U.S. Food and Drug Administration (FDA) granted Generally Recognized as Safe (GRAS) status to many forms of cellulose for a variety of applications. The FDA, through a Select Committee on GRAS Substances (SCOGS), conducted an evaluation of cellulose and its derivatives in 1973 [11]. After a comprehensive review of available information on cellulose and certain cellulose derivatives, the committee concluded that: *"There is no evidence in the available information […] that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when it is used at levels that are now current or that might reasonably be expected in future."* The present study compares a commercially representative form of fibrillated cellulose to conventional cellulose that is established as GRAS, offering a comparison of the relative hazards of cellulose substances prepared by different manufacturing techniques.

2. Materials and methods

2.1. Material preparation and characterization

Food-grade Solka Floc (grade FCC200, CAS number 9004-34-6) was purchased from Solvaira Specialty LP and offered to the control animals as a control fiber. Solka Floc is commonly used as a dietary fiber in animal feed and human dietary studies, *e.g.*, [12], and is sold commercially for food use. Fibrillated cellulose, produced through mechanical homogenization of a wood pulp starting material, was purchased from the University of Maine Process Development Center [8].

Fibrillated cellulose was assessed for impurities following the methods and specifications outlined the Food Chemicals Codex, including chloride, lead, sulfur, ash, loss on drying, pH, and water-soluble substances [13]. A Certificate of Analysis for Solka Floc was provided by the manufacturer and included impurity and microbial contaminant testing following the Food Chemicals Codex. In addition, microbial contaminant testing was performed to determine microbiological contamination by standard plate count, yeast and mold contamination by plate count method, and salmonella, using methods recommended by the Food Chemicals Codex [13].

Both materials were also characterized for a variety of physical and chemical endpoints, including morphology, size, size distribution, surface charge and elemental composition. Unless otherwise indicated, samples were prepared by diluting cellulose in distilled water to 2 wt%. Samples were manually separated into small chunks, and 2 g of 10 wt% cellulose were slowly added to water. A Disruptor Genie (60 kHz; 240 W; 3000 rpm) was used to vortex the solution for 10 min during dilution.

2.1.1. Light microscopy

Light microscopy images were collected by adding 1 drop (~10 μL) of the diluted sample suspension to a glass microscope slide. The drop was then smeared with the dropper tip and allowed to dry at room temperature (20 °C). Samples were imaged with a DMi1 Inverted Microscope (Leica Microsystems, Buffalo Grove, IL) in phase contrast field mode for phase contrast images, and in oblique field mode for dark-field images. All measurements were taken with Cell Sens 1.13 software (Olympus Corp.).

2.1.2. Electron microscopy

For transmission electron microscopy (TEM) images, a drop of cellulose suspension (~10 μL diluted to 0.2 wt%) was placed onto plastic paraffin film (Bemis NA Inc., Neenah, WI). A TEM grid (Lacey Carbon Film, Copper (LC200-Cu), Electron Microscopy Sciences, Hatfield, PA) was inverted onto the suspension and allowed to dry for 5 min. Grids were imaged with a JEOL Transmission Electron Microscope (JEM-1010, Peabody, MA) using Image Capture Engine software (version AMT 602.600.15).

For scanning electron microscopy (SEM) images, a drop of cellulose suspension (~10 μL diluted to 0.2 wt%) was deposited onto a SEM pin stub (Aluminum Specimen Mounts, Electron Microscopy Sciences, Hatfield, PA) and allowed to dry. The pin stub was sputter coated with a 20 nm-thick layer of carbon to decrease sample charging (Sputter Coater Leica ACE600). Pins were imaged with FEI Focused Ion Beam Scanning Electron Microscope (Versa 3D, Hillsboro, OR) using xT Microscope Control software (version 6.3.2, FEI Corporation).

2.1.3. Length and width analysis

The widths of fibrillated cellulose and Solka Floc were assessed using electron microscopy micrographs. Average agglomerate diameters were calculated from light microscopy micrographs, using Olympus Cell Sens Dimension software. Briefly, micrographs were loaded into the software and internally calibrated. A polyline tool was used to measure particle width or aggregate diameter, taking a minimum of 50 measurements per image. For fibril width measurements of fibrillated cellulose, measurements focused on the finest fraction of fibrils evident in transmission electron micrographs. For width measurements of Solka Floc, scanning electron micrographs were used.

2.1.4. Dynamic light scattering analysis

Samples were diluted to 0.002 wt% in triplicate for hydrodynamic diameter, zeta potential, and polydispersity measurements. Hydrodynamic diameter, polydispersity, and zeta potential measurements were taken using a Zetasizer Nano ZS (Malvern Instruments Ltd., Westborough, MA). Measurements were completed in triplicate with sample parameters for absorbance and refractive index set to 0.01 nm and 1.580, respectively.

2.1.5. Elemental mapping

Energy-dispersive X-ray spectroscopy (EDXS) was performed during SEM analysis to determine elemental composition using xT Microscope Control software (version 6.3.2). Elemental maps were constructed for representative samples of fibrillated cellulose and Solka Floc, overlaid with the SEM image.

2.2. Diet formulation, stability, and characterization

2.2.1. Formulation

Open Standard Diet D11112219N Rodent Diet and customized basal diets were supplied by Research Diets Inc. (New Brunswick, NJ) (Supplemental Table 1). Control and test diets were formulated by adding the Solka Floc control cellulose or the fibrillated cellulose test substance [6] to the standard basal diet to achieve the target dose. Comparable fat, protein, and carbohydrate contents were maintained

across dose groups.

2.2.2. Homogeneity and stability

Samples from the respective test diets were collected at the first presentation of the diet, and 4, 7, and 10 days after preparation. All samples were stored frozen. The homogeneity of the test and control substance distribution, as well as the concentration of the test diets, were evaluated in samples collected from the initial diet preparation. For each concentration, at least three samples were taken indiscriminately from the top, middle, and bottom of the feed to test for homogeneity. Analytical characterizations of the cellulose substance in the diets were conducted in compliance with Good Laboratory Practice (GLP). Diet pellets were ground to a fine powder, and 20 – 30 mg added to 15 mL of ether, then vortexed. Samples were centrifuged for 15 min at 2850 rpm, and supernatant discarded. Samples were set in 50 °C water bath for 30 min to evaporate ether, then 10 mL of a 10:1 ratio of 80 % acetic acid:concentrated nitric acid added and mixed. Tubes were then placed into a 100 °C oven for 45 min, cooled, then centrifuged for 40 min at 2850 rpm. Supernatants were discarded, and pellets were washed twice with deionized water. 10 mL of 70 % sulfuric acid was added, and after mixing, tubes were left to stand for 1 h. 10 mL of anthrone reagent (0.2 g anthrone in 100 mL of ice-cold 95 % sulfuric acid) was added to 1 mL aliquots of samples, then heated in a 100 °C oven for 10 min. Samples were cooled then absorbance at 630 nm measured in a spectrophotometer.

2.3. Study design

The study was conducted according to OECD Test Guideline 408 [14] and US FDA guidance [15], under standards of GLP [16] at Product Safety Labs (Dayton, New Jersey), a member of the Association for Assessment and Accreditation of Laboratory Animal Care following National Research Council [17] guideline standards.

One hundred and twenty six- to seven-week old Sprague Dawley CD® IGS rats (from Charles River Laboratories, Raleigh, North Carolina) were acclimated in suspended stainless steel cages for five days prior to random assignment to control or treatment groups. Each group was assigned 10 female and 10 male rats. The temperature in the animal room ranged from 19 – 23 °C and was on a 12 h light/dark cycle. The basal diet was available *ad libitum* during acclimation. Body weights and clinical observations were recorded twice prior to study start. Target doses of 2 %, 3 %, and 4 % cellulose were selected on the basis of two range finding studies (data not shown), which determined that there were no adverse effects associated with feeding 5 % dietary fibrillated cellulose over 7 days or with feeding up to 1.2 % dietary fibrillated cellulose over 14 days.

The study included three control groups fed diets containing 2 %, 3 %, and 4 % Solka Floc so that any adverse effects resulting from higher or lower dietary fiber intake could be distinguished from effects related to the fibrillated test material itself (in comparison to Solka Floc at the same concentration). The diets were provided *ad libitum*, except for a fasting period prior to blood collection.

2.4. Clinical observations

All animals were observed at least twice daily for viability, and cage-side observations were recorded daily. On Day 0 (prior to the first treatment with the test substance) and weekly thereafter, a detailed clinical observation was conducted while handling the animal. Observers noted (among other potential observations): visible changes to skin, fur, eyes, or mucous membranes; secretions and excretions; autonomic activity (*e.g.*, lacrimation, piloerection, pupil size, unusual respiratory pattern); changes in gait, locomotion (speed and vigor of movement), posture, and response to handling, as well as the presence of clonic or tonic movements, stereotypies (*e.g.,* excessive grooming, repetitive circling), vocalizations, and bizarre behavior (*e.g.,* self-mutilation, walking backwards).

2.4.1. Body weight and body weight gain

Individual body weights were recorded twice during acclimation. Control and test animals were weighed on Day 0 (prior to study start) and weekly thereafter (intervals of 7 days ± 1). Body weight gain was calculated for selected intervals and for the study overall.

2.4.2. Food consumption and efficiency

Individual food consumption was measured and recorded at the same time as body weight measurements were performed. Food efficiency, calculated as the mean daily body weight gain divided by the mean daily food consumption, was also recorded for each animal (mg/kg/day). Animals were housed individually in suspended steel cages; a drop pan caught any uneaten food that fell through the bottom of the cage and was weighed for accurate calculation of food consumption.

2.5. Ophthalmology

During the acclimation period and prior to test termination, the eyes of rats were examined by focal illumination, indirect ophthalmoscopy and, if necessary, slit-lamp microscopy. Mydriatic eye drops were administered prior to ophthalmoscopy, and examinations occurred in subdued light.

2.6. Clinical pathology

Clinical pathology evaluations included assessment of hematology, serum chemistry, urinalysis, and post-mortem anatomic pathology.

One day prior to sample collection for clinical pathology evaluation, the animals were placed in metabolism cages to fast overnight. Blood samples for hematology and clinical chemistry were collected *via* sublingual bleeding under isoflurane anesthesia during approximately Week 12 of the test period. Approximately 500 µL of blood was collected in pre-calibrated tubes with K2-ethylenediaminetetraacetic acid (K2EDTA) for hematology assessments and stored under refrigeration. Approximately 1000 µL of blood was collected into a tube containing no preservative for clinical chemistry assessments. These samples were centrifuged in a refrigerated centrifuge, the serum collected and stored in a -80 °C freezer until analysis. Blood samples used to determine the prothrombin time and activated partial thromboplastin time (coagulation) were collected *via* the inferior vena cava under isoflurane anesthesia at terminal sacrifice. Approximately 1.8 mL of blood was collected in pre-calibrated tubes with 3.2 % sodium citrate. Samples were centrifuged in a refrigerated centrifuge, the plasma collected and stored in a -80 °C freezer until analysis. For urinalysis, animals were fasted at least 15 h prior to urine collection. Urine samples were stored under refrigeration until analysis. At terminal sacrifice, all rats were euthanized by exsanguination from the abdominal aorta under isoflurane anesthesia. All animals in the study underwent gross necropsy.

2.6.1. Hematology

Complete blood counts and hematology parameters were measured using an ADVIA 120 Hematology system. White blood cell count (WBC), red blood cell count (RBC), hemoglobin (HBG), hematocrit (HCT), mean corpuscular cell volume (MCV), mean corpuscular cell hemoglobin (MCH), mean corpuscular cell hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (PLT), absolute neutrophil (ANEU), absolute lymphocyte (ALYM), absolute monocyte (AMON), absolute eosinophil (AEOS), absolute basophil (ABAS), absolute large unstained cell (ALUC), absolute reticulocyte (ARET), and percent reticulocyte (% RET) were evaluated. Coagulation was determined on a Siemens Systmex CA620 automated coagulation system.

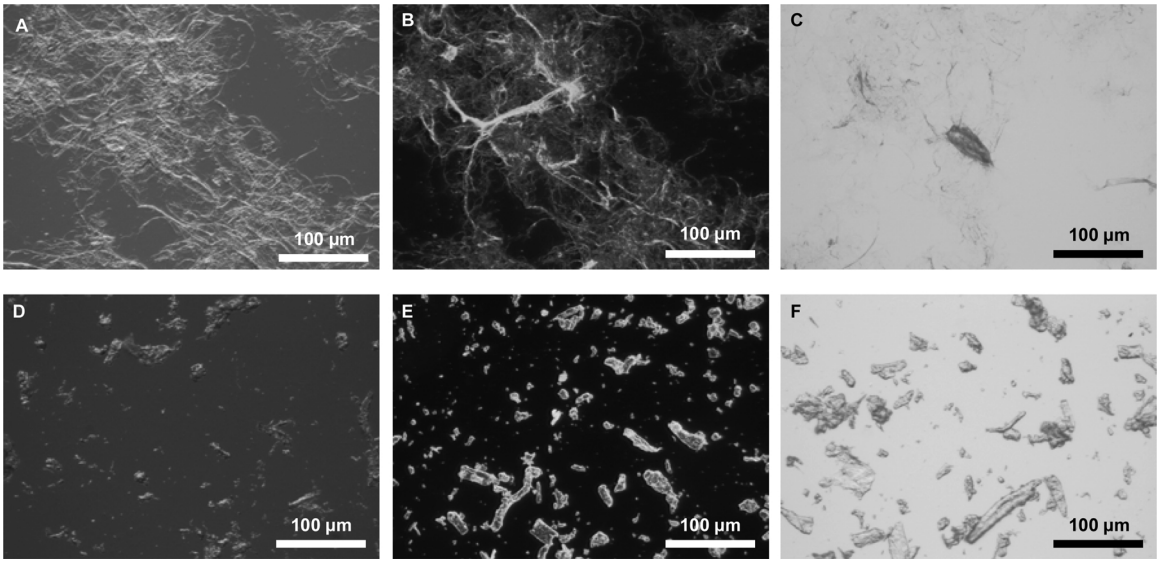

Fig. 1. Micrographs of fibrillated cellulose imaged in (A) bright field, (B) dark field and (C) phase contrast modes. Micrographs of Solka Floc imaged in (D) bright field, (E) dark field and (F) phase contrast modes. Scale bars are indicated within each image.

2.6.2. Serum chemistry

Serum sodium (Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphorus (PHOS), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), urea nitrogen (BUN), cholesterol (CHOL), creatinine (CREAT), glucose (GLU), total protein (TP), total bilirubin (TBIL), triglycerides (TRIG), sorbitol dehydrogenase (SDH), total bile acids (TBA), and globulin (GLOB) were measured on a COBAS C311 automated clinical chemistry analyzer.

2.6.3. Urinalysis

Urine quality, color, and clarity were evaluated visually. Other parameters such as pH, ketone, glucose, bilirubin, specific gravity, blood, volume, protein, urobilinogen, were measured using a Siemens Multistix SG 10, and urine sediment was evaluated microscopically.

2.6.4. Anatomic pathology

Necropsy included examination of the external surface of the body, all orifices, musculoskeletal system, and the cranial, thoracic, abdominal, and pelvic cavities, including associated organs and tissues. All gross lesions were recorded. The adrenals, kidneys, testes, brain, liver, thymus, epididymides, ovaries with oviducts, uterus, heart, and spleen were weighed wet.

2.7. Histopathology

Histopathological examination was performed on the preserved organs and tissues of the animals from the 4 % control and 4 % fibrillated cellulose groups. Any tissues and organs with macroscopic observations in other control or treatment groups were also evaluated, as were gross lesions of note in all control and test groups. The following tissues were preserved in 10 % neutral buffered formalin: prostate and seminal vesicles, adrenals, aorta, bone (femur), bone marrow (from femur and sternum), brain (sections including medulla/pons, cerebellar, and cerebral cortex), cecum, cervix, colon, duodenum, esophagus, Harderian gland, heart, ileum with Peyer's patches, jejunum, kidney, larynx, liver, lungs, lymph node mandibular, lymph node mesenteric, mammary gland, nasal turbinates, nose, ovaries, oviducts, pancreas, parathyroid, peripheral nerve (sciatic), pharynx, pituitary gland, rectum, salivary glands (sublingual, submandibular, and parotid), skeletal muscle, skin, spinal cord (cervical, mid-thoracic, and lumbar), spleen, sternum, stomach, thymus, thyroid, trachea, urinary bladder, uterus, and vagina. The epididymides, eyes, optic nerve, and testes were preserved in modified Davidson's fixative, then stored in 70 % ethanol. The fixed tissues were trimmed, processed, embedded in paraffin, sectioned with a microtome, placed on glass microscope slides, stained with hematoxylin and eosin, and examined by light microscopy Slide preparation and evaluation was conducted in compliance with GLP by a board-certified veterinary pathologist. All slides were evaluated and findings entered into Pristima® software. Each tissue was assessed for macroscopic observations, then each tissue was assessed based on any remarkable incidences, defined as: 1 = minimal or present; 2 = mild; 3 = moderate, 4 = marked, and 5 = severe.

2.8. Statistical analysis

To compare control and test groups, parametric one-way analyses of variance (ANOVAs) were performed for each quantitative parameter with homogeneous variance (assessed using Levene's test). Welch's ANOVAs were performed for parameters violating the homogeneity of variance assumption. Separate analyses were performed on the data collected for males and females. Parameters found significant in the ANOVAs were further analyzed using post-hoc Tukey's tests (for parameters with homogenous variance) or Games-Howell tests (for parameters with unequal variance). Differences were considered significant at $p < 0.05$. All statistical analyses were performed using RStudio (Version 1.2.1335).

3. Results

3.1. Material preparation and characterization

Light (Fig. 1) and electron microscopy (Fig. 2) micrographs show the fibrillar morphology of fibrillated cellulose, consisting of an entangled network of fibers and fibrils of varying widths. The finest fraction of fibrils had an average fibril width of 25.06 ± 6.29 nm and formed aggregates with an average size of 227.7 ± 103.3 µm when dried, as measured through light and electron micrographs (Fig. 3). Dynamic light scattering (DLS) measurements recorded an average hydrodynamic diameter of 3330 ± 407 nm and polydispersity index of 0.836 ± 0.190 for fibrillated cellulose. Surface charge measurements resulted in an average zeta potential of −37.5 ± 1.67 (Table 1).

Solka Floc has a lower aspect ratio than fibrillated cellulose and

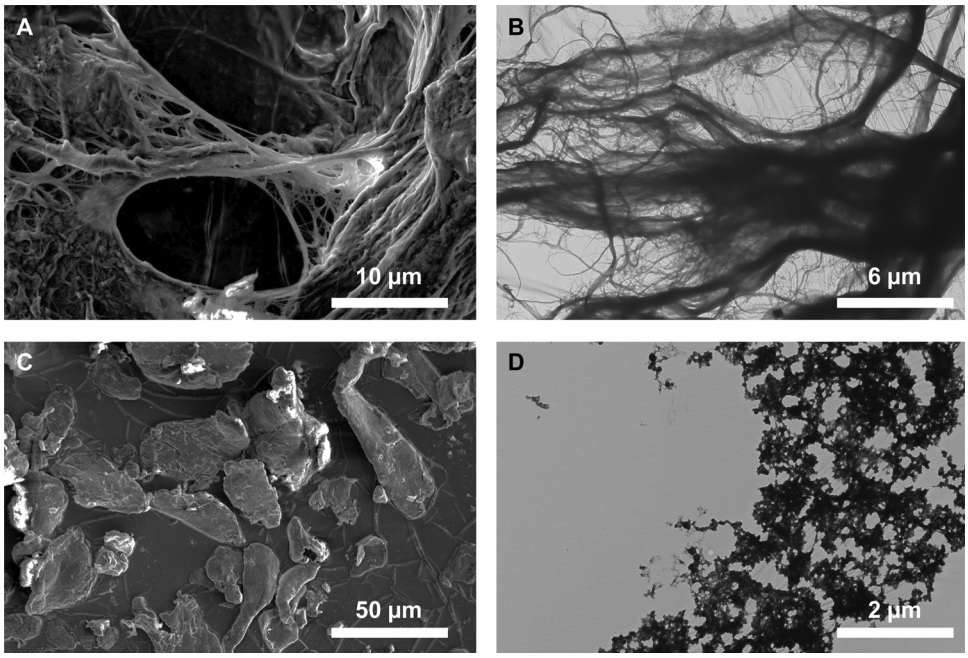

Fig. 2. Electron microscopy images of the cellulose samples used in this study. (A) Scanning electron micrograph of Fibrillated Cellulose, (B) transmission electron micrograph of fibrillated cellulose, (C) scanning electron micrograph of Solka Floc, and (D) transmission electron micrograph of Solka Floc. Scale bar is indicated in each of the images.

does not form an entangled network of fibers (Fig. 1). Generally, it has an amorphous morphology that is microns in length and width, with an average width of 3.72 ± 0.728 µm and aggregate size of 58.6 ± 10.5 µm (Fig. 3). DLS measurements recorded an average hydrodynamic diameter of 625 ± 41.0 nm and polydispersity index of 0.594 ± 0.05 for Solka Floc. Surface charge measurements resulted in an average zeta potential of -24.3 ± 1.95 mV (Table 1).

Elemental mapping of fibrillated cellulose and Solka Floc using EDXS revealed similar elemental compositions for both cellulose materials. Samples contained 88 % and 82 % carbon, 9 % and 16 % oxygen, and 3 % and 2 % calcium for fibrillated cellulose and Solka Floc, respectively (Supplemental Fig. 1).

Table 1
Quantitative analyses of fibrillated cellulose and Solka Floc physicochemical properties. The table includes hydrodynamic diameter (as a measure of size), zeta potential (as a measure of surface charge), polydispersity index (as a measure stability). All data were collected using DLS technique.

Physiochemical Property	Fibrillated Cellulose	Solka Floc
Hydrodynamic diameter (nm)	3330 ± 407	625 ± 41.0
Zeta potential (mV)	−37.5 ± 1.67	−24.3 ± 1.95
Polydispersity index (unitless)	0.836 ± 0.190	0.594 ± 0.05

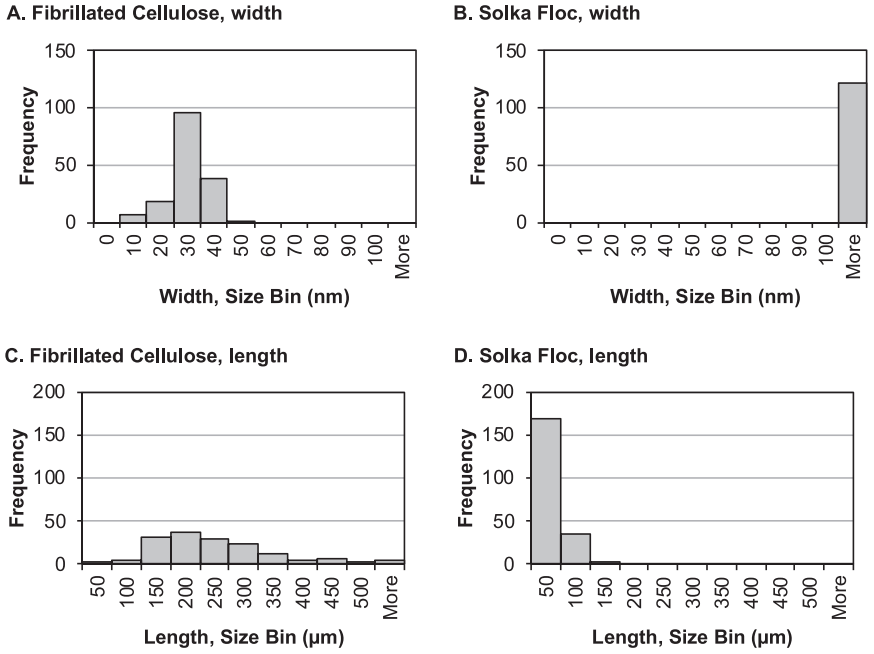

Fig. 3. Particle size distributions for the average width measurements of (A) fibrillated cellulose (25.06 ± 6.29 nm) and (B) Solka Floc (3.72 ± 0.728 µm). Particle size distributions for the average aggregate length measurements of (C) fibrillated cellulose (227.7 ± 103.3 µm) and (D) Solka Floc (58.6 ± 10.5 µm).

3.2. Diet characterization

The diet was stable, homogenously mixed, and the celluloses were administered at the expected concentrations of 2, 3, and 4 %. Based on the body weight and food consumption measurements, the mean overall dietary intakes of the 2 %, 3 %, and 4 % diets of Solka Floc were calculated to be 1070, 1536, and 2119 mg/kg/day, respectively, for the male rats and 1311.7, 1920.2, and 2597.5 mg/kg/day, respectively, for the female rats. For the 2 %, 3 %, and 4 % diets of fibrillated cellulose, mean dietary intakes were calculated to be 1044, 1550, and 2194 mg/kg/day for the male rats and 1302, 1886, and 2667 mg/kg/day for the female rats.

Solka Floc and fibrillated cellulose were considered stable in the dietary feed throughout the study, ranging from 103.6 to 108.3% over the 10 measured days. Homogeneity analysis demonstrated that Solka Floc and fibrillated cellulose were homogeneously distributed through the diets with concentrations averaging 99.9 %, 91.9 %, and 106.1 % of Solka Floc target concentrations (2 %, 3 %, and 4 %), and 105.0 %, 104.8 %, and 98.3 % of fibrillated cellulose target concentration (2 %, 3 %, and 4 %).

3.3. Clinical observations

There were no treatment-related mortalities attributed to the consumption of Solka Floc or fibrillated cellulose at any concentration. One rat in the 4 % fibrillated cellulose group was found dead on Day 90 due to a spontaneous death; necropsy and histopathological examination did not reveal any evidence of treatment-related toxicity, and no other rat in the group suffered adverse effects. One rat in the 4 % Solka Floc group was humanely sacrificed on Day 66 due to self-inflicted mechanical damage. There were no consistent clinical observations for prolonged periods of time that indicated any adverse results in control or test animals.

3.3.1. Body weight and body weight gain

There were no differences in body weight parameters in male or female rats between control and fibrillated cellulose groups ($p > 0.05$) (Fig. 4). Overall and mean weekly body weights, as well as mean daily body weight gain, were not significantly different between control and treatment groups, nor between different dietary concentrations.

3.3.2. Food consumption and food efficiency

There were no differences in daily average food consumption or food efficiency in male or female rats between control and treatment groups ($p > 0.05$) (Table 2).

3.4. Ophthalmology

One 2 % Solka Floc female had extensive chorioretinal scarring in the left eye, and one 3 % Solka Floc female had chorioretinal scarring in the right eye; however, as isolated incidents, these observations were not considered attributable to the diet. No incidents were identified in fibrillated cellulose treatment groups.

3.5. Clinical pathology

The clinical pathology investigations indicate no toxicologically significant results ($p > 0.05$). A statistically significant increase ($p < 0.05$) in white blood cells was shown in 2 % fibrillated cellulose males compared to 2 % Solka Floc males (Supplemental Table 2), and an increase in sodium levels in 4 % fibrillated cellulose males compared to 4 % Solka Floc males (Supplemental Table 3). These changes were not considered biologically significant because values were within normal ranges, as seen in other 90-day studies with Sprague-Dawley rats, and were not considered related to the differences in cellulose because these differences were not seen between any other groups.

There were no other significant adverse fibrillated cellulose effects on the hematology factors (Supplemental Table 2), the serum chemistry (Supplemental Table 3), or the urine parameters (not shown) ($p > 0.05$). Hematology, coagulation, and serum chemistry values fall within normal range of Sprague-Dawley rats fed similar diets (*e.g.* [18,43]). There were no adverse effects nor lesions observed during the gross necropsy. There were no significant differences in organ weight between control and fibrillated cellulose treatment groups (Tables 3 and 4).

3.6. Histopathology

Vacuolation of periportal hepatocytes (the presence of variably-sized, clear cytoplasmic vacuoles present within the cytoplasm of periportal hepatocytes) in the liver were present in both 4 % Solka Floc and 4 % fibrillated cellulose groups. There was little variation in incidence and severity between the groups examined, and vacuolation was not accompanied by hepatocyte degeneration or other pathologic observations in the liver. There were no other consistent macroscopic or microscopic effects noted in the animals examined. In the absence of any apparent altered liver function or clinical histopathology observations, is not considered to be an adverse result. All macroscopic observations were isolated and considered to be common background changes often noted in laboratory rats, with no relation to test substance administration.

4. Discussion and conclusion

Dietary fiber is an important component of diets and provides many health benefits, such as risk reduction for various diseases including heart disease, diabetes, and hypertension [19]. A number of studies have concluded that cellulose is safe for use in foods ([11,10,20]), and microcrystalline cellulose, powdered cellulose, and other cellulose derivatives are authorized as food additives *ad libitum*, to an amount required to achieve the desired technical effect, in the United States, European Union, Canada, and many other countries. This study demonstrates that, similar to conventional forms of cellulose such as Solka Floc, dietary administration of 2 %, 3 %, and 4 % fibrillated cellulose for 90-days does not result in any treatment-related adverse effects. Effects assessed included mortality, clinical symptoms, body weight, food consumption, food efficiency, ophthalmology, hematology, serum chemistry, urinalysis, anatomic pathology, organ weight, and histopathology.

The results of this study add to a rich body of literature on the safety of various celluloses. Fibrillated cellulose, conventional celluloses, micro and nano-forms of cellulose, and bacterial celluloses share the same fundamental molecular structure, a linear homopolymer of β-1,4-linked anhydro-D-glucose units, and exhibit similar general morphology and chemical identity. These physical and chemical similarities, in addition to the lack of adverse biological effects observed in published cellulose dietary studies, suggest that studies of conventional celluloses and other unmodified forms of cellulose can be used as supporting evidence toward fibrillated celluloses being safe to eat, even at relatively high concentrations. A review of studies examining the sub-acute (< 28 days), subchronic (< 90 days), and chronic (> 90 days) oral toxicity of different forms of unmodified cellulose revealed no indication of adverse effects, even when cellulose contributed a relatively high percentage of the diet. DeLoid et al. conducted a dietary study assessing safety in rats gavaged twice weekly over a period of five weeks with 1 % nanocellulose fibrils in either water or cream; in accordance with this study, no evidence of toxicity was observed after assessing hematology, serum markers, or histology [21]. In another study, groups of 100 rats were fed diets of 30 % dry, gel, or fibrous forms of cellulose for 72 weeks (Paynter 1963 in SCOGS 1973). After this chronic feeding, there were no differences in survival, appearance, behavior, or food consumption. Andrade et al. [22] fed mice diets

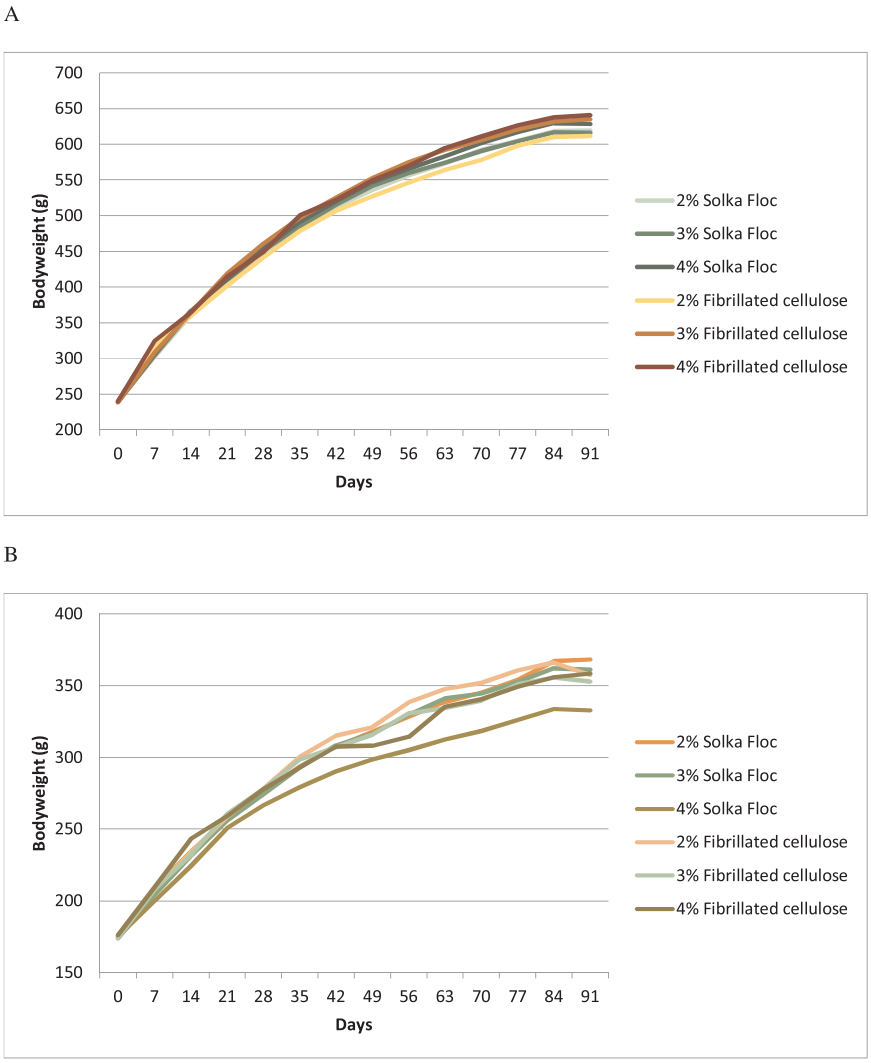

Fig. 4. Average male (A) and female (B) rat body weight, day 0–91.

Table 2
Daily average consumption of cellulose (mg cellulose/kg rat/day).

	Consumption (mg/kg/day)	
	Males	*Females*
2 % Solka Floc	1070.4 ± 48.3	1311.7 ± 52.3
2 % Fibrillated cellulose	1043.7 ± 47.3	1302.2 ± 84.7
3 % Solka Floc	1535.8 ± 54.8	1920.2 ± 119.5
3 % Fibrillated cellulose	1550.4 ± 40.2	1886.4 ± 144.3
4 % Solka Floc	2119.2 ± 128.9	2597.5 ± 135.3
4 % Fibrillated cellulose	2194.2 ± 61.4	2666.6 ± 143.8

containing 7 %, 14 %, or 21 % cellulose nanofibrils derived from peach palm residue. After 30 days, the researchers conducted biological, biochemical, and histological tests and determined there was no evidence of toxicity. A study examining the sub-acute oral toxicity of a mixture of 60 % bacterial cellulose (a fermentation-derived cellulose fiber) from *Acetobacter aceti*, 20 % Na-carboxymethylcellulose, and 20 % sucrose reported no adverse effects [23]. The researchers also examined the effects of a 28-day exposure to this mixture in rats at dietary levels ranging from 0 to 5%, with no adverse clinical effects noted, and no change in mortality, body weight, food or water consumption observed. Results from urinalysis, ophthalmology, hematology, blood chemistry and histopathology were all similar to controls with the exception of a slight increase in cecum weight in exposed animals.

The OECD TG 408 protocol was also recently used to evaluate dietary exposure to other sources of cellulose materials as dietary fibers, such can pecan shell fiber and bacterial cellulose, with similar negative findings [18,24]. Other naturally derived products are similarly tested using the 13-week study design at relatively high concentrations with no observed toxicity, including krill powder and krill oil ([25,26]), algae [27], bacterial carotenoid [28], cucumber extract [29], insect larvae [30], and rice extract [31].

Animals sometimes increase food intake to compensate for the low energy density of a higher cellulose diet [32]. In this study, overall food intake and efficiency were similar amongst groups fed different concentrations of cellulose, indicating that the concentrations of cellulose used do not substantially affect the energy density of the feed over a 90-day period.

Some studies have shown that chronic intake of 10–25 % cellulose fiber can change the structure of the mammalian gastrointestinal tract, affecting food transit time, nutrient absorption, and cholesterol absorption [32–35]. Here there were no changes in food intake, clinical chemical observations, nutritional deficiencies, or gastrointestinal lesions observed in the histopathology, nor indication of any inflammatory or proliferative changes that suggest longer term effects; this study demonstrates no indications of adverse effects as a result of gastrointestinal changes.

Animal and human data, as analyzed by the European Food Safety Authority (EFSA), demonstrate that microcrystalline and powdered

Table 3
Mean ± standard deviation organ weights (g) in male rats on day 90.

Organ	Solka Floc			Fibrillated cellulose		
	2 %	3 %	4 %	2 %	3 %	4 %
Adrenal glands	0.0915 ± 0.956	0.0655 ± 0.0120	0.0601 ± 0.0125	0.0558 ± 0.0129	0.0643 ± 0.0091	0.0606 ± 0.0116
Brain	2.317 ± 0.102	2.237 ± 0.206	2.332 ± 0.082	2.310 ± 0.095	2.293 ± 0.079	2.349 ± 0.080
Epididymides	1.5952 ± 0.1788	1.6097 ± 0.1488	1.6159 ± 0.1911	1.5317 ± 0.1896	1.5033 ± 0.1510	1.5348 ± 0.1034
Heart	1.606 ± 0.112	1.604 ± 0.144	1.637 ± 0.151	1.626 ± 0.162	1.669 ± 0.153	1.544 ± 0.121
Kidneys	3.611 ± 0.328	3.669 ± 0.414	3.722 ± 0.355	3.542 ± 0.597	3.517 ± 0.277	3.517 ± 0.224
Liver	15.022 ± 1.309	14.933 ± 2.388	16.574 ± 3.711	15.009 ± 1.720	15.232 ± 2.941	15.288 ± 2.134
Spleen	0.950 ± 0.156	0.948 ± 0.170	1.016 ± 0.381	1.027 ± 0.202	0.974 ± 0.143	0.972 ± .137
Testes	3.575 ± 0.236	3.547 ± 0.282	3.723 ± 0.402	3.596 ± 0.351	3.415 ± 0.608	3.617 ± 0.331
Thymus	0.2762 ± 0.0492	0.2979 ± 0.0574	0.3007 ± 0.0609	0.3623 ± 0.0659	0.3058 ± 0.0821	0.3282 ± 0.0936

Table 4
Mean ± standard deviation organ weights (g) in female rats on day 90.

Organ	Solka Floc			Fibrillated cellulose		
	2 %	3 %	4 %	2 %	3 %	4 %
Adrenal glands	0.0713 ± 0.0137	0.0694 ± 0.0116	0.0720 ± 0.0104	0.0706 ± 0.0082	0.0666 ± 0.0082	0.0711 ± 0.0133
Brain	2.100 ± 0.125	2.118 ± 0.077	2.093 ± 0.080	2.136 ± 0.122	2.121 ± 0.123	2.129 ± 0.066
Heart	1.119 ± 0.151	1.082 ± 0.165	1.083 ± 0.154	1.099 ± 0.159	1.079 ± 0.131	1.201 ± 0.095
Kidneys	2.431 ± 0.58	2.340 ± 0.474	2.207 ± 0.298	2.356 ± 0.348	2.218 ± 0.317	2.485 ± 0.302
Liver	10.109 ± 2.113	9.675 ± 2.341	9.603 ± 1.630	10.955 ± 4.200	9.514 ± 1.861	10.780 ± 1.698
Ovaries with oviducts	0.1243 ± 0.0318	0.1122 ± 0.0211	0.1204 ± 0.0208	0.1329 ± 0.0195	0.1271 ± 0.0262	0.1145 ± 0.0187
Spleen	0.629 ± 0.139	0.597 ± 0.079	0.539 ± 0.047	0.707 ± 0.395	0.570 ± 0.092	0.597 ± 0.071
Thymus	0.3017 ± 0.1080	0.3085 ± 0.0854	0.2581 ± 0.0676	0.2815 ± 0.0799	0.3212 ± 0.0649	0.3038 ± 0.0476
Uterus	0.749 ± 0.167	0.827 ± 0.250	0.844 ± 0.323	0.707 ± 0.245	0.671 ± 0.153	0.781 ± 0.189

cellulose are not absorbed intact in the gastrointestinal tract [36]. This study did not determine the toxicokinetics of fibrillated cellulose, as these carbon-based materials pose detection challenges *in vivo*, particularly during dietary exposure. Studies to determine the absorption, distribution, metabolism, and excretion kinetics are ongoing. Cellulose, an insoluble dietary fiber, moves through the gut quickly, reducing the amount of time available for colonic bacterial fermentation of non-digested foodstuff. Cellulose is resistant to degradation in the human gut due to a lack of enzymes specialized in cellulose breakdown [37,38]. Since fibrillated cellulose has the same molecular structure as conventional celluloses, it is anticipated to be similarly resistant to breakdown in the gut. In addition, studies demonstrate that fibrillated cellulose is resistant to breakdown under simulated digestive conditions [39]. It is likely that fibrillated cellulose will alter microbial diversity in the gut, much like other fibers can alter bacterial fermentation, colony size, and species diversity and composition ([40,38,41,42]). In general, consuming a diet consisting of diverse dietary fibers is more supportive of a varied gastrointestinal microbial community compared to a low fiber refined diet [38].

In this study, the 90-day subchronic toxicity test showed no systemic toxicity attributable to dietary consumption of fibrillated cellulose. Under the conditions of this study and based on the toxicological endpoints evaluated, the no-observed-adverse-effect level (NOAEL) for fibrillated cellulose was 2194.2 mg/kg/day (males) and 2666.6 mg/kg/day (females), corresponding to the highest dose tested (4 %) for male and female Sprague Dawley rats. Other studies on conventional celluloses indicate that much higher concentrations may be tolerated, with one study demonstrating no adverse effects even at 30 % diet for 72 weeks. Therefore, we conclude that fibrillated cellulose behaves similarly to conventional cellulose in the GI tract and raises no safety concerns when used as a food ingredient at this concentration. The differences in manufacturing, physical and chemical properties of fibrillated cellulose from conventional cellulose does not result in any biological differences in the gastrointestinal tract.

Conflict of interests

The authors declare no conflict of interest.

CRediT authorship contribution statement

Kimberly J. Ong: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Project administration. **James D. Ede:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Project administration. **Cassidy A. Pomeroy-Carter:** Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Christie M. Sayes:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision. **Marina R. Mulenos:** Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Jo Anne Shatkin:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2020.01.003.

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