CAELUS HEALTH

31 March 2022

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

Dear Dr. Gaynor:

Re: GRAS Notice for *A. soehngenii* CH106

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285, Caelus Health [based at Rondweg 50, 3474 KG Zegveld, The Netherlands], as the notifier, is submitting one hard copy and one electronic copy (on CD), of all data and information supporting the company's conclusion that *A. soehngenii* CH106, as manufactured by Caelus, is GRAS on the basis of scientific procedures, for use in various conventional food and beverage products; these food uses of *A. soehngenii* CH106 are therefore not subject to the premarket approval requirements of the *Federal Food, Drug and Cosmetic Act*. Information setting forth the basis for Caelus' GRAS conclusion, as well as a consensus opinion of an independent panel of experts, also are enclosed for review by the agency.

Should you have any questions or concerns regarding this GRAS notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

Luc Sterkman, MD, CEO
Caelus Health
Rondweg 50
3474 KG Zegveld
The Netherlands
Tel: +31(0)622979249
l.sterkman@caelushealth.coma

GRAS NOTICE FOR *ANAEROBUTYRICUM SOEHNGENII* CH106 FOR USE IN CONVENTIONAL FOOD AND BEVERAGE PRODUCTS IN THE UNITED STATES

SUBMITTED TO:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

SUBMITTED BY:
Caelus Health
Rondweg 50
3474 KG Zegveld
The Netherlands

DATE:
30 March 2022

GRAS Notice for *Anaerobutyricum soehngenii* CH106 for Use in Conventional Food and Beverage Products in the United States

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GRAS Notice for *Anaerobutyricum soehngenii* CH106 for Use in Conventional Food and Beverage Products in the United States

Part 1. §170.225 Signed Statements and Certification

In accordance with 21 CFR 170 Subpart E consisting of §170.203 through §170.285 (U.S. FDA, 2021), Caelus Health (Caelus) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that the intended uses of *Anaerobutyricum soehngenii* CH106, as manufactured by Caelus, in various conventional food and beverage products as described in Part 1.3 below, are not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* based on Caelus' view that these notified uses of *A. soehngenii* CH106 for Use in Conventional Food and Beverage Products in the United States are Generally Recognized as Safe (GRAS). In addition, as a responsible official of Caelus, the undersigned hereby certifies that all data and information presented in this notice represents a complete and balanced submission that is representative of the generally available literature. Caelus considered all unfavorable as well as favorable information that is publicly available and/or known to Caelus, and that is pertinent to the evaluation of the safety and GRAS status of *A. soehngenii* CH106 as a food ingredient for addition to *A. soehngenii* CH106 food products, as described herein.

Signed,

30 March 2022

Luc Sterkman, MD, CEO
Tel: +31(0)622979249
l.sterkman@caelushealth.com

Date

1.1 Name and Address of Notifier

Caelus Health
Rondweg 50
3474 KG Zegveld
The Netherlands

1.2 Common Name of Notified Substance

Anaerobutyricum soehngenii CH106

1.3 Conditions of Use

A. soehngenii CH106 is intended to be added as a food ingredient to various beverage, cereal and grain, milk, milk analogue, nut, confectionary, and meal replacement products to be marketed in the U.S. The food ingredient is intended to be added at a maximum use level of 1.0×10^{10} total fluorescent units (TFU)/serving across all food categories. A summary of the food categories in which *A. soehngenii* CH106 is intended for use is provided in Table 1.3-1 below. Food uses are organized according to 21 CFR §170.3

(U.S. FDA, 2021). The ingredient is not subject to 21 §170.270 as it is not intended for use in meat and poultry or meat and poultry containing products that are subject to USDA oversight.

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for *A. soehngenii* CH106 in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2021)	Food Uses*	Maximum Intended Use Level (TFU/serving)
Beverages and Beverage Bases	Sport or Electrolyte Drinks, Fluid Replacement Drinks	1.0×10^{10}
Breakfast Cereals	Ready-to-Eat Breakfast Cereals	1.0×10^{10}
Dairy Product Analogs	Non-Dairy Yogurts	1.0×10^{10}
Frozen Dairy Desserts	Ice Cream	1.0×10^{10}
Grain Products and Pastas	Cereal and Granola Bars	1.0×10^{10}
	Energy Bars, Protein Bars, and Meal Replacement Bars	1.0×10^{10}
Milk Products	Fermented Milks, Plain	1.0×10^{10}
	Plain or Flavored Yogurt	1.0×10^{10}
Nut and Nut Products	Nut Spreads	1.0×10^{10}
Soft Candy	Chocolate	1.0×10^{10}

CFR = *Code of Federal Regulations*; TFU = total fluorescent units; U.S. = United States.
* *A. soehngenii* CH106 is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2021), Caelus has concluded that the intended uses of *A. soehngenii* CH106 as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Caelus Health
Rondweg 50
3474 KG Zegveld
The Netherlands

Should the FDA have any questions or additional information requests regarding this Notification, Caelus will supply these data and information upon request.

1.6 *Freedom of Information Act*, 5 U.S.C. 552

It is Caelus' view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempted from the *Freedom of Information Act*, 5 U.S.C. 552.

Part 2. §170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

2.1.1 Name and Taxonomic Lineage

Common Name: *Anaerobutyricum soehngenii* CH106

Alternate Name(s): *A. soehngenii* CH106; *Eubacterium hallii* R6M8; *Anaerobutyricum soehngenii* R6M8

Trade Name: GlucoBiome™

Taxonomic Lineage:

Kingdom: *Bacteria*
Phylum: *Firmicutes*
Class: *Clostridia*
Order: *Clostridiales*
Family: *Lachnospiraceae*
Genus: *Anaerobutyricum*
Species: *soehngenii*
Strain: CH106

2.1.2 History of *A. soehngenii* CH106

Anaerobutyricum soehngenii CH106 has been deposited in the restricted collection of Westerdijk Fungal Biodiversity Institute on 13 November 2018 (CBS 145175). Samples from this deposition have been used to establish an on-site working cell bank (WCB) for production of commercial fermentation lots of *A. soehngenii* CH106.

The original strain, *A. soehngenii* L2-7 (previously classified as *E. hallii* L2-7)[1], was isolated from infant feces and phylogenetically characterized by Barcenilla *et al*. (2000). Butyrate-producing bacteria were identified from isolated colonies and grouped by 16S rRNA sequence analysis, 80% of which fell into the XIVa cluster of Gram-positive bacteria defined by Collins *et al.* (1994) and later termed *Lachnospiracaea* (Rajilić-Stojanović and de Vos, 2014). *A. soehngenii* belongs to a group of human gut microbes that produce butyrate from intestinal carbon sources. The L2-7 strain was initially classified as a *Eubacterium* and identified as a butyrate-producing acetate consumer (see Part 2.1.4 for further details). In studies conducting 16S rRNA analyses by Shetty *et al.* (2018) it was determined that *E. hallii* L2-7 would be reclassified as *Anaerobutyricum soehngenii* sp. nov. with L2-7 as type strain [DSM 17630^T; KCTC 15707^T]. The L2-7 strain, now *A. soehngenii* L2-7, was treated with ethyl methanesulfonate (EMS) to induce random mutagenesis with the intention to increase the safety of the organism by disruption of the gene that conferred tetracycline antibiotic resistance. The resultant tetracycline-susceptible strain was classified as *A. soehngenii* CH106.

[1] For taxonomic information detailing the revised nomenclature, see Shetty *et al*. (2018).

2.1.3 Description

The *A. soehngenii* CH106 ingredient is prepared as a lyophilized, white to off-white, odorless powder containing not less than 5.0 x 10^9 TFU/g and 1.0 x 10^8 active fluorescent units (AFU)/g *A. soehngenii* CH106/g of powder. Proximate analysis of the ingredient is provided below in Table 2.1.3-1.

Table 2.1.3-1 Proximate Composition of *A. soehngenii* CH106

Parameter	Typical Range	Method
Humidity	2.00–5.00 g/100 g	Rapporti ISTISAN 1996/34
Protein	2.00–3.50 g/100 g	Internal method based on AOAC 990.06 2002, 992.15, 1992, 992.23, 1992
Fat	<0.050–0.700 g/100 g	Rapporti ISTISAN 1996/34
Ash	1.5–9.0/100 g	Internal method based on AOAC 945.46, 923.03, 938.08, 920.93A
Carbohydrates	85.00–92.50 g/ 100 g	Internal method based on AOAC 986.25
Energy Value	350–400 kcal/100 g 1,465–1,674 kJ/100 g	Internal method based on AOAC 986.25

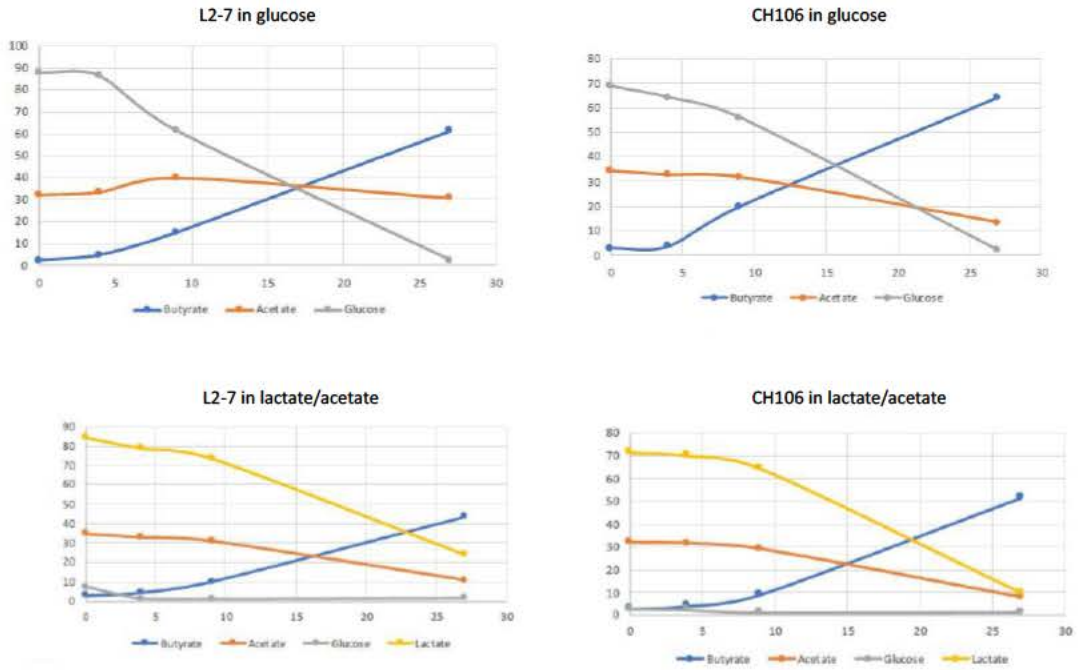
AOAC = Association of Official Analytical Chemists.

2.1.4 Phenotypic Identity

A. soehngenii CH106 is a strictly anaerobic, Gram-positive, rod-shaped bacterium belonging to the *Clostridium* cluster XIVa family of the Firmicutes phylum and is an abundant intestinal microbe (Rajilić-Stojanović and de Vos, 2014). The *A. soehngenii* species has been characterized to produce butyrate from glucose as well as lactate in the presence of acetate (Flint *et al.,* 2012; Shetty *et al.,* 2017a). As indicated above, *A. soehngenii* CH106 was generated by EMS treatment from the original strain, *A. soehngenii* L2-7, and the only resulting phenotypic change was tetracycline sensitivity due to a frame shift mutation in the TetO gene. Susceptibility of the CH106 strain to tetracycline was demonstrated by conducting a minimum inhibitory concentration (MIC) evaluation, the results of which are described in Part 6.5.2. All other EMS-induced mutations were synonymous or were non-synonymous with no impact on gene function and phenotype.

The growth characteristics of the *A. soehngenii* CH106 strain were tested by e-comparison of the nutrient utilization and metabolite production profiles of L2-7 and CH106 to confirm similarity. Experiments were conducted with both strains grown in glucose media or an acetate/lactate blended media, and the changes in the consumption of glucose, lactate, acetate and the production of butyrate were found to be similar between strains, as shown in Figure 2.1.4-1.

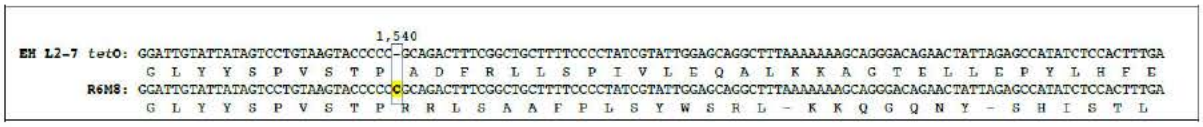
Figure 2.1.4-1 Growth Comparison of *A. soehngenii* Strains L2-7 and CH106



2.1.5 Genotypic Identity

The genome of *A. soehngenii* L2-7 has been sequenced under the previous name of *E. hallii* (Shetty *et al.*, 2017b). The genome sequence is stored at GenBank/EMBL-EBI under accession number LT907978 (assembly version EH1). *A. soehngenii* L2-7 is registered at the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 17630. Its 16S rRNA has been sequenced and deposited at GenBank (Accession Number AJ270490), as described in Barcenilla *et al*. (2000), Shetty *et al*. (2018), and Sayers *et al.* (2019). *Anaerobutyricum* species can readily be identified from stool samples by 16S rRNA sequence analysis (Shetty *et al*., 2018). Genetic mutation of the L2-7 strain to generate the CH106 strain was accomplished using EMS treatment to disrupt tetracycline resistance, among other mutations. Mutations generated by EMS are random in nature and generally produce point mutations but can also generate larger deletions of 4 to 6 base pairs. Colonies that exhibited the desired trait of sensitivity to tetracycline were selected and tested. Strain CH106 was selected as the best candidate based on the following characteristics: a) the level of tetracycline sensitivity, b) mutation stability, and c) characteristics of the additional mutations. There were 12 mutations observed in non-coding regions of the genome and 25 mutations (4 synonymous and 21 non-synonymous) in coding regions. Of the 21 non-synonymous mutations, 16 were missense mutations, 2 were nonsense mutations, 1 resulted in an amino acid (AA) deletion (3 nucleotide deletion), and 1 insertion resulted in a frame shift. The latter included an insertion (cytosine) at position 1540 in the coding sequence of the TetO gene generating a frameshift, resulting in a stop codon early in the protein sequence (see Figure 2.1.5-1). This results in a truncated TetO protein that is not functional since it does not contain the essential so-called 507 loop of the TetO protein (Li *et al*., 2013), thereby yielding a tetracycline-sensitive strain. Conduct of a reversion assay to determine mutation frequency confirmed that the mutation in the TetO gene was stable (*i.e.,* the calculated reversion rate was below 5.9 x 10^{-8}/cell division).

Figure 2.1.5-1 Sequence Comparison of the TetO Gene Region from *A. soehngenii* Strains L2-7 and CH106



Following EMS mutagenesis, a full sequence analysis was conducted on strain CH106. Briefly, DNA was extracted from strain CH106 for the generation of paired-end sequence reads prior to generating FASTQ read sequences. Concurrent quality control assessment steps were applied to the generated reads (8,648,950 total), yielding an average quality score of 36.16, or >99.99% accuracy of inferred base call. Genome alignment was conducted using the reference sequence for *A. soehngenii* L2-7 (Shetty *et al.*, 2017b), to which 99.83% of reads from the test article strain CH106 were mapped. Functional annotation of the genome was generated using the UniProtKB Swiss-Prot database, and functional elements included in the annotation consisted of tRNA, tmRNA, rRNA, ncRNA, CRISPR, and CDS. The taxonomic distinction between the test article strain, *A. soehngenii* CH106, and a panel of 10 other *A. soehngenii* strains was made; the reported Average Nucleotide Identity (ANI) score of 99.99 % between strains CH106 and L2-7 well exceeds the ANI lower limit ≥95%.

2.2 Manufacturing

2.2.1 Additives and Processing Aids

The nutrient media used to culture *A. soehngenii* CH106 contains ingredients commonly employed in microorganism propagation media. The yeast extract and peptones (soy peptone has been used in earlier batches but due to its allergenic potential has been replaced by non-allergenic pea peptone; other plant or yeast-based peptones can also be used) provide various nutrients required for microbial growth. Carbon source requirements are met by the addition of sucrose, and the remaining ingredients contribute to the ionic strength and buffering capacity. A complete list of the ingredients of the fermentation media, the cell wash buffer, and the cryoprotectant is included in Table 2.2.1-1.

Table 2.2.1-1 Additives and Processing Aids Used in the Manufacture of *A. soehngenii* CH106 and Regulatory Status in Food in the United States

Ingredient	Function	Regulatory Status in Food in the U.S.
Preculture and Production Media		
Yeast extract	Fermentation nutrient	GRAS, 21 CFR §184.1983 (U.S. FDA, 2021)
Peptones	Fermentation nutrient	GRAS, 21 CFR §184.1553 (U.S. FDA, 2021)
Sodium bicarbonate	Fermentation nutrient	GRAS, 21 CFR §184.1736 (U.S. FDA, 2021)
Sodium acetate	Fermentation nutrient	GRAS, 21 CFR §184.1721 (U.S. FDA, 2021)
Potassium phosphate dibasic	Fermentation nutrient	GRAS, 21 CFR §182.6285 (U.S. FDA, 2021)
Potassium phosphate monobasic	Fermentation nutrient	GRAS, FCC Listed Substance
Ammonium chloride	Fermentation nutrient	GRAS, 21 CFR §184.1138 (U.S. FDA, 2021)
Sodium chloride	Fermentation nutrient	Food for human consumption
Magnesium sulphate heptahydrate	Fermentation nutrient	GRAS, 21 CFR §184.1443 (U.S. FDA, 2021)
Sucrose	Fermentation nutrient	GRAS, FCC Listed Substance
Cysteine	Fermentation nutrient	GRAS, 21 CFR §184.1271 (U.S. FDA, 2021)
Calcium chloride	Fermentation nutrient	GRAS, 21 CFR §184.1193 (U.S. FDA, 2021)

Table 2.2.1-1 Additives and Processing Aids Used in the Manufacture of *A. soehngenii* CH106 and Regulatory Status in Food in the United States

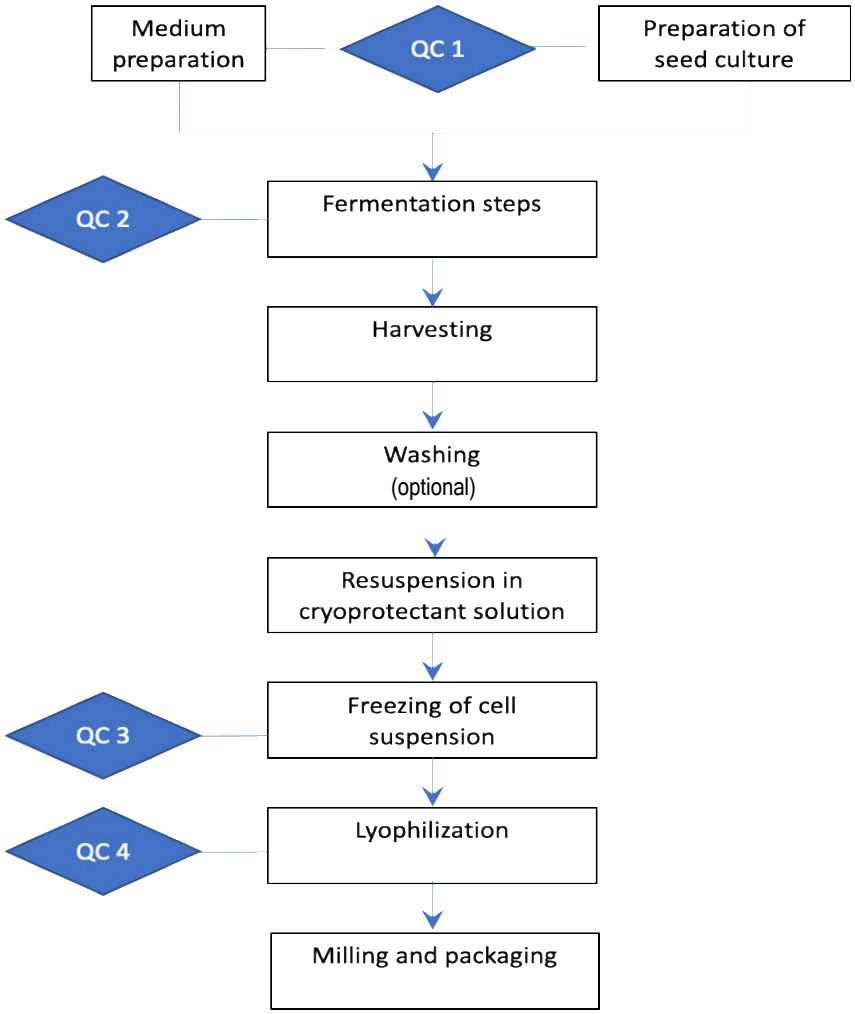
Ingredient	Function	Regulatory Status in Food in the U.S.
Wash Buffer		
Sodium chloride	Processing aid	Food for human consumption
Disodium phosphate	Processing aid	GRAS, 21 CFR §182.6290 (U.S. FDA, 2021)
Potassium dihydrogen phosphate	Processing aid	GRAS, FCC Listed Substance
Sucrose	Processing aid	GRAS, FCC Listed Substance
Cysteine HCl	Processing aid	GRAS, 21 CFR §184.1271 (U.S. FDA, 2021)
Cryoprotectant		
Sucrose	Additive	GRAS, FCC Listed Substance
Maltodextrin	Additive	GRAS, 21 CFR §184.1444 (U.S. FDA, 2021)
Sodium chloride	Additive	Food for human consumption
Proline/Arginine	Additive	Food additives permitted for direct addition to food for human consumption 21 CFR §172.320 (U.S. FDA, 2021)
Lyophilization Agent		
Maltodextrin	Additive	GRAS, 21 CFR §184.1444 (U.S. FDA, 2021)
Silicon dioxide	Additive	Food additives permitted for direct addition to food for human consumption 21 CFR §172.480 (U.S. FDA, 2021)
Magnesium stearate	Additive	GRAS, 21 CFR §184.1440 (U.S. FDA, 2021)

CFR = *Code of Federal Regulations*; FCC = *Food Chemicals Codex*; GRAS = Generally Recognized as Safe; HCl = hydrochloric acid; U.S. = United States.

2.2.2 Manufacturing Process

Production of *A. soehngenii* CH106 by fermentation is conducted according to current Good Manufacturing Practice (cGMP) and Hazard Analysis and Critical Control Point conditions. A validated WCB is maintained at the production facility to store inoculum for culture initiation. Briefly, a seed culture is initiated using an aliquot from the WCB, and the seed culture is then used to inoculate the production culture in the fermentation vessel where the product organism is grown; production yield can be scaled up according to demand. Cells are separated from the growth medium and stored in a manner dependent on the final formulation. The storage method does not impact the structure or functionality of the product. The composition of each batch of media prepared is consistent across all levels of culture, from seed culture to production culture. A flow chart of the production process is presented below in Figure 2.2.2-1, with further details of each step of the production process outlined below.

Figure 2.2.2-1 Flow Chart Manufacturing Process for *A. soehngenii* CH106



QC1 = strain identity and purity confirmation by polymerase chain reaction (PCR), media ingredients verified; QC2 = cell count and viability determination; QC3 = viability determination and contamination test; QC4 = contamination test, determination of viability, water activity, and moisture content.

Preparation of Seed Culture

An aliquot of the WCB is thawed at 37°C and used to inoculate freshly prepared culture medium. This culture is incubated at 37°C for 24 hours to generate the seed culture.

Preparation of Pre-Culture

Pre-culture inoculum from the seed culture is added at a ratio of between 1:50 and 1:200, based on fermentation timing, and cells are propagated between 37°C and 39°C until end log-phase, as determined by culture optical density. In general, a lower seed culture optical density requires a higher seeding ratio to maintain cell density, but the ratio and fermentation time do not influence the structure, function, or physical appearance of the final product. For very large-scale production, the preparation of the preculture at a larger scale is repeated.

Initiation of Fermentation Culture

The production fermenter is prepared with fresh media and inoculated with pre-culture at a ratio of between 1:50 and 1:200; the ratio is dictated by fermentation conditions and timing. The culture undergoes fermentation at 37°C to 39°C until harvesting cell density is reached.

Cell-Product Harvest

Optimized cell harvesting conditions are determined by culture optical density and pH. Cells are harvested by pellet centrifugation or crossflow filtration concentration. Cells are washed with an isotonic buffered solution to clear the pellet of all residual medium components and metabolites produced during culture and are dispensed in a cryoprotectant media as a cell suspension. Cryoprotectants and stabilizing components are all food-grade and do not affect the structure or composition of the final product, which is optimized for subsequent lyophilization.

Lyophilization

The cryoprotectant cell suspension is snap frozen and freeze-dried in a lyophilizer. This process results in a cake that is then ground to powder and mixed with maltodextrin-based bulking agent to achieve optimized cell density and conditions for storage. This lyophilized powder is used to prepare the final product.

Quality Control

Throughout the production process, line samples are taken for quality control analysis to ensure process controls in place are met to guarantee optimized fermentation process reproducibility and efficiency. Quality control (QC) steps are defined in Figure 2.2.2-1, above, and involve calibration of all equipment, temperature control, pH monitoring, monitoring of optical density, centrifugation speed, *etc*. Only if QC requirements are met is the production process allowed to move to the next phase.

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

The product specifications for the *A. soehngenii* CH106 ingredient, including physical characteristics and contaminant limits for microbes and heavy metals, accompanied by their respective methods of analyses are presented below, in Table 2.3.1-1.

While plate counts resulting in colony forming units (CFU) have long been the golden standard for microbial analysis and are still very useful for enumerating pathogenic or other undesired microbes, for strict anaerobes that are often hard to enumerate reproducibly on plates, other methods have been developed to accurately assess their cell number. Flow cytometry using specific functional dyes has been developed as the method of choice and is used here for the enumeration of *A. soehngenii* CH106 [International Organization for Standardization (ISO) 19344-International Dairy Federation (IDF) 232 – ISO IDF, 2015; Foglia *et al.,* 2020]. This method, which is based on membrane integrity (Nebe-von Caron and Badley, 1995) results in 3 populations that can be detected separately, representing dead cells, damaged cells, and intact cells. The latter group consists of bacteria that are generally able to reproduce and form colonies, but can become dormant, resulting in viable but non culturable cells, and would be missed with traditional plate counts.

In the specific use described here, total cells of *A. soehngenii* is defined as total fluorescent units (TFU) which represents the sum of dead cells and damaged cells (DC) and of intact cells which represent the active cells (*i.e.*, viable cells). The latter is referred to as active fluorescent units (AFU) in the formula TFU = AFU + DC.

CFU values will still be maintained where the method does not involve flow cytometric measurements (*e.g.*, determination of microbiological parameters); however, in the case where amounts are related to safety levels, TFUs are used rather than CFUs. This is considered a valid adaptation as the mentioned CFUs are by definition lower than the administered TFUs. Therefore, the number of CFUs would give an underestimation of the total number of cells that can safely be added to food.

Table 2.3.1-1 Specifications for *A. soehngenii* CH106

Specification Parameter	Limit/Description	Method of Analysis
Physical Parameters		
Appearance	Free flowing powder	Visual inspection
Color	White to off-white	Visual inspection
Odor	Characteristic	Organoleptic
Identity	>98% *A. soehngenii* CH106	16S rRNA sequencing
Moisture content	<7 %	ISTISAN 1996/34 Met B Pag 7
Water activity	<0.15 %	MI_009_2011_Rev0
Total cell count	≥5.0 x 10^9 TFU/g	Flow cytometry, ISO 19344-IDF 232
Viable cell count	≥1.0 x 10^8 AFU/g	Flow cytometry, ISO 19344-IDF 232
Microbiological Parameters		
Aerobic mesophilic bacteria	<5.0 x 10^3 CFU/g	EU PHARMA 01/2021:20612
Yeasts and molds	<100 CFU/g	EU PHARMA 01/2021:20612
Salmonella	Absent in 10 g	EU PHARMA 01/2021: 20612 + 01/2021: 20613 + 01/2014: 20631
Listeria monocytogenes	Absent in 10 g	UNI EN ISO 11290-1:2017
Bacillus cereus	<500 CFU/g	AFNOR BKR 23/06-02/10
Enterobacteriaceae	<100 CFU/g	UNI EN ISO 21528-2:2017/EC1:2018
Staphylococcus aureus	<100 CFU/g	EU PHARMA 01/2021: 20612 + 01/2021: 20613
Sulfite reducing anaerobes	<100 CFU/g	EU PHARMA 7.0/2011 and s.m.i.
Heavy Metal Parameters		
Arsenic	<0.1 ppm	UNI EN 13805:2014 + UNI EN 15763:2010
Cadmium	<0.1 ppm	
Lead	<0.1 ppm	
Mercury	<0.1 ppm	

AFNOR = Association Française de Normalisation; AFU = active fluorescent units; CFU = colony forming units; EU PHARMA = European Pharmacopeia; IDF = International Dairy Federation; ISO = International Organization for Standardization; ppm = parts per million; TFU = total fluorescent units.

Under normal growth conditions and with certified ingredients, the heavy metal content is expected to stay below the indicated values. To assure that this is the case these will be measured intermittently but will not be measured for each individual batch.

2.3.2 Batch Analysis

Analysis of 3 non-consecutive lots of *A. soehngenii* CH106 demonstrates that the manufacturing process as described in Part 3.2 produces a consistent product that meets the defined product specifications. A summary of the analyses for the 3 lots of *A. soehngenii* CH106 is presented below in Table 2.3.2-1.

Table 2.3.2-1 Product Analysis of 3 Non-Consecutive Lots of *A. soehngenii* CH106

Specification Parameter	Limit	Lot Number		
		GBCH106-NL0320	GBCH106-NL0820	GBCH106-IT1021
Appearance	Free flowing powder	Free-flowing powder	Free-flowing powder	Free-flowing powder
Color	White to off white	White	White	Off-white
Odor	Characteristic	Odorless	Odorless	Odorless
Identity	>98% *A. soehngenii* CH106	100	100	100
Moisture content	<7%	2.18	3.5	5.95
Water activity	<0.15%	0.101	0.147	0.04
Total cell count	≥5 x 10^9 TFU/g	6.91 x 10^9	1.18 x 10^{10}	4.56 x 10^{10}
Viable cell count	≥1.0 x 10^8 AFU/g	7.83 x 10^8	4.18 x 10^9	5.53 x 10^8
Microbiological Parameters				
Aerobic mesophilic bacteria	<5.0 x 10^3 CFU/g	<10	<10	180
Yeasts and molds	<100 CFU/g	90	<10	<10
Salmonella	Absent in 10 g	Absent	Absent	Absent
Listeria monocytogenes	Absent in 10 g	Absent	Absent	Absent
Bacillus cereus	<500 CFU/g	<100	<100	<100
Enterobacteriaceae	<100 CFU/g	<10	<10	<10
Staphylococcus aureus	<100 CFU/g	Absent	Absent	Absent
Sulfite reducing anaerobes	<100 CFU/g	<10	<10	<10
Heavy Metals				
Arsenic	<0.1 ppm	0.035	0.014	<0.02
Cadmium	<0.1 ppm	0.011	0.005	0.0125
Lead	<0.1 ppm	0.016	0.07	<0.02
Mercury	<0.1 ppm	<0.001	<0.001	<0.005

AFU = active fluorescent units; CFU = colony forming units; ppm = parts per million; TFU = total fluorescent units.

2.4 Stability

The stability of *A. soehngenii* CH106 is at least 14 months, with a target of 24 months. Shelf-life stability of batch GBCH106-NL0320 and GBCH106-IT1021 was tested on the manufactured product, packed in sealed aluminum bags. Shelf-life stability of batch GBCH106-NL0820 was tested on capsules, containing the manufactured product, in combination with maltodextrin and supplemented with magnesium stearate and silicon dioxide to enhance flowability for capsulation.

Ongoing stability testing is being conducted in climate chambers under 3 different conditions: temperature of 4°C, temperature of 25°C with a relative humidity of 60%, and a temperature of 40°C with a relative humidity of 75%. The results obtained to date demonstrate that the product is stable at 4°C (Table 2.4-1). Stability studies at 25°C are ongoing. Overall limited loss in viability is observed so far (Table 2.4-2). Viability is rapidly lost at 40°C (data not shown). Improvements in the formulation have been made to generate a product with a shelf life of at least 12 months at 25°C, but these have not been incorporated in the current batches. Notably, it has been found that replacement of proline by arginine as a stabilizing agent and use of ultra-dry bulking agent (moisture below 3%) have a major beneficial impact on shelf life at room temperature.

Table 2.4-1 Shelf-Life Stability of *A. soehngenii* CH106 at 4°C

Sample ID	Date of Manufacture	TFU	Original AFU	Recount Date	AFU on Recount Date	SD	%SD	Survival	Time Elapsed (months)
GBCH106-NL0320	06-03-2020	6.91×10^9	7.83×10^8	29-10-2021	5.66×10^8	4.34×10^7	8%	72%	19
GBCH106-NL0820	20-08-2020	1.18×10^{10}	4.18×10^9	29-10-2021	4.66×10^9	6.18×10^8	13%	100%	14
GBCH106-IT1021	01-11-2021	4.56×10^{10}	5.53×10^8	17-11-2021	5.51×10^8	1.84×10^8	33%	99%	0.5

AFU = active fluorescent units; TFU = total fluorescent units; SD = standard deviation.

Table 2.4-2 Shelf-Life Stability of A. soehngenii CH106 at 25°C, 60% RH*

Sample ID	Date of First Measurement	TFU	Original AFU	Recount Date	AFU on Recount Date	Survival	Time Elapsed (months)
GBCH106-NL0320	04-01-2021	6.89×10^9	6.43×10^8	02-06-2021	6.85×10^8	100%	5
GBCH106-NL0820	08-01-2021	1.28×10^{10}	3.46×10^9	11-05-2021	1.76×10^9	51%	4
GBCH106-IT1021	05-11-2021	4.56×10^{10}	5.53×10^8	17-11-2021	5.39×10^8	97%	0.5

AFU = active fluorescent units; TFU = total fluorescent units.
* Stability studies at 25°C were initiated at a later date, starting with material that was stored at 4°C.

Part 3. §170.235 Dietary Exposure

3.1 Estimated Intake of *A. soehngenii* CH 106

3.1.1 Methods

As outlined in Part 1.3, *A. soehngenii* CH106 is intended to be added as a food ingredient to various beverage, cereal and grain, milk, milk analogue, nut, confectionary, and meal replacement products to be marketed in the U.S. The food ingredient is intended to be added at a maximum use level of 1.0×10^{10} TFU/serving across all food categories.

Anticipated intakes of *A. soehngenii* CH106 under the intended conditions of use were evaluated using a serving basis approach. The maximum number of servings of foods containing *A. soehngenii* CH106 that an individual may consume in a day as determined based on the intended conditions of use of *A. soehngenii* CH106 and data from the U.S. Department of Agriculture (USDA) Food Patterns Equivalents Database (FPED) (USDA ARS, 2021a). FPED is based on Day 1 dietary intake data (weighted) from the National Health and Nutrition Survey (NHANES) 2017-2018 for individuals 2 years of age and over (USDA ARS, 2021a). This database converts the foods and beverages in the Food and Nutrient Database for Dietary Studies component of the NHANES survey to the 37 USDA Food Patterns components (USDA ARS, 2021a). The Food Patterns are measured as "cup equivalents" of Fruit, Vegetables, and Dairy; "ounce equivalents" of Grains and Protein Foods; "teaspoon equivalents" of Added Sugars; "gram equivalents" of Solid Fats and Oils; and the "number" of Alcoholic Drinks (USDA ARS, 2021b). For the purposes of the current assessment, each of these unit "equivalents" were considered to be equal to a "serving".

Overall, mean amounts of Food Pattern Equivalents for total grains, nuts and seeds, fluid milk, and yogurt were utilized to determine the number of daily servings of foods in which *A. soehngenii* CH106 is proposed to be used (UDSA ARS, 2021a). The number of servings for each food group were summed to determine the total number that may be consumed in a day in various U.S. population groups. No representative food components of chocolate or sport, electrolyte, and fluid replacement drinks were available from the FPED. Nevertheless, the maximum number of servings derived using this approach is considered suitably conservative as all grains, nuts and seeds, fluid milk, and yogurt were assumed to contain *A. soehngenii* CH106 at the proposed use level of 1.0×10^{10} TFU/serving.

3.1.2 Results

3.1.2.1 Number of Servings

The maximum number of servings of foods containing *A. soehngenii* CH106 consumed in a day under the proposed food uses, determined using data from the USDA FPED 2017-2018, are presented by U.S. population group in Table 3.1.2.1-1. Males aged 12 to 19, and between 30 and 59 years were determined to consume the greatest number of combined servings of "food components", at 10 servings/day. The lowest number of servings consumed among all food components evaluated combined was of 6 servings/day in females 2 to 5 years of age.

It is highly unlikely that *A. soehngenii* CH106 would be consumed by an individual from all of the proposed uses (*i.e.*, grains, nuts and seeds, fluid milk, and yogurt) in 1 day; as such, these are considered to be highly conservative estimates.

Table 3.1.2.1-1 Maximum Number of Servings of Foods Containing *A. soehngenii* CH106 Consumed in a Day Under the Proposed Food Uses by U.S. Population Group (USDA FPED 2017-2018)

Age Group (Years)	Servings/Day[a]				
	Total Grains	Protein (Nuts and Seeds)	Dairy (Fluid Milk)	Dairy (Yogurt)	Total
Males					
2 to 5	5.53	0.42	1.33	0.10	**7**
6 to 11	7.57	0.50	1.14	0.06	**9**
12 to 19	8.12	0.43	1.05	0.03*	**10**
20 to 29	7.94	0.60*	0.70	0.04*	**9**
30 to 39	8.55	0.87	0.56	0.06*	**10**
40 to 49	7.82	0.99	0.61	0.08*	**10**
50 to 59	7.71	1.03	0.71	0.05*	**10**
60 to 69	7.53	0.86	0.57	0.04*	**9**
≥70	6.28	0.95	0.93	0.06	**8**
2 to 19	7.39	0.45	1.14	0.05	**9**
≥20	7.7	0.88	0.67	0.06	**9**
≥2	7.62	0.77	0.79	0.05	**9**
Females					
2 to 5	4.63	0.40	1.20	0.11	**6**
6 to 11	6.74	0.37	1.03	0.09	**8**
12 to 19	6.28	0.46	0.61	0.03	**7**
20 to 29	6.13	0.54	0.51	0.03	**7**
30 to 39	6	0.63	0.46	0.06	**7**
40 to 49	5.54	0.61	0.49	0.08	**7**
50 to 59	5.47	0.75	0.52	0.08	**7**
60 to 69	5.18	1.20	0.50	0.10	**7**
≥70	5.02	0.82	0.65	0.06	**7**
2 to 19	6.08	0.42	0.88	0.07	**7**
≥20	5.58	0.75	0.52	0.07	**7**
≥2	5.69	0.67	0.60	0.07	**7**
Females and Males					
2 to 19	6.75	0.43	1.01	0.06	**8**
≥20	6.6	0.81	0.59	0.06	**8**
≥2	6.64	0.72	0.69	0.06	**8**

Table 3.1.2.1-1 Maximum Number of Servings of Foods Containing *A. soehngenii* CH106 Consumed in a Day Under the Proposed Food Uses by U.S. Population Group (USDA FPED 2017-2018)

Age Group (Years)	Servings/Day[a]				
	Total Grains	Protein (Nuts and Seeds)	Dairy (Fluid Milk)	Dairy (Yogurt)	Total

FPED = Food Patterns Equivalents Database; NHNAES = National Health and Nutrition Examination Survey; U.S. = United States; USDA = United Stated Department of Agriculture.
*Indicates an estimate with a relative standard error >30%.
[a] The total number of servings of foods potentially containing *A. soehngenii* CH106 consumed per day was determined using the USDA FPED 2017-2018. This database provides individual-based mean Food Patterns Equivalent intakes for 37 USDA Food Patterns Components by gender and age in the U.S. population, based on weighted dietary intake data from Day 1 of the NHANES 2017-2018. The proposed food uses of *A. soehngenii* CH106 were matched as closely as possible to Food Patterns Components. For the purposes of the current assessment, unit equivalents for each of the Components were considered to be equal to a "serving".

3.1.2.2 Intake Estimates for A. soehngenii CH106

The total number of servings for all combined food groups, as presented in Table 3.1.2.1-1, were combined with the proposed use level to determine the estimated daily intake level of *A. soehngenii* CH106 on an absolute basis; data on default body weights were combined with this information in order to calculate the equivalent intakes on a body weight basis. The results are presented in Table 3.1.2.2-1.

At a maximum intended use level of 1.0×10^{10} TFU/serving, the daily intake of *A. soehngenii* CH106 in high-end consumers is estimated to be 1.0×10^{11} TFU/day (see Table 3.1.2.2-1 below). When converted to a body weight basis, the highest estimated daily intake of *A. soehngenii* CH106 in heavy consumers of 5.1×10^{9} TFU/kg body weight/day is calculated for male children 2 to 5 years of age consuming up to 7 servings/day of foods containing the ingredient (assuming a default body weight of 13.8 kg in children 2 years of age). In adults, the estimated daily intake of *A. soehngenii* CH106 in heavy consumers on a body weight basis is calculated to be approximately 1.3×10^{9} TFU/kg body weight/day, assuming the consumption of up to 10 serving/day of foods containing the ingredient in an 80-kg adult.

Table 3.1.2.2-1 Summary of the Estimated Daily Intake of *A. soehngenii* CH106 from Proposed Conditions of Use and Maximum Number of Daily Servings in the U.S. by Population Group

Age Group (Years)	Maximum Intended Use Level of *A. soehngenii* CH106 (TFU/serving)	Absolute Intakes		Body Weight Intakes	
		Maximum Number of Servings/Day (USDA FPED 2017-2018)[a]	Maximum Estimated Daily Intake (TFU/day)[b]	Lowest Default bw (kg) (U.S. EPA, 2011)	Maximum Estimated Daily Intake (TFU/kg bw/day)[c]
Males					
2 to 5	1.0×10^{10}	7	7.0×10^{10}	13.8	5.1×10^{9}
6 to 11	1.0×10^{10}	9	9.0×10^{10}	31.8	2.8×10^{9}
12 to 19	1.0×10^{10}	10	1.0×10^{11}	56.8	1.8×10^{9}
20 to 29	1.0×10^{10}	9	9.0×10^{10}	80.0	1.1×10^{9}
30 to 39	1.0×10^{10}	10	1.0×10^{11}	80.0	1.3×10^{9}
40 to 49	1.0×10^{10}	10	1.0×10^{11}	80.0	1.3×10^{9}
50 to 59	1.0×10^{10}	10	1.0×10^{11}	80.0	1.3×10^{9}

Table 3.1.2.2-1 Summary of the Estimated Daily Intake of *A. soehngenii* CH106 from Proposed Conditions of Use and Maximum Number of Daily Servings in the U.S. by Population Group

Age Group (Years)	Maximum Intended Use Level of *A. soehngenii* CH106 (TFU/serving)	Absolute Intakes		Body Weight Intakes	
		Maximum Number of Servings/Day (USDA FPED 2017-2018)[a]	Maximum Estimated Daily Intake (TFU/day)[b]	Lowest Default bw (kg) (U.S. EPA, 2011)	Maximum Estimated Daily Intake (TFU/kg bw/day)[c]
60 to 69	1.0×10^{10}	9	9.0×10^{10}	80.0	1.1×10^{9}
≥70	1.0×10^{10}	8	8.0×10^{10}	80.0	1.0×10^{9}
2 to 19	1.0×10^{10}	9	9.0×10^{10}	-[d]	-[d]
≥20	1.0×10^{10}	9	9.0×10^{10}	80.0	1.1×10^{9}
≥2	1.0×10^{10}	9	9.0×10^{10}	-[d]	-[d]
Females					
2 to 5	1.0×10^{10}	6	6.0×10^{10}	13.8	4.3×10^{9}
6 to 11	1.0×10^{10}	8	8.0×10^{10}	31.8	2.5×10^{9}
12 to 19	1.0×10^{10}	7	7.0×10^{10}	56.8	1.2×10^{9}
20 to 29	1.0×10^{10}	7	7.0×10^{10}	80.0	8.8×10^{8}
30 to 39	1.0×10^{10}	7	7.0×10^{10}	80.0	8.8×10^{8}
40 to 49	1.0×10^{10}	7	7.0×10^{10}	80.0	8.8×10^{8}
50 to 59	1.0×10^{10}	7	7.0×10^{10}	80.0	8.8×10^{8}
60 to 69	1.0×10^{10}	7	7.0×10^{10}	80.0	8.8×10^{8}
≥70	1.0×10^{10}	7	7.0×10^{10}	80.0	8.8×10^{8}
2 to 19	1.0×10^{10}	7	7.0×10^{10}	-[d]	-[d]
≥20	1.0×10^{10}	7	7.0×10^{10}	80.0	8.8×10^{8}
≥2	1.0×10^{10}	7	7.0×10^{10}	-[d]	-[d]
Females and Males					
2 to 19	1.0×10^{10}	8	8.0×10^{10}	-[d]	-[d]
≥20	1.0×10^{10}	8	8.0×10^{10}	80.0	1.0×10^{9}
≥2	1.0×10^{10}	8	8.0×10^{10}	-[d]	-[d]

AFU = active fluorescent units; bw = body weight; FPED = Food Patterns Equivalents Database; TFU = total fluorescent units; U.S. = United States; USDA = United Stated Department of Agriculture.

[a] See Table 4.3.2-1.

[b] Calculation: Maximum intended use level of *A. soehngenii* CH106 (AFU/serving) * Total number of servings.

[c] Calculation: Maximum estimated daily intake of *A. soehngenii* CH106 (AFU/day) / Lowest default bw (kg).

[d] Not calculated due to the variation in body weights within the broad age range.

3.1.3 Summary and Conclusions

A. soehngenii CH106 is intended to be added as a food ingredient to a range of food and beverage products at a maximum use level of 1.0×10^{10} TFU/serving. Dietary intakes of *A. soehngenii* CH106 were estimated based on the proposed conditions of use in combination with the maximum number of servings of foods containing *A. soehngenii* CH106 consumed in a day (determined to be 10 servings/day in adults based on data from the USDA FPED 2017-2018).

In the assessment, the consumption of 10 servings/day of foods containing *A. soehngenii* CH106 at a maximum use level of 1.0×10^{10} TFU/serving results in the dietary intake of 1.0×10^{11} TFU/day on an absolute basis, equivalent to 1.3×10^{9} TFU/kg body weight/day in an 80-kg adult. On a body weight basis, the highest estimated daily intake of *A. soehngenii* CH106 was 5.1×10^{9} TFU/kg body weight/day, calculated in male children 2 to 5 years of age (7 servings/day; 13.8-kg child 2 years of age). Several conservative assumptions were included in this assessment. For example, in deriving the maximum number of servings of foods containing *A. soehngenii* CH106 consumed in a day, it was assumed that all relevant food components from the FPED (grains, nuts and seeds, fluid milk, and yogurt) contained *A. soehngenii* CH106 at the maximum proposed level of use. As a result, the maximum number of daily servings derived in the current assessment (10 servings/day in adults) is considered highly conservative as it assumes that an individual consumes 10 distinct food products containing this ingredient every day.

Part 4. §170.240 Self-Limiting Levels of Use

No known self-limiting levels of use are associated with *A. soehngenii* CH106.

Part 5. §170.245 Experience Based on Common Use in Food Before 1958

Not applicable.

Part 6. §170.250 Narrative and Safety Information

6.1 Introduction

Currently, there are no federal regulations or guidelines provided by the U.S. FDA specifying requirements for the evaluation of the safe use of microorganisms as food ingredients. The safety evaluation of *A. soehngenii* CH106 therefore was conducted in a manner consistent with up-to-date guidance documents that outline best practices and/or specific jurisdictional requirements for the safety assessment of microorganisms intended for use in food. The guidance documents and expert reviews from regulatory authorities and industry specialists that were utilized in the safety evaluation of *A. soehngenii* CH106 included the European Food Safety Authority (EFSA) Qualified Presumption of Safety guidelines (EFSA, 2007), the guidelines for the Evaluation of Probiotics in Food (FAO/WHO, 2002) issued by the Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food, and the safety decision tree for evaluating microbial cultures intended for human and animal consumption published by Pariza *et al.* (2015).

Caelus has conducted a battery of toxicological studies using *A. soehngenii* CH106, including *in vitro* studies assessing genotoxicity and mutagenicity, and a 90-day toxicology study in rats, to support the safety of *A. soehngenii* CH106. The details of these studies are summarized in Part 5.5. While there are yet no completed clinical trials that have been conducted using *A. soehngenii* CH106 (see 5.6.2), human studies evaluating the safety, colonization, and metabolic impact of the parent strain, *A. soehngenii* L2-7, have been conducted. Since *A. soehngenii* L2-7 only differs from *A. soehngenii* CH106 by the functional TetO gene, and is otherwise phenotypically identical, these human studies using *A. soehngenii* L2-7 are considered relevant to support the safety of *A. soehngenii* CH106, as discussed in Part 6.4.

Additionally, a comprehensive search of the published literature was conducted to identify publications relevant to the safety of *A. soehngenii* or *Eubacterium hallii* (*i.e*., its former name, see Section 2.0), inclusive to 23 February 2021. The search was conducted using the electronic databases Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. Summaries of studies deemed relevant to the safety assessment of *A. soehngenii* CH106 are provided in the relevant sections below.

A. soehngenii is a commensal bacterium present in the human intestinal tract which contributes to metabolic regulation by consuming glucose, acetate, and lactate and producing butyrate and propionate (Engels *et al.,* 2016; Shetty *et al*., 2018). *A. soehngenii* L2-7, the parent strain of *A. soehngenii* CH106, was originally isolated from the feces of a healthy infant (Barcenilla *et al*., 2000; Shetty *et al.,* 2017a,b); however, *A. soehngenii* CH106, the tetracycline-susceptible derivative of strain L2-7, has not been found in the human intestine naturally and is not currently marketed in food or supplement products in any other jurisdiction. It has been reported that *A. soehngenii* represents as much as 3% of total fecal bacteria in healthy individuals (Louis *et al.,* 2010). *A. soehngenii* and related species are obligate anaerobes that are important contributors to the metabolism of simple sugars and are part of complex microbial populations that form inter-organism nutritional webs involved in cross-feeding interactions with other gut microbes (see Belzer *et al.,* 2017). Production of butyrate from acetate and lactate are important metabolic functions of these species, but the amount that is being produced can vary depending on the contribution of precursors from other organisms present in the gut microbiota (Duncan *et al.,* 2004; Moens *et al.,* 2017; Bunesova *et al.,* 2018).

6.2 Absorption, Distribution, Metabolism and Excretion

Strain *A. soehngenii* CH106 is not expected to colonize the gastrointestinal (GI) tract. The mucosa lining the GI tract in healthy individuals is typically impenetrable to bacteria; any microorganisms passing through the gut are not expected to translocate into systemic circulation. It is expected that *A. soehngenii* CH106 will traverse the intestine for ultimate excretion in feces and any non-viable cells are expected to be metabolized by the gut microbiota.

The parent strain of *A. soehngenii* CH106, *A. soehngenii* L2-7, is naturally present in the GI tract as a commensal microbe and is not absorbed from the intestine. *A. soehngenii* L2-7 cells administered to study participants (n=24; overweight/obese, males with metabolic syndrome) survived passage through the digestive tract, and cell counts were elevated from baseline in fecal samples during administration ($p<0.05$), as determined by quantitative polymerase chain reaction (qPCR) analysis of extracted fecal DNA (Gilijamse *et al*., 2020). However, the concentration of *A. soehngenii* L2-7 cells returned to pretreatment levels within 2 weeks of treatment cessation, as measured *via* fecal excretion (see Part 6.4 for details). These data suggest that while *A. soehngenii* L2-7 cells survived passage of the gut, oral consumption of these cells did not impact the abundance of L2-7 cells in the gut post-treatment, and therefore, the colonization of *A. soehngenii* L2-7 in the gut is transient.

These observations are further supported by 2 unpublished studies: 1 in a novel *in vitro* simulated GI tract system, and another a clinical study in healthy adults. The *in vitro* Simulator of the Human Intestinal Microbial Ecosystem (SHIME) was used to evaluate the upper gastrointestinal tract (UGIT) survivability of the CH106 strain under a variety of conditions. These data indicate that *A. soehngenii* CH106 from oral consumption are sufficient for transit through the UGIT and for butyrate production in the small intestine segment of the model. These data concerning the survival and metabolic activity of *A. soehngenii* CH106 in the artificial small intestine system corroborate the clinical findings described by Gilijamse *et al.* (2020), in that *A. soehngenii* CH106 is capable of survival throughout the GI tract and shows metabolic activity.

For the trial described by Gilijamse *et al.* (2020), an extensive metagenome-based analysis was conducted by Clinical Microbiomics (Copenhagen DK) to evaluate the impact of oral consumption of *A. soehngenii* L2-7 on subject microbiome population dynamics. In this 28-day study, subjects (n=9/group) consumed *A. soehngenii* L2-7 in a daily beverage, each group receiving a different dosage (low, 10^7 TFU/day; medium, 10^9 TFU/day; and high, 10^{11} TFU/day). Single-nucleotide variants (SNVs) distinguishing between the administered *A. soehngenii* strain and endogenous *Anaerobutyricum* spp. were identified. From these analyses the authors were able to quantitatively discriminate between administered and endogenous *A. soehngenii* L2-7 at an SNV level and quantitatively estimate the replication activity of *A. soehngenii* in fecal samples as well as determine the ratio between administered and endogenously present *Anaerobutyricum* species. A clear correlation was detected between the dosage level and the ratio of endogenous *Anaerobutyricum* species to the administered *A. soehngenii* L2-7 strain after 4 weeks of administration. The analysis also showed considerable replication activity of the administered *A. soehngenii* L2-7 strain, indicating its metabolic activity. Moreover, 2 weeks after cessation of daily dosing with *A. soehngenii* L2-7 the strain could no longer be detected in fecal samples of study subjects in any of the dosing groups by using a sensitive qPCR approach. This showed that, although the bacteria were metabolically active and showed replication activity, they were not able to permanently colonize the GI tract of the study subjects (Gilijamse *et al*., 2020).

These data demonstrate that *A. soehngenii* L2-7 is capable of surviving transit of the GI tract and that it is metabolically active in producing butyrate, as observed *in vitro*. Since the only major difference between the L2-7 and CH106 strains of *A. soehngenii* is the inactivation/interruption of the TetO gene in the CH106 strain, and they are otherwise phenotypically similar, *A. soehngenii* CH106 is likewise expected to survive passage of the digestive tract and be excreted in the feces.

6.3 Toxicological Studies

6.3.1 Repeat Dose Studies

In a 90-day study by Seegers *et al.* (2021), healthy Wistar [Crl: WI(Han)] rats (n=10/sex/group; 5 to 6 weeks old; M, 224 g ± 20%; F, 157 g ± 20%) were administered *A. soehngenii* CH106 *via* gavage in doses of 0 (control), 8.0 x 10^9 (low-), 4.0 x 10^{10} (mid-), or 2.0 x 10^{11} (high-dose) CFU/animal/day. These doses were equivalent to 3.0 to 4.5 x 10^{10}, 1.5 to 2.2 x 10^{11}, and 7.5 x 10^{11} to 1.1 x 10^{12} CFU/kg body weight/day, respectively, for males and females at the beginning of the trial, and 1.9 to 3.4 x 10^{10}, 9.5 x 10^{10} to 1.7 x 10^{11}, and 4.7 to 8.5 x 10^{11} CFU/kg body weight/day, respectively, for males and females at the end of the trial. The study was conducted in accordance with Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) Test Guidance (TG) No. 408 for a 90-day study, in a GLP-certified facility.

General clinical observations were recorded daily, and more comprehensive clinical observations were conducted weekly throughout the study (*e.g.,* body weight and food consumption); ophthalmic observations were documented at study initiation and again at the end of the dosing period. Animals were subjected to an Irwin test on Study Days 86 and 87 to assess sensory reactivity and motor activity. A functional battery test was conducted on each animal, once prior to study initiation and once after Week 11, to conduct a number of behavioral observations during out-of-cage functional tests. Sanguineous blood samples were collected on Day 90 for analysis of hematological metrics as follows: hematocrit, hemoglobin, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, platelet count, white blood cell count, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and large unstained cells. Biochemical blood parameters measured included: alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, creatinine, total protein, albumin, urea, bilirubin, total bile acids, cholesterol (total, high-density lipoprotein and low-density lipoprotein), triglycerides, glucose, sodium, potassium, prothrombin time, and activated partial thromboplastin time. Urinalyses for specific gravity, nitrite, pH-value, protein, glucose, ketone bodies, urobilinogen, bilirubin, erythrocytes, and leukocytes were conducted on urine samples collected at end of study. At necropsy, organs were weighed and tissue samples were collected for macroscopic examination and histopathology. All study-time measures were compared to control group levels.

No clinical indications of test item-related toxicity were observed during the study. The functional observation battery did not identify any test item-dependent changes in behavior or general ophthalmologic health. Although 2 mortalities were observed on Day 4 and Day 9, they were determined to be due to natural events and gavage dosing error, respectively, and thus they were not considered to be test item-related. No significant effects on food consumption were reported and body weights increased normally over study duration; however, in females a slight but significantly lower body weight change was observed in all dose groups when compared to the control. While this effect was considered to be test-item related, the body weights remained within normal range (Charles River, 2011), and therefore, it was not considered to be adverse.

Animals in the high-dose group exhibited significantly decreased white blood cell counts and animals in all dose groups had significantly increased red blood cell counts, hematocrit, and hemoglobin content compared to control. Additional hematology and biochemistry metrics reported the following significant effects: decreased total bile acids in mid- and high-dose male rats, decreased creatinine levels in mid-dose females, and decreased blood glucose levels in high-dose females. However, the levels of all significant changes reported from hematology and biochemistry analyses remained within generally accepted basal levels, and no related effects were observed upon histochemical analyses post-mortem. No test item-related toxicologically relevant effects were observed during urinalysis, organ weights analysis, macroscopic examination, or histopathology. Based on the results of this study, the no-observed-adverse-effect level (NOAEL) was determined by the authors to be 2.0×10^{11} CFU/day, equivalent to 4.7×10^{11} CFU/kg body weight/day, the highest dose tested in rats.

6.3.2 Genotoxicity and Mutagenicity

Bacterial Reverse Mutation Assay

A bacterial reverse mutation assay was conducted with *A. soehngenii* CH106 using 4 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537) and 1 strain of *Escherichia coli* WP2 uvrA, in accordance with OECD Guideline for Testing of Chemicals (Sect. 4, No. 471) (Seegers *et al.,* 2021). *A. soehngenii* CH106 was tested at concentrations of 0, 31.6, 100, 316, 1,000, 2,500, and 5,000 µg/plate, with and without metabolic activation (±S9 mix), in triplicate. Negative results were reported for *A. soehngenii* CH106 at all concentrations, with and without metabolic activation, and the compound was determined to be non-mutagenic under the conditions of this study.

Micronucleus

An *in vitro* micronucleus assay was conducted in accordance with OECD Guideline for Testing of Chemicals (Sect. 4, No. 487) using human peripheral blood lymphocytes; *A. soehngenii* CH106 was added at doses of 100, 250, 500, 1,000, 2,500, or 5,000 µg/mL with and without metabolic activation (S9 mix) (Seegers *et al.,* 2021). The clastogenic positive controls used were methylmethanesulfonate for +S9 and cyclophosphamide for -S9 conditions, and colchicine was used as an aneugenic positive control for assay in -S9 conditions. Two distinct experimental designs were used in the micronucleus test: Experiment I – 4-hour cell incubation with *A. soehngenii* CH106, ±S9 conditions, followed by incubation with cytochalasin B for 42 hours; and Experiment II – 1-hour cell incubation with *A. soehngenii* CH106, -S9 conditions, followed incubation with cytochalasin B for 43 hours. Experiment II was conducted after Experiment I, and only on the condition that negative or equivocal results were obtained from Experiment I. The results demonstrate that *A. soehngenii* CH106 did not induce structural or numerical chromosomal damage in human lymphocytes. Therefore, *A. soehngenii* CH106 is considered to be non-mutagenic based on the conditions of this study.

Taken together, the results of the repeat dose studies and the genotoxicity and mutagenicity studies show that the product is non-toxicogenic.

6.4 Human Studies

6.4.1 Studies Conducted with *A. soehngenii* L2-7

Gilijamse *et al.* (2020) conducted a single blind phase I/II dose range-finding efficacy study to determine a level of dietary intake of *A. soehngenii* L2-7 that would attenuate peripheral insulin insensitivity, in which subjects were administered 1.0 x 10^7 (low-dose), 1.0 x 10^9 (mid-dose), or 1.0 x 10^{11} (high-dose) CFU/day of *A. soehngenii* L2-7 for 4 weeks. Viability tests were performed at the start and at regular intervals during the trial. At the time, viability was measured by a method that is referred to as Most Probable Number (MPN). Briefly this method involves a serial dilution of the original sample to a level where growth no longer occurs, *i.e*., to a level where no viable cells are present anymore. The number of cells reported here refers to the live cell count that was established using the MPN method. Similar to the plate count method (CFU) this method only takes viable and culturable cells into account and not viable but non-culturable cells. Generally, the total cell number exceeds the viable cell count by a factor of 1.5. Therefore, the actual dose in TFUs is at least twice the value as indicated in CFU. Safety related endpoints were included as part of this study. Participants [n=24; overweight/obese, insulin-resistant, Caucasian males, 21 to 69 years of age, 25 to 43 kg/m^2 body mass index (BMI)] had blood drawn following overnight fasting periods at treatment initiation and cessation. Blood was analyzed for plasma biochemistry and hematology metrics concerning lipid and glucose metabolism, inflammation, hepatic enzymes, and renal function. The presence of *A. soehngenii* L2-7 in feces was detected and quantified using shotgun metagenomic screening of DNA isolated from fecal samples collected at baseline, after 4 weeks of treatment, and 2 weeks post-treatment (*i.e.,* 6 weeks following treatment initiation).

No serious adverse reactions or side effects were reported at any dose or any timepoint during the study. A significant decrease in hemoglobin was reported in the high-dose group but was determined to be "clinically insignificant" by Gilijamse *et al.* (2020) because the values were still within normal expected physiological range. There were no changes in hematology, hepatic or renal metrics, cholesterol, and inflammatory markers across all dose groups, as compared to baseline. From the data presented in this study, it can be concluded that *A. soehngenii* L2-7 was well-tolerated at intakes of up to 1.0 x 10^{11} CFU/day for 4 weeks.

A second study with *A. soehngenii* L2-7 involved a single intake of 1.0 x 10^{11} cells/day administered directly to the duodenum, and aimed at studying the effects on duodenal transcriptome profiles and metabolic responses to better understand the mode of action (Koopen *et al*., 2021). This study was a phase II randomized double-blind placebo-controlled cross-over study in 12 male adults with metabolic syndrome. The study authors reported that infusion of *A. soehngenii* L2-7 was well-tolerated and no (severe) adverse events occurred during the entire study. As well, safety laboratory parameters (inflammatory, kidney, and liver parameters) were determined to be stable during the study. Energy and macronutrient intake did not differ in the week after administration and no differences in body weight, blood pressure, glucose, insulin, homeostatic model assessment of insulin resistance (HOMA-IR), or serum cholesterol, compared to controls.

Perraudeau *et al*. (2020) conducted a multicenter, double-blind, randomized, placebo-controlled study in which a combination product, containing a close relative to *A. soehngenii* L2-7 (referred to as *Anaerobutyricum hallii* in the publication*)*, was administered to subjects with type-2 diabetes (inclusion criteria: treated with diet and exercise, and/or metformin, and/or sulfonylurea; stable blood glucose ≥3 months) for 12 weeks to investigate the effects of butyrate-producing microorganisms on gut health, glucose homeostasis, and several metabolic and inflammatory markers. Subjects were distributed among 3 different formulation groups and 1 control group; this summary will focus only on the group that received the formulation containing *A. soehngenii*, group WBF-011 (n=23; 56.5% female, 51.3 ± 1.7 years old,

BMI 31.9 ± 1 kg/m^2). Findings in the WBF-011 group were compared to the placebo group (n=26; 57.7% female, 53.7 ± 1.5 years old, BMI 33.4 ± 1.1 kg/m^2). Subjects consumed the formulation (*i.e.,* WBF-011) orally twice daily for 12 weeks. The daily dose of WBF-011 contained inulin, *A. hallii* (9.0 x 10^8 CFU), *Akkermansia muciniphila* (3.3 x 10^9 CFU)*, Clostridium beijerinckii* (1.6 x 10^{10} CFU)*, Clostridium butyricum* (2.0 x 10^9 CFU)*,* and *Bifidobacterium infantis* (1.2 x 10^9 CFU). Subjects began the study treatment phase following an 8-hour fast where samples were collected, and clinical observations were made (baseline); this fast and observe protocol was repeated on Week 4, Week 12 (end of dosing period), and Week 16 (washout period). Observations included fasting glucose and insulin, as measured during the meal-tolerance test. The authors reported that there were no statistically significant changes in the metabolic or inflammatory markers, such as C-reactive protein, interleukin-10, and tumor necrosis factor *alpha* (TNFα), evaluated in this study between placebo and WBF-011, and that WBF-011 was well-tolerated by test subjects; only minor GI symptoms (diarrhea, nausea, and vomiting) were reported. Based on these results, it can be concluded that oral consumption of the *A. soehngenii* relative *A. hallii* at 9.0 x 10^8 CFU/day is well-tolerated.

6.5 Assessment for Virulence Potential and Antibiotic Resistance

6.5.1 Bioinformatic Analyses

Analysis of the *A. soehngenii* CH106 genome was conducted to screen for potential antibiotic resistance and virulence factor genes using the homology search tools Resfinder and Virulence Factor DataBase (VFDB), respectively. A summary of the results is provided below.

Resfinder v. 28.10.2020 was used to identify potential antimicrobial resistance genes, both acquired and novel, by comparison across 3 gene resistance databases present in ResFams (v.1.2): Comprehensive Antibiotic Resistance Database (CARD), Lactamase Engineering Database (LacED), and Jacoby and Bush's collection of curated β-lactamase proteins (www.dantaslab.org/resfams). To identify antimicrobial resistance sequence matches, thresholds of >60% coverage and >70% identity were applied based on relevant EFSA guidance (EFSA, 2021). Although 9 genes with antibiotic resistance properties were identified based on low E-values (<1 x 10^{-4}), only 1 gene met the threshold qualifications of >60% coverage and >70% identity. The single qualifying gene, AS_CH106_02437, was a significant match to the tetracycline resistance gene TetO. However, as discussed in Part 2.0, in *A. soehngenii* CH106 this gene contains a mutation (G insertion, pos. 2648641) that produces a frame shift introducing an early stop codon in the coding region of the gene. Of the remaining 8 genes identified with low E-values, 4 were adenosine 5'-triphosphate (ATP)-binding cassette transporters, 2 were transcriptional regulatory proteins that are part of the vancomycin resistance gene cluster, and 1 was loosely connected to a β-lactamase (no % identity provided). Although coverage was >89% for all 8 genes, identity was low in all cases, ranging between 29.0 to 38.2%. Based on this assessment, it was concluded that none of the identified genes in the *A. soehngenii* CH106 genome are antimicrobial resistance risk factors.

The VFDB is an open source "reference database for bacterial virulence factors" that has been frequently updated since the database's assembly (Chen *et al.,* 2005). The *A. soehngenii* CH106 genome was screened against the most up to date version of the VFDB as of 06 November 2020 and thresholds of >60% coverage and >80% identity for virulence factors identity were applied based on relevant EFSA guidance (EFSA, 2021). There were no genes with >60% coverage or >80% identity to known virulence factors identified in the *A. soehngenii* CH106 genome.

From these bioinformatic analyses of the *A. soehngenii* CH106 genome, it was determined that the strain does not possess any gene sequences indicative of antibiotic resistance or virulence factors. Furthermore, the prophage-like regions that may be associated with horizontal gene transfer in bacteria, which were identified in the *A. soehngenii* CH106 genome, were confirmed to not contain antibiotic resistance genes or virulence factors.

The bioinformatic analyses, combined with the results from human studies, confirms that *A. soehngenii* is a non-pathogenic bacterium.

6.5.2 Minimum Inhibitory Concentration Evaluation of Clinically Important Antibiotics

Assessing the resistance of microbial cultures intended for human consumption to antimicrobial drugs, a characteristic that is largely strain-specific, is necessary to ensure that genetic material conferring antimicrobial resistance is not at risk of being transferred to pathogenic organisms. The potential for genetic material transfer from microbes to other species in the GI tract exists which allows for the possible transfer of antimicrobial resistance factors. The antibiotic susceptibility profile of a microorganism may be characterized using a validated microdilution procedure that determines the MICs for a selection of clinically important antibiotics. Qualification of a microorganism as displaying acquired resistance is then typically determined by comparing the MIC values with established breakpoint values for the species of interest (Klare *et al.,* 2007; Vankerckhoven *et al.,* 2008; EFSA, 2012). The methods reported in ISO 10932:2010 and Clinical and Laboratory Standards Institute (CLSI) M-11 for anaerobic bacteria were employed to assess the susceptibility of *A. soehngenii* CH106 to a selection of 16 antibiotics: ampicillin, penicillin, clindamycin, linezolid, vancomycin, ciprofloxacin, gentamicin, streptomycin, kanamycin, erythromycin, quinupristin-dalfopristin, neomycin, tetracycline, chloramphenicol, rifampicin, and trimethoprim. The concentration range tested for each antibiotic is provided in Table 6.5.2-1. The parent strain *A. soehngenii* L2-7 was included as a comparator and *Eggerthella lenta* DSM 2243 (previously known as *Eubacterium lentum*) was utilized as a positive control. All tests were carried out in duplicate. The MIC results for the antibiotics tested are reported in Table 6.5.2-1.

Table 6.5.2-1 Minimum Inhibitory Concentration Test Results for 16 Antibiotics

Antibiotic	MIC Value (µg/mL)				
	***A. soehngenii* CH106 (test strain)**	***A. soehngenii* L2-7 (comparator)**	***E. lenta* DSM 2243 (positive control)**	**Antibiotic Concentration Range Tested**	**EFSA Cut-Off Values (EFSA, 2012)**
Gentamicin	32	128	16	0.5 to 256	4
Kanamycin	512	512	16	2 to 1,024	16
Streptomycin	>256	>256	>256	0.5 to 256	8
Neomycin	>64	>64	32	0.12 to 64	NR
Tetracycline	0.12	8	0.12	0.12 to 64	2
Erythromycin	0.25	0.25	0.12	0.016 to 8	0.5
Clindamycin	0.03	0.03	0.25	0.03 to 16	0.25
Chloramphenicol	0.5	0.12	4	0.12 to 64	2
Ampicillin	0.25	0.25	0.06	0.03 to 16	1
Penicillin	0.12	0.03	0.12	0.03 to 16	NR
Vancomycin	0.25	0.25	1	0.25 to 128	2
Quinupristin-Dalfopristin	0.12	0.12	2	0.016 to 8	NR
Linezolid	0.5	0.25	0.25	0.03 to 16	NR

Table 6.5.2-1 Minimum Inhibitory Concentration Test Results for 16 Antibiotics

Antibiotic	MIC Value (µg/mL)				
	A. soehngenii CH106 (test strain)	*A. soehngenii* L2-7 (comparator)	*E. lenta* DSM 2243 (positive control)	Antibiotic Concentration Range Tested	EFSA Cut-Off Values (EFSA, 2012)
Trimethoprim	>64	32	>64	0.12 to 64	NR
Ciprofloxacin	16	8	0.5	0.25 to 128	NR
Rifampicin	0.12	0.12	0.12	0.12 to 64	NR

EFSA = European Food Safety Authority; MIC = minimum inhibitory concentration; NR = not required.

The reported MIC values for the test strain *A. soehngenii* CH106 were compared to that of the positive control, *E. lenta* DSM 2243, and the microbiological cut-off values published by EFSA for other Gram-positive species (EFSA, 2012). The observed *E. lenta* DSM 2243 antibiotic sensitivity was in compliance with that published by Gardiner *et al.* (2015). High MIC values for the aminoglycosides gentamycin, kanamycin, streptomycin, and neomycin were reported in all 3 strains, including the reference strain *E. lenta* DSM 2243. To note, these antibiotics mainly act on Gram-negative aerobic bacteria (Dalu, 2005). The observed resistance to the class of aminoglycosides may be considered intrinsic and is not uncommon in anaerobic bacteria given that aminoglycosides require active electron transport for cellular uptake, negatively impacting therapeutic activity in these anaerobic bacteria (Martin *et al.,* 1972; Ramirez and Tolmasky, 2010). *A. soehngenii* CH106 was susceptible to the other EFSA-recommended antibiotics (tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin, and vancomycin) as well as the majority of other antibiotics tested (penicillin, quinupristin-dalfopristin, linezolid, and rifampicin). To note, these data demonstrate that *A. soehngenii* CH106 is susceptible to tetracycline (MIC = 0.12 µg/mL) when compared with the parent strain *A. soehngenii* L2-7 (MIC = 8 µg/mL), confirming the introduction of tetracycline sensitivity into the CH106 strain *via* interruption of the TetO gene by EMS mutation. *A. soehngenii* CH106 demonstrated resistance to trimethoprim (similar to the positive control) and ciprofloxacin (similar to the parent strain); it is well-known that anaerobic bacteria are typically resistant to trimethoprim, and quinolones such as ciprofloxacin are recognized as not active on most anaerobes (Rosenblatt and Stewart, 1974; Appelbaum, 1995; Brook *et al.,* 2013). The observed resistance to trimethoprim and ciprofloxacin may be considered intrinsic and is not uncommon in Gram-positive anaerobic bacteria.

6.6 Allergenicity

To confirm that *A. soehngenii* CH106 would not pose a risk for allergenicity, a genomics and bioinformatics analysis was conducted to evaluate the allergic potential of the proteins present in *A. soehngenii* CH106, most notably, those impacted by amino acid substitutions resultant of the EMS mutation. The GenBank *A. soehngenii* CH106 genome assembly, containing 3,316 protein coding sequences (CDS), was analyzed for potential allergenic proteins using the following sequence homology tools: Allermatch (http://allermatch.org), Allergome (http://allergome.org), Allerbase (http://bioinfo.unipune.ac.in/AllerBase/PHP_codes/igeepi.php), and Immune Epitope Database (IEDB) (http://www.iedb.org). These databases of known allergens were compared to the amino acid sequences of the CDS identified in the CH106 strain genome using basic local alignment search tool (BLAST) algorithms to predict sequence homology. Contiguous protein segments were analyzed for linear epitopes indicative of allergenic peptides (80 AA frame, >35% identity). The potential immunoglobulin E (IgE) cross-reactivity with *A. soehngenii* CH106 proteins was also evaluated. The protein sequences identified in the Allermatch BLASTP search were further analyzed using AllerBase and the Immune Epitope Database (IEDB) to evaluate sequence homology to known allergens. Additionally, the predicted proteins from curated genomes of 23

highly diverse allergenic species from animals, plants, and arthropods, as well as humans, to AOL sequences and compiled identities were used. In the full-length sequence homology search, 179 hits were identified, of which 88 hits had greater than 35% identity; 10 of these hits were unique proteins. The identity of these matches ranged between 36.1 to 53.1%. Next, the proteome was searched using the 80-mer approach, which identified 7,966 hits; 19 hits shared greater than 35% identity, and 9 of these matches were also identified in the full-length search. A 6 amino acid exact match search was also performed. The majority of the identified exact matches were less than 6 amino acids, and 4 matches had an exact match of 7 to 10 amino acids. Upon closer evaluation, the exact matches were to enolase or heat shock proteins. The heat shock protein had a maximum identity score of 68 to 74% (E-value: 10^{-31} to 10^{-36}) in the 80-mer search and 39 to 53% identity in the full length search. The enolase matches shared 63 to 73% identity (E-value: 10^{-33} to 10^{-36}) in the 80-mer search and 48 to 51% identity in the full length search. Taken together, the outcome of the 80-mer search indicates sequence homology to a known allergen; however, when evaluated with the full-length search results, the identity scores of 50% or less suggest that no potential for cross-reactivity exists (Aalberse, 2000). Furthermore, an additional consideration in the cross-reactivity is the protein structure and IgE binding in which no matches were identified in the IEDB search. Taken together, the *in silico* findings indicate that proteins expressed by *A. soehngenii* CH106 do not contain allergenic potential, and would pose a low risk for allergenicity in the final consumer.

Care has been taken to avoid allergenic substances in the production process of *A. soehngenii* CH106. Therefore, the product can be classified as non-allergenic.

6.7 Margin of Safety Estimates

A NOAEL for *A. soehngenii* CH106 of 2.0 x 10^{11} CFU/day, equivalent to 4.7 x 10^{11} CFU/kg body weight/day, was determined based on the highest dose tested in the 90-day study in rats by Seegers *et al.* (2021). As indicated in Part 2.3.1 above, CFUs are by definition lower than the administered TFUs. Therefore, the number of CFUs determined as the NOAEL is an underestimation of the total number of cells that can safely be added to food. Considering this, the NOAEL value of 4.7 x 10^{11} CFU/kg body weight/day is already 2 orders of magnitude higher than the worst-case estimates for dietary intakes of *A. soehngenii* CH106 among heavy consumers (1.3 x 10^{9} TFU[2]/kg body weight/day in an 80-kg adult, 5.1 x 10^{9} TFU/kg body weight/day, in male children 2 to 5 years of age; see Part 3.0).

[2] Total fluorescent units (TFU) represents the sum of dead cells and damaged cells (DC) and of intact cells which represent the active cells.

6.8 Application of the Decision Tree Approach (Pariza *et al.,* 2015)

The decision tree for determining the safety of microbial cultures to be consumed by humans or animals published by Pariza *et al.* (2015) was applied to evaluate the safety of *A. soehngenii* CH106 for human consumption. Based upon safety considerations evaluated under the Pariza decision tree paradigm, the following conclusions on *A. soehngenii* CH106 were noted:

- The phenotypic and genomic identity of *A. soehngenii* CH106 is well-characterized, and no phenotypic or genotypic attributes could be identified to suggest that the strain may display pathogenic or toxicogenic potential.

- *A. soehngenii* CH106 was derived from a species that is a human commensal, and members of this species are expected to be present within the GI tract of humans from birth through adulthood.

- *A. soehngenii* CH106 was without evidence of toxicity in a subchronic oral toxicity evaluation using mature Wistar rats conducted under cGLP and using OECD 408 guidelines.

- *A. soehngenii* CH106 was concluded to be derived from a safe lineage based upon findings reported in human studies of the parent strain *A. soehngenii* L2-7. Based on phenotypic and genotypic characterization of *A. soehngenii* CH106, the GRAS panel concluded that studies conducted using the parent strain were relevant to the safety evaluation of *A. soehngenii* CH106.

Utilization of the Pariza decision tree resulted in the following conclusion regarding *A. soehngenii* CH106: "*The strain is deemed safe for use in the manufacture of food and dietary supplements for human consumption*". The decision tree assessment is as follows:

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? (If YES, go to 2. If NO, the strain must be characterized and unambiguously identified before proceeding).

   Answer: Yes

   Confirmation of the taxonomic identity of A. soehngenii CH106 was accomplished using both pan genome analysis of 10 A. hallii strains plus A. soehngenii L2-7 for comparison, and computation of an ANI score using whole-genome sequence alignment against A. soehngenii L2-7 (ANI = 99.99%)*; both of which confirm the CH106 strain as an A. soehngenii species.*

2. Has the strain genome been sequenced? (If YES, go to 3. If NO, the genome must be sequenced before proceeding to 3.)

   Answer: Yes

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? (If YES, go to 4. If NO, go to 15.)

   Answer: Yes

   The A. soehngenii CH106 genome sequence was searched for genes potentially associated with virulence using VFDB (v.06.11.2020)*. No genes with >60% coverage or >80% identity to known virulence factors were identified in the A. soehngenii CH106 genome.*

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? (If YES, go to 5. If NO, go to 15.)

   Answer: Yes

   Assessment of the A. soehngenii CH106 genome sequence using Resfinder v. 28.10.2020 and MIC testing of 16 antibiotics confirmed that the strain is free of functional and transferable antibiotic resistance gene DNA.

5. Does the strain produce antimicrobial substances? (If NO, go to 6. If YES, go to 15.)

   Answer: No

   The A. soehngenii species is not associated with the production of any known antimicrobial substances used in medical or veterinary medicine.

6. Has the strain been genetically modified using rDNA techniques? (If YES, go to 7a or 7b. If NO, go to 8a or 8b.)

   Answer: No

8a. For strains to be used in human food: Was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an "incidental isolate")? (If YES, go to 9a. If NO, go to 13a.)

   Answer: No

13a. For strains to be used in human food: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? (If YES, go to 15. If NO, go to 14a.)

   Answer: No

   In a 90-day study in rats by Seegers et al. (2021), the NOAEL was determined by the authors to be in excess of 2.0×10^{11} *CFU/day, equivalent to* 4.7×10^{11} *CFU/kg body weight/day, the highest dose tested.*

14a. The strain is deemed to be safe for use in the manufacture of food and dietary supplements for human consumption.

6.9 GRAS Panel Evaluation

Caelus has concluded that *A. soehngenii* CH106 is GRAS for use in non-exempt term infant formula and specified conventional food products, as described in Part 1.3, on the basis of scientific procedures. This GRAS conclusion is based on data generally available in the public domain pertaining to the safety of *A. soehngenii* CH106, as discussed herein, and on consensus among a panel of experts (the GRAS Panel) who are qualified by scientific training and experience to evaluate the safety of food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine); Michael W. Pariza Ph.D. (University of Wisconsin-Madison); and James T. Heimbach Ph.D. (JHeimbach LLC).

The GRAS Panel, convened by Caelus, independently and critically evaluated all data and information presented herein, and also concluded that *A. soehngenii* CH106 is GRAS for use in conventional food products as described in Part 1.3, based on scientific procedures. A summary of data and information reviewed by the GRAS Panel, and evaluation of such data as it pertains to the proposed GRAS uses of *A. soehngenii* CH106, is presented in Appendix A.

6.10 Conclusion

Based on the above data and information presented herein, Caelus has concluded that *A. soehngenii* CH106 is GRAS, on the basis of scientific procedures, for use in food and beverage products as described in Part 1.3. General recognition of Caelus' GRAS conclusion is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified by experience and scientific training, to evaluate the use of *A. soehngenii* CH106 in food, who similarly concluded that the proposed uses of *A. soehngenii* CH106 are GRAS on the basis of scientific procedures.

A. soehngenii CH106 therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the *Code of Federal Regulations*.

Part 7. §170.255 List of Supporting Data and Information

7.1 References

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Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Section Title
170—Food additives	170.3	Definitions
	170.30	Eligibility for classification as generally recognized as safe (GRAS)
	Subpart E (170.203 through 170.285)	Generally Recognized as Safe (GRAS) Notice
172—Food additives permitted for direct addition to food for human consumption	172.320	Amino acids
	172.480	Silicon dioxide
182—Substances generally recognized as safe	182.6285	Dipotassium phosphate
	182.6290	Disodium phosphate
184—Direct food substances affirmed as generally recognized as safe	184.1138	Ammonium chloride
	184.1193	Calcium chloride
	184.1271	L-Cysteine
	184.1440	Magnesium stearate
	184.1443	Magnesium sulfate
	184.1444	Maltodextrin
	184.1553	Peptones
	184.1721	Sodium acetate
	184.1736	Sodium bicarbonate
	184.1983	Bakers yeast extract

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APPENDIX A
GRAS Panel Statement

GRAS Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Use of *Anaerobutyricum soehngenii* CH106 as an Ingredient in Conventional Food and Beverage Products

07 December 2021

INTRODUCTION

At the request of Caelus, a panel of independent scientists, qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients (the GRAS Panel), was convened to conduct a critical and comprehensive evaluation of the available pertinent data and information on *Anaerobutyricum soehngenii* CH106 and to determine whether the intended uses of *A. soehngenii* CH106 in various conventional food and beverage products, as described in Table A-1, are Generally Recognized as Safe (GRAS) based on scientific procedures. For purposes of the GRAS Panel's evaluation, "safe" or "safety" means there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use, as defined by the U.S. Food and Drug Administration (FDA) in 21 CFR §170.3(i) (U.S. FDA, 2021). The GRAS Panel consisted of the below-signed qualified scientific experts: Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine); Michael W. Pariza Ph.D. (University of Wisconsin-Madison), and James T. Heimbach Ph.D. (JHeimbach LLC).

The GRAS Panel was selected and convened in accordance with the U.S. Food and Drug Administration's (FDA's) draft guidance for industry on *Best Practices for Convening a GRAS Panel* (U.S. FDA, 2017). Prior to convening the GRAS Panel, all reasonable efforts were made to identify and select a balanced GRAS Panel with expertise in appropriate scientific disciplines deemed necessary for the safety evaluation of *A. soehngenii* CH106, and efforts were placed on identifying conflicts of interest or relevant appearance issues that would potentially bias the outcome of the deliberations of the GRAS Panel; no such conflicts of interest or appearance of conflicts were identified. The GRAS Panel received reasonable honoraria as compensation for its time, and honoraria provided to the GRAS Panel were not contingent upon the outcome of the GRAS Panel's deliberations.

The GRAS Panel, independently and collectively, critically evaluated a comprehensive package of scientific information and data pertinent to the safety of *A. soehngenii* CH106 that had been compiled from the published literature up to 27 October 2021. This information was presented in a dossier titled "*Generally Recognized as Safe (GRAS) Status of A. soehngenii CH106 for Use in Conventional Food and Beverage Products in the United States*" dated 18 November 2021. The information critically evaluated by the GRAS Panel included information pertaining to the method of manufacture, product specifications and analytical data, the conditions of intended use of *A. soehngenii* CH106, dietary intake estimates for the intended uses, and a comprehensive assessment of the available scientific literature pertaining to the safety of *A. soehngenii* CH106.

Following independent and collaborative critical evaluation of the data and information presented within the GRAS dossier, the GRAS Panel met *via* teleconference on 07 December 2021. At the conclusion of this meeting, the GRAS Panel unanimously agreed that *A. soehngenii* CH106, meeting appropriate food-grade specifications and manufactured in accordance with current Good Manufacturing Practice (cGMP), is GRAS for use as an ingredient in conventional food and beverage products under the proposed conditions of use, as described in Table A-1. The GRAS Panel's conclusion on the GRAS status of *A. soehngenii* CH106 is based on scientific procedures, and a summary of the basis for the GRAS Panel's conclusion is provided below.

CHARACTERIZATION OF *ANAEROBUTYRICUM SOEHNGENII* CH106

The food ingredient that is the subject of this GRAS evaluation is a lyophilized powder preparation of *A. soehngenii* CH106. *A. soehngenii* is a strictly anaerobic, Gram-positive, rod-shaped bacterium belonging to the *Clostridium* cluster XIVa of the Firmicutes phylum that is an abundant intestinal microbe, important to gut microbiome health (Collins *et al.,* 1994; Rajilić-Stojanović and de Vos, 2014; Barcenilla *et al.,* 2000). The *A. soehngenii* species produces butyrate from glucose as well as lactate in the presence of acetate (Flint *et al.,* 2012; Shetty *et al.,* 2017a). *A. soehngenii* CH106 was generated from parent strain, *A. soehngenii* L2-7, and the only phenotypic change was introduction of tetracycline sensitivity. The growth characteristics of the *A. soehngenii* CH106 strain were tested empirically in side-by-side comparisons of the L2-7 and CH106 strains to confirm similarity in nutrient utilization and metabolite production.

The whole genome of *A. soehngenii* L2-7 has been sequenced under the previous name of *Eubacterium hallii* (Shetty *et al*., 2018). The complete genome sequence is stored at GenBank/EMBL-EBI under accession number LT907978 (assembly version EH1). *A. soehngenii* L2-7 is registered at the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 17630. Its 16s rRNA has been fully sequenced and deposited at GenBank (Accession Number AJ270490), as described in Barcenilla *et al*. (2000), Shetty *et al*. (2018), and Sayers *et al.* (2019). Confirmation of the taxonomic identity of *A. soehngenii* CH106 was accomplished using genome analysis of 10 *A. soehngenii* strains, and computation of an ANI score using whole-genome sequence alignment against *A. soehngenii* L2-7 (ANI = 99.99%), both of which confirm the CH106 strain as an *A. soehngenii* species. Based on these data, the GRAS Panel concluded that *A. soehngenii* CH106 has been adequately characterized at the species and strain level.

MANUFACTURING AND SPECIFICATIONS

Production of *A. soehngenii* CH106 by fermentation is conducted according to cGMP and Hazard Analysis and Critical Control Point conditions. A validated WCB is maintained at the production facility to store inoculum for culture initiation. Briefly, a seed culture is initiated using an aliquot from the WCB, the seed culture is then used to inoculate the production culture in the fermentation vessel where the product organism is grown; production yield can be scaled up according to demand. Cells are separated from the growth medium and stored in a manner dependent on final formulation, although storage method does not impact the structure of functionality of the product. The composition of each batch of media prepared is consistent across all levels of culture, from seed culture to production culture. The final *A. soehngenii* CH106 product is a lyophilized powder.

While plate counts resulting in colony forming units (CFU) have long been the gold standard for microbial analysis, flow cytometry was determined to be the appropriate method for the enumeration of *A. soehngenii* CH106 herein. In the specific use, total cells of *A. soehngenii* CH106 was defined as total fluorescent units (TFU) which represents the sum of dead cells and damaged cells (DC) and of intact cells which represent the active cells. The latter are referred to as active fluorescent units (AFU) in the formula

TFU = AFU + DC. As such, product specifications for *A. soehngenii* CH106 were set at not less than 5.0 x 10^9 TFU/g and 1.0 x 10^8 AFU/g of powder. Analyses of the *A. soehngenii* CH106 product from 3 non-consecutive lots confirmed that the ingredient is manufactured in a reproducible manner that is compliant with and meets the established product specifications including cell count and suitable limits on microorganisms and heavy metals. Stability data demonstrate that *A. soehngenii* CH106 is stable for at least 14 months when stored at 4°C.

INTENDED USES AND CONSUMPTION ESTIMATES

A. soehngenii CH106 is intended for use as an ingredient in conventional foods and beverages as outlined in Table A-1, at a maximum use level of 1.0 x 10^{10} TFU/serving across all food categories. Dietary intakes of *A. soehngenii* CH106 were estimated based on the proposed conditions of use in combination with either (i) the intended directions of use of products containing *A. soehngenii* CH106 (up to 3 servings/day—typical scenario) or (ii) the maximum number of servings of foods containing *A. soehngenii* CH106 consumed in a day (determined to be 10 servings/day in adults based on data from the USDA FPED 2017-2018—worst-case scenario).

In the typical scenario assessment, the consumption of 3 servings/day of foods containing *A. soehngenii* CH106 at a maximum use level of 1.0 x 10^{10} TFU/serving results in the dietary intake of 3.0 x 10^{10} TFU/day on an absolute basis. On a body weight basis, using default body weights established by the U.S. EPA for the U.S. population, the estimated daily intake of *A. soehngenii* CH106 is 3.8 x 10^8 TFU/kg body weight/day in an 80 kg adult. Considering all age groups 2 years of age and above, the highest estimated daily intake of *A. soehngenii* CH106 on a body weight basis was of 2.2 x 10^9 TFU/kg body weight/day in children 2 years of age (default body weight of 13.8 kg).

In the worst-case scenario assessment, the consumption of 10 servings/day of foods containing *A. soehngenii* CH106 at a maximum use level of 1.0 x 10^{10} TFU/serving results in the dietary intake of 1.0 x 10^{11} TFU/day on an absolute basis, equivalent to 1.3 x 10^9 CFU/kg body weight/day in an 80-kg adult. On a body weight basis, the highest estimated daily intake of *A. soehngenii* CH106 was 5.1 x 10^9 CFU/kg body weight/day, calculated in male children 2 to 5 years of age (7 servings/day; 13.8 kg child 2 years of age).

DATA PERTAINING TO SAFETY

History of Safe Use

A. soehngenii is a commensal bacterium present in the human intestinal tract which contributes to metabolic regulation by consuming glucose, acetone, and lactate to produce butyrate and propionate in humans (Engels *et al.,* 2016; Shetty *et al*., 2018). *A. soehngenii* L2-7, the parent strain of *A. soehngenii* CH106, was originally isolated from the feces of a healthy infant (Barcenilla *et al*., 2000; Shetty *et al.,* 2017a,b); however, *A. soehngenii* CH106, the tetracycline susceptible derivative of strain L2-7, has not been found in the human intestine naturally and is not currently marketed in food or supplement products in any other jurisdiction.

Metabolic Fate and Colonization

The mucosa lining the gastrointestinal tract in healthy individuals is impenetrable to bacteria, and any microorganisms passing through the gut are not expected to translocate into systemic circulation. Data demonstrate that the parent strain, *A. soehngenii* L2-7, is capable of surviving transit of the gastrointestinal

tract and that it is metabolically active in producing butyrate as observed *in vitro*. Thus, *A. soehngenii* CH106 is likewise expected to survive passage of the digestive tract and be excreted in the feces. Moreover, any colonization of the gut is expected to be transient.

Antibiotic Resistance and Toxigenicity

Analysis of the *A. soehngenii* CH106 genome was conducted to screen for potential antibiotic resistance and virulence factor genes using the homology search tools Resfinder and Virulence Factor DataBase (VFDB), respectively. Resfinder v. 28.10.2020 was used to identify potential antimicrobial resistance genes, both acquired and intrinsic, by comparison across 3 gene resistance databases present in ResFams (v. 1.2): Comprehensive Antibiotic Resistance Database (CARD), Lactamase Engineering Database (LacED), and Jacoby and Bush's collection of curated β-lactamase proteins (www.dantaslab.org/resfams). To identify antimicrobial resistance sequence matches, thresholds of >60% coverage and >70% identity were applied based on relevant European Food Safety Authority (EFSA) guidance (EFSA, 2021). Based on the results of this assessment, it was concluded that the strain is free of functional and transferable antibiotic resistance gene DNA. The VFDB is an open source "reference database for bacterial virulence factors" that has been frequently updated since the database's assembly (Chen *et al.,* 2005). The A. soehngenii CH106 genome sequence was searched for genes potentially associated with virulence using VFDB (v.06.11.2020). No genes with >60% coverage or >80% identity to known virulence factors were identified in the *A. soehngenii* CH106 genome.

The antibiotic susceptibility profile of a microorganism may be characterized using a validated microdilution procedure that determines the minimum inhibitory concentrations (MICs) for a selection of clinically important antibiotics. Qualification of a microorganism as displaying acquired resistance is then typically determined by comparing the MIC values with established breakpoint values for the species of interest (Klare *et al.,* 2007; Vankerckhoven *et al.,* 2008; EFSA, 2012). The methods reported in ISO 10932:2010 and CLSI M-11 for anaerobic bacteria were employed to assess the susceptibility of *A. soehngenii* CH106 to a selection of 16 antibiotics. Results of this assessment further confirmed that *A. soehngenii* CH106 is free of functional and transferable antibiotic resistance gene DNA.

Toxicological Studies

The strain specific safety of *A. soehngenii* CH106 was evaluated in a battery of toxicology studies (standard genotoxicity tests and 90-day repeated dose oral toxicity studies in rats) published by Seegers *et al.* (2021). All strain specific toxicological studies were conducted consistent with OECD GLP and OECD Study specific Guidelines.

A. soehngenii CH106 was not genotoxic in a bacterial reverse mutation assay and was not mutagenic in an *in vitro* micronucleus assay conducted using human peripheral blood lymphocytes.

The 90-day oral toxicology study was conducted in Wistar [Crl:WI (Han)] rats (n=40/sex; 5-6 weeks old), which were administered *A. soehngenii* CH106 *via* gavage in doses of 0 (control), 8.0 x 10^9(low-), 4.0 x 10^{10}(mid-), or 2.0 x 10^{11} (high-dose) CFU/animal/day. These doses were equivalent to 3.0 to 4.5 x 10^{10}, 1.5 to 2.2 x 10^{11}, and 7.5 x 10^{11} to 1.1 x 10^{12} CFU/kg body weight/day for males and females, respectively, at the beginning of the trial and 1.9 to 3.4 x 10^{10}, 9.5 x 10^{10} to 1.7 x 10^{11}, and 4.7 to 8.5 x 10^{11} CFU/kg body weight/day, for males and females, respectively, at the end of the trial. The study authors concluded that there were no test item-related adverse effects observed on mortality, clinical observations, functional observation battery, ophthalmologic health, hematology, clinical biochemistry, organ weights, urinalysis, gross pathology, or histopathology examination. No significant effects on food consumption were reported

and body weights increased normally over study duration; however, in females a slight but significantly lower body weight change was observed in all dose groups when compared to the control. While this effect was considered to be test-item related, the body weights remained within normal range (Charles River, 2011), and therefore, was not considered to be adverse. A no-observed-adverse-effect level (NOAEL) of 2.0 x 10^{11} CFU/day, equivalent to 4.7 x 10^{11} CFU/kg body weight/day, the highest dose tested, was concluded.

Human Studies with *A. soehngenii*

A. soehngenii CH106 was derived from the parent strain L2-7 using chemical mutagenesis procedures for deletion of the tetracyline resistance phenotype from the strain. The GRAS Panel noted that any gene mutations, chromosome rearrangements or epigenetic changes that may have occurred during selective mutagenesis of a pure culture in a closed system are highly unlikely to induce a non-pathogenic microbe to become pathogenic. As discussed by Pariza *et al*. (2015) pathogenicity is a complex process that depends on the acquisition and expression of genes responsible for virulence and toxigenicity, which require selective evolutionary pressures within a host organism (*i.e*., mammalian gastrointestinal tract) and/or acquisition of genes from related pathogenic/virulent donor organisms. These conclusions are further corroborated by observations that L2-7 and CH106 strains display similar growth, nutrient utilization, and metabolite production. The GRAS Panel therefore considered studies conducted in humans using the L2-7 strain as relevant to the safety evaluation of the tetracycline sensitive strain CH106.

Gilijamse *et al.* (2020) conducted a single blind escalated intake study to determine the effect of *A. soehngenii* L2-7 on attenuation of peripheral insulin insensitivity in which subjects were administered 1.0 x 10^7 (low-dose), 1.0 x 10^9 (mid-dose), or 1.0 x 10^{11} (high-dose) CFU/day of *A. soehngenii* L2-7 for 4 weeks. Safety related endpoints were included as part of this study, including clinical measures (*e.g.,* bodyweight, BMI, blood pressure, hematology), GI symptoms (*e.g.,* flatulence, cramps, gastric reflux), as well as functional renal and hepatic parameters (*e.g.,* ALT, AST) and inflammatory markers (*e.g.,* C-reactive protein). Participants [n=24; overweight/obese, insulin-resistant, Caucasian males, 21 to 69 years of age, 25 to 43 kg/m^2 body mass index (BMI)] had blood drawn following overnight fasting periods at treatment initiation and cessation. No serious adverse reactions or side effects were reported at any dose or any timepoint during the study. A significant decrease in hemoglobin was reported in the high dose group but was determined to be "*clinically insignificant*" by Gilijamse *et al.* (2020) because the values were still within normal expected physiological range. There were no changes in hematology, hepatic or renal metrics, cholesterol, and inflammatory markers across all dose groups, as compared to baseline. There were no test article-dependent changes in endogenous glucose production or rate of disposal, therefore neither adipose tissue insulin sensitivity, hepatic insulin sensitivity, or intrahepatic triglyceride were significantly affected at any dose as compared to baseline. The authors reported no significant changes in resting energy expenditure or insulin-mediated lipolysis at any dose. From the data presented in this study, it can be concluded that *A. soehngenii* L2-7 was well tolerated at intakes of up to 1.0 x 10^{11} CFU/day for 4 weeks. The GRAS Panel considered the target population evaluated in this study to be largely representative of the population of consumers likely to consume food products containing *A. soehngenii* CH106 and it therefore was regarded as pivotal to the safety evaluation.

A second study with *A. soehngenii* L2-7 involved a single intake of 1.0 x 10^{11} cells/day directly administered to the duodenum, and measured effects on duodenal transcriptome profiles and metabolic responses (Koopen *et al*., 2021). This study was a randomized double-blind placebo-controlled cross-over study in 12 male adults with metabolic syndrome. The study authors reported that infusion of *A. soehngenii* L2-7 was well tolerated and no (severe) adverse events occurred during the entire study. Safety laboratory parameters (inflammatory, kidney, and liver parameters) were reported to be stable during the study. Energy and macronutrient intake did not differ in the week after administration and no differences in body

weight, blood pressure, glucose, insulin, homeostatic model assessment of insulin resistance (HOMA-IR) or cholesterol levels compared to controls. Based on the acute duration of this study, the GRAS Panel considered findings reported by the authors to be corroborative of safety.

Perraudeau *et al*. (2020) conducted a multicenter, double-blind, randomized, placebo-controlled study in which a combination product (WBF-011) containing *Anaerobutyricum hallii* DSM 3353T – a strain with close phylogenetic relationship to *A. soehngenii* L2-7 (Shetty *et al*., 2018) – was administered to subjects with type-2 diabetes (inclusion criteria: treated with diet and exercise, and/or metformin, and/or sulfonylurea; stable blood glucose ≥3 months) for 12 weeks to investigate the effects of butyrate-producing probiotics on gut health, glucose homeostasis, and several metabolic and inflammatory markers. Subjects consumed the probiotic formulation (*i.e.,* WBF-011) orally twice daily for 12 weeks. WBF-011 contained inulin, *A. soehngenii* (9.0×10^8 CFU), *Akkermansia muciniphila* (3.3×10^9 CFU), *Clostridium beijerinckii* (1.6×10^{10} CFU), *Clostridium butyricum* (2.0×10^9 CFU), and *Bifidobacterium infantis* (1.2×10^9 CFU). Subjects began the study treatment phase following an 8 hour fast where samples were collected, and clinical observations recorded; this fast and observation protocol were repeated on Week 4, Week 12 (end of intervention period), and Week 16 (washout period). Observations included fasting glucose and insulin, as measured during the meal-tolerance test. The authors reported that there were no statistically significant changes in the metabolic or inflammatory markers, such as C-reactive protein, interleukin-10, and TNFα between placebo and WBF-011, and that WBF-011 was well tolerated by test subjects; only minor gastrointestinal symptoms (diarrhea, nausea, and vomiting) were reported and occurred less frequently in the WBF-011 group compared to controls. Under the conditions of this study the related species *A. hallii* was well tolerated at intake levels of 9.0×10^8 CFU/day, providing corroborating information to support the safety of *A. soehngenii* CH106.

The Panel was aware of ongoing research[1] being conducted on *A. soehngenii* CH106; however, this information was not pivotal to the GRAS conclusion and would serve as corroborating evidence of safety once completed.

Allergenicity

Caelus conducted a genomics and bioinformatics study to evaluate the allergic potential of proteins found in *A. soehngenii* CH106. The GenBank *A. soehngenii* CH106 genome assembly, containing 3,316 protein coding sequences (CDS), was analyzed for potential allergenic proteins using the following sequence homology tools: Allermatch, Allergome, Allerbase, and Immune Epitope Database (IEDB). A stepwise approach was conducted using the full length, 80-mer, and 6-mer exact match. In the full-length sequence homology search, 179 hits were identified, of which 88 hits had greater than 35% identity; 10 of these hits were unique proteins. The identity of these matches ranged between 36.1 to 53.1%. Next, the proteome was searched using the 80-mer approach, which identified 7966 hits; 19 hits shared greater than 35% identity, and 9 of these matches were also identified in the full-length search. A 6 amino acid exact match search was also performed. The majority of the identified exact matches were less than 6 amino acids, and 4 matches had an exact match of 7 to 10 amino acids. Upon closer evaluation, the exact matches were to enolase or heat shock proteins. The heat shock protein had a maximum identity score of 68 to 74% (E-value: 10^{-31} to 10^{-36}) in the 80-mer search and 39 to 53% identity in the full-length search. The enolase matches shared 63 to 73% identity (E-value: 10^{-33} to 10^{-36}) in the 80-mer search and 48 to 51% identity in the full-length search. Taken together, the outcome of the 80-mer search indicates sequence homology to a known allergen; however, when evaluated with the full-length search results, the identity scores of 50% or

[1] Efficacy and Safety of 12-weeks Supplementation of Eubacterium Hallii on Insulin Sensitivity and Glycaemic Control - Full Text View - ClinicalTrials.gov

less suggest that no potential for cross-reactivity exists (Aalberse, 2000). Furthermore, an additional consideration in the cross-reactivity is the protein structure and IgE binding in which no matches were identified in the Immune Epitope Database search. The weight of the available evidence from the *in silico* studies indicates that proteins expressed by *A. soehngenii* CH106 do not contain allergenic potential and would pose a low risk for allergenicity in the final consumer.

Margin of Safety Estimates

A NOAEL for *A. soehngenii* CH106 of 2.0 x 10^{11} CFU/day, equivalent to 4.7 x 10^{11} CFU/kg body weight/day, was determined in the 90-day oral toxicity study in rats by Seegers *et al.* (2021). This NOAEL value of 4.7 x 10^{11} CFU/kg body weight is two orders of magnitude higher than the worst-case estimates for dietary intakes of *A. soehngenii* CH106 among heavy consumers (1.3 x 10^{9} TFU[2]/kg body weight/day in an 80-kg adult, 5.1 x 10^{9} TFU/kg body weight/day, in male children 2 to 5 years of age).

Application of the Decision Tree Approach (Pariza *et al*., 2015)

The GRAS Panel agreed that available data and information characterizing the identity and hazard of *A. soehngenii* CH106 were suitable for evaluation of safety using the decision tree approach for microbial cultures intended for human and animal consumption (Pariza *et al.,* 2015). Based upon safety considerations evaluated under the Pariza decision tree paradigm, the following conclusions on *A. soehngenii* CH106 were noted:

- The phenotypic and genomic identity of *A. soehngenii* CH106 is well characterized, and no phenotypic or genotypic attributes could be identified to suggest that the strain may display pathogenic or toxicogenic potential.
- *A. soehngenii* CH106 was derived from a species that is a human commensal, and members of this species are expected to be present within the gastrointestinal tract of humans from birth through adulthood.
- *A. soehngenii* CH106 was without evidence of toxicity in a subchronic oral toxicity evaluation using mature Wistar rats conducted under cGLP and using OECD 408 guidelines.
- *A. soehngenii* CH106 was concluded to be derived from a safe lineage based upon findings reported in human studies of the parent strain *A. soehngenii* L2-7. Based on phenotypic and genotypic characterization of *A. soehngenii* CH106, the GRAS panel concluded that studies conducted using the parent strain were relevant to the safety evaluation of *A. soehngenii* CH106.

Utilization of the Pariza decision tree resulted in the following conclusion regarding *A. soehngenii* CH106: "*The strain is deemed safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.*" See Attachment B for the decision tree assessment.

[2] Total fluorescent units (TFU) represents the sum of dead cells and damaged cells (DC) and of intact cells which represent the active cells

CONCLUSION

We, the undersigned independent qualified members of the GRAS Panel, have individually and collectively critically evaluated the data and information summarized above, and other data and information that we deemed pertinent to the safety of the proposed use as an ingredient in select food and beverage products of *Anaerobutyricum soehngenii* CH106.

We unanimously conclude that the proposed use as an ingredient in food and beverage products of Caelus' *Anaerobutyricum soehngenii* CH106, produced in a manner consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications as presented in the supporting dossier "*Generally Recognized as Safe (GRAS) Status of Anaerobutyricum soehngenii* CH106 *for Use in Conventional Food and Beverage Products in the United States*", is safe.

We further unanimously conclude that the proposed use as an ingredient in food and beverage products of Caelus' *Anaerobutyricum soehngenii* CH106, produced in a manner that is consistent with cGMP and meeting appropriate food grade specifications as presented in the supporting dossier, is Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

14 December 2021

Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Virginia Commonwealth University School of Medicine

Date

14 December 2021

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin-Madison

Date

12/14/21

James T. Heimbach, Ph.D.
Heimbach LLC

Date

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ATTACHMENT A: INTENDED FOOD USES AND USE LEVELS FOR *ANAEROBUTYRICUM SOEHNGENII* CH106 IN THE UNITED STATES

Table A-1 Summary of the Individual Proposed Food Uses and Use Levels for *A. soehngenii* CH106 in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2021)	Food Uses*	Maximum Intended Use Level (TFU/serving)
Beverages and Beverage Bases	Sport or Electrolyte Drinks, Fluid Replacement Drinks	1.0×10^{10}
Breakfast Cereals	Ready-to-Eat Breakfast Cereals	1.0×10^{10}
Dairy Product Analogs	Non-Dairy Yogurts	1.0×10^{10}
Frozen Dairy Desserts	Ice Cream	1.0×10^{10}
Grain Products and Pastas	Cereal and Granola Bars	1.0×10^{10}
	Energy Bars, Protein Bars, and Meal Replacement Bars	1.0×10^{10}
Milk Products	Fermented Milks, Plain	1.0×10^{10}
	Plain or Flavored Yogurt	1.0×10^{10}
Nut and Nut Products	Nut Spreads	1.0×10^{10}
Soft Candy	Chocolate	1.0×10^{10}

CFR = *Code of Federal Regulations*; TFU = total fluorescent units; U.S. = United States.

* *Anaerobutyricum soehngenii* CH106 is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.

ATTACHMENT B: DECISION TREE FOR DETERMINING THE SAFETY OF MICROBIAL CULTURES TO BE CONSUMED BY HUMANS (PARIZA *ET AL.,* 2015)

The decision tree for determining the safety of microbial cultures to be consumed by humans or animals published by Pariza *et al.* (2015) was applied as follows to evaluate the safety of *Anaerobutyricum soehngenii* CH106 for human consumption:

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? (If YES, go to 2. If NO, the strain must be characterized and unambiguously identified before proceeding).

   Answer: Yes

   Confirmation of the taxonomic identity of A. soehngenii CH106 was accomplished using both pan genome analysis of 10 A. hallii strains plus A. soehngenii L2-7 for comparison, and computation of an ANI score using whole-genome sequence alignment against A. soehngenii L2-7 (ANI = 99.99%)*; both of which confirm the CH106 strain as a A. soehngenii species.*

2. Has the strain genome been sequenced? (If YES, go to 3. If NO, the genome must be sequenced before proceeding to 3.)

   Answer: Yes

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? (If YES, go to 4. If NO, go to 15.)

   Answer: Yes

   The A. soehngenii CH106 genome sequence was searched for genes potentially associated with virulence using VFDB (v.06.11.2020)*. No genes with >60% coverage or >80% identity to known virulence factors were identified in the A. soehngenii CH106 genome.*

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? (If YES, go to 5. If NO, go to 15.)

   Answer: Yes

   Assessment of the A. soehngenii CH106 genome sequence using Resfinder v. 28.10.2020 and MIC testing of 16 antibiotics confirmed that the strain is free of functional and transferable antibiotic resistance gene DNA.

5. Does the strain produce antimicrobial substances? (If NO, go to 6. If YES, go to 15.)

   Answer: No

   The A. soehngenii species is not associated with the production of any known antimicrobial substances used in medical or veterinary medicine.

6. Has the strain been genetically modified using rDNA techniques? (If YES, go to 7a or 7b. If NO, go to 8a or 8b.)

Answer: No

8a. For strains to be used in human food: Was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an "incidental isolate")? (If YES, go to 9a. If NO, go to 13a.)

Answer: No

13a. For strains to be used in human food: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? (If YES, go to 15. If NO, go to 14a.)

Answer: No

In a 90-day study in rats by Seegers et al. (2021), the NOAEL was determined by the authors to be in excess of 2.0×10^{11} *CFU/day, equivalent to* 4.7×10^{11} *CFU/kg body weight/day, the highest dose tested.*

14a. The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.

Caelus Pharmaceuticals BV
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3474KG Zegveld, The Netherlands

November 2, 2022

Dear Sir/Madam

In a letter received from the division of Food Ingredients on the GRAS Notification, registered under number GRN 001065, dated October 20, 2022 Caelus was requested to provide a response to a number of questions and comments that were raised. Below we list the questions/comments and provide a response to these.

Questions/Comments Regarding GRN 001065:

1. In Table 1.3-1, you provide maximum use levels of Anaerobutyricum soehngenii "CH106" expressed on the basis of total fluorescent units (TFU)/serving of food. Please specify a serving size for each food category listed in Table 1.3-1 or provide the reference that was used as the basis for determining serving sizes.

Response:
Table 1.3-1 has been updated to include averages serving sizes for each food category, based on the Reference Amounts Customarily Consumed (RACC)[1].

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for *Anaerobutyricum soehngenii* CH106 in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020)	Food Uses*	Average serving size (g/ml) (based on RACC)[a]	Maximum Intended Use Level (TFU/serving)
Beverages and Beverage Bases	Sport or Electrolyte Drinks, Fluid Replacement Drinks	360	1.0×10^{10}
Breakfast Cereals	Ready-to-Eat Breakfast Cereals	40	1.0×10^{10}
Dairy Product Analogs	Non-Dairy Yogurts	170	1.0×10^{10}
Frozen Dairy Desserts	Ice Cream	160	1.0×10^{10}
Grain Products and Pastas	Cereal and Granola Bars	40	1.0×10^{10}
	Energy Bars, Protein Bars, and Meal Replacement Bars	40	1.0×10^{10}
Milk Products	Fermented Milks, Plain	240	1.0×10^{10}
	Plain or Flavored Yogurt	170	1.0×10^{10}
Nut and Nut Products	Nut Spreads	30	1.0×10^{10}
Soft Candy	Chocolate	30	1.0×10^{10}

CFR = Code of Federal Regulations; CFU = colony forming units; RACC= Reference Amounts Customarily Consumed per Eating Occasion; U.S. = United States.
* *A. soehngenii* CH106 is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.
[a] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2021b). https://www.fda.gov/media/102587/download

[1] Reference Amounts Customarily Consumed (RACC): Guidance for Industry: Reference Amounts Customarily Consumed: List of Products for Each Product Category: (fda.gov)

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2. In Table 2.2.1-1, you cite 21 CFR 172.320 to support the regulatory status of the use of proline and arginine as cryoprotectants. We note that 21 CFR 172.320 authorizes the use of these amino acids as nutrients added to food and therefore is not applicable to their use as cryoprotectants. Please address the regulatory status of proline and arginine for the intended use as cryoprotectants. In addition, please clarify whether the cryoprotectants and other residual medium components added during the manufacturing process are present in the final ingredient or if they are removed.

Response:
The FDA does not maintain a specific list of allowed substances for use as cryoprotectants. The process of lyophilization is based on removing liquid. This can have a detrimental effect on live cells such as bacteria. To protect these cells from damage certain substances are added that are then designated as cryoprotectants. L-proline and L-arginine are used to enable the cryopreservation process and have no technical function in the finished ingredient or in foods to which the ingredient is added. From a regulatory perspective, L-proline and L-arginine are considered processing-aids that are used to produce *A. soehngenii* CH106 (*i.e.*, they have no technical function in the food and are present at insignificant levels). Processing-aids used for cryopreservation are selected from the FDA's 'substances added to food (formerly EAFUS)' database and include sucrose, maltodextrin, sodium chloride, L-proline and L-arginine. All cryoprotectants, as well as residual medium components are present in the final ingredient. For this reason, only medium components have been selected that are food grade and non-allergenic.

3. In Table 2.3.1-1, you list the specification parameters and the corresponding analytical methods.

- Please state that all the analytical methods are validated and are fit for their intended uses.
- Please provide the limits of quantitation (LOQ) and limits of detection (LOD) of the methods used to test for heavy metals.

The test facility at which the analyses are carried out has stated that all analytical methods are validated and fit for their intended use.
The methods used for detection of heavy metals (UNI EN 13805:2014 + UNI EN 15763:2010) do not state a limit of detection but do allow the determination of a reliable limit of quantitation. These are as follows:
Arsenic: 0.02 mg/kg
Cadmium: 0.005 mg/kg

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Lead: 0.01 mg/kg
Mercury: 0.001 mg/kg

4. In Table 2.3.2-1, you provide the results from analyses of three non-consecutive batches of A. soehngenii "CH106", including the results for heavy metals. We note that the batch analyses show that the results for mercury are consistently < 0.005 mg/kg; however, the specification limit is < 0.1 mg/kg. We recommend that you lower the specification limit for mercury or justify the need for the specification limit of < 0.1 mg/kg.

Response:
We recognize that the specified limit is higher than what is regularly encountered. Therefore a maximum threshold for mercury of 0.01 mg/kg, 10 x lower than mentioned in the original GRAS notification, is acceptable.

5. In footnote a to Table 3.1.2.2-1, you refer to Table 4.3.4-1 that is not included in GRN 001065. Please clarify.

Response:
This is a typo. The footnote should state "a See Table 3.1.2.1-1".

6. For the administrative record, please confirm that *A. soehngenii* "CH106" is nonpathogenic and non-toxigenic and please briefly discuss (with relevant references, as appropriate) the phenotypic characteristics of *A. soehngenii* "CH106" (e.g., production of antimicrobials, production of secondary metabolites, antimicrobial resistance), and whether these pose a safety concern.

Response:
Caelus confirms that *A. soehngenii* "CH106" is nonpathogenic and non-toxigenic. As stated in section 2.1.2 *A. soehngenii* CH106 is a direct descendant of *A. soehngenii* strain L2-7 which was completely sequenced (Shetty et al., 2017). The genome sequence showed the absence of any virulence, pathogenic and toxicogenic factors. As a result of the presence of the tetO gene strain L2-7 is resistant to tetracycline. Strain CH106 was selected for its tetracycline sensitivity. Sequence analysis confirmed a mutation in the tetO gene, rendering the strain tetracycline sensitive.
A. soehngenii is known for its ability to produce both propionate and butyrate (Engels et al., 2016, Seegers et al., 2021). Both are widely published as nutritive substances that are common to the diet and the presence of these metabolites do not pose a safety concern.

- Shetty et al., *Complete genome sequence of Eubacterium hallii strain L2-7*. Genome announcements. 2017, 5(43):11-12. DOI: 10.1128/genomeA.01167-17
- Engels et al., *The common gut microbe Eubacterium hallii also contributes to intestinal propionate formation*. Front. Microbiol., 2016. 7:1-12. DOI: 10.3389/fmicb.2016.00713
- Seegers et. al, 2021, *Remarkable Metabolic Versatility of the Commensal Bacteria Eubacterium hallii and Intestinimonas butyriciproducens: Potential Next-Generation Therapeutic Microbes.* In: Probiotic Bacteria and Postbiotic Metabolites: Role in Animal and Human Health, Springer Singapore. DOI: 10.1007/978-981-16-0223-8

7. Please confirm whether any raw materials used in the manufacturing process are allergens or derived from major allergens and whether this poses a safety concern.

Response:
Caelus confirms that the raw materials used in the manufacturing process are not derived from a major allergen that is the subject of the Food Allergen Labeling and Consumer Protection Act (FALCPA).

8. For the administrative record, please briefly describe how the stability of *A. soehngenii* "CH106" is ensured.

Response:
A master cell bank is maintained of the original strain A. soehngenii CH106 at two locations. This master cell bank has been verified and tested for purity using strain specific PCR and 16S rRNA sequence analysis. The master cell bank is used for the generation of a working cell bank, which is also verified and tested for purity through strain specific PCR and 16S rRNA sequence analysis.
Each batch is produced from a single inoculum that is taken from the working cell bank. QC analysis of the final product incorporates strain specific PCR and 16S rRNA analysis to verify the strain integrity and purity.

9. On page 12, the notifier describes the harvesting of the cell product and states that "Cells are washed with an isotonic buffered solution to clear the pellet of all residual medium components and metabolites produced during culture…"
However, we note on page 11, the washing step is listed as optional. Please clarify the discrepancy between the notifier's discussion of the washing step on page 12 and the flowchart on page 11.

Response:

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Initially the cells were washed because the resulting powder that was obtained from unwashed cells had a slightly unpleasant odor. Since we found that through encapsulation the odor can be contained this is no longer of concern. Therefore, whenever the product is used in a capsulated format, the washing step is not required. When the powder is used for other, non-capsulated food products, washing will be required.
Residual medium components do not pose a threat as they are all food grade and allergen free (see also response to question 7).

10. On page 14, the notifier lists the specification parameter for *Salmonella* as "Absent in 10 g." For the administrative record, please confirm whether the analytical methods listed under EU PHARMA 01/2021:20612, 01/2021: 20613, and 01/2014: 20631 have been validated for testing a 10 g sample size of *Salmonella*. If they have not, we recommend that *Salmonella* testing be performed on a sample size of 25 g in order to effectively detect whether any *Salmonella* is present in the final ingredient.

Response:
The test facility at which the analyses are carried out has stated that the analytical methods listed under EU PHARMA 01/2021:20612, 01/2021: 20613, and 01/2014: 20631 have been validated for testing a 10 g sample size of *Salmonella*.

11. On page 14, the notifier lists a specification for "sulfite reducing anaerobes." For the administrative record, please discuss why the notifier is testing for "sulfite reducing anaerobes."

Response:
Testing of sulfite reducing bacteria is a standard item for testing of anaerobic bacterial cultures. Although based on ingredients that are used for the manufacturing (*i.e.*, no animal derived components) there are no reasons to believe that these organisms would be present it is nonetheless maintained as a standard test.

12. For the administrative record, please confirm whether *A. soehngenii* "CH106" is capable of DNA transfer to other organisms. On page 30, the notifier describes resistance to the class of aminoglycosides, trimethoprim, and ciprofloxacin as "intrinsic." Please clarify what the notifier means by "intrinsic" (i.e., genome encoded). If the resistance is due to the genome, please clarify if the genes encoding resistance are located near any transposable elements that could transfer to commensal organisms.

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Response:
A. Soehngenii CH106 is not capable of DNA transfer to other organisms.
The only antibiotic resistance gene present on the genome of *A. soehngenii* CH106 is the *tet*O gene, which was rendered inactive. No other antibiotic resistance genes are present on the genome. As a result, there is no risk of transfer of antibiotic resistance. Intrinsic resistance to specific antibiotics refers to resistance due to the nature of the bacterium (Gram positive) and its growth conditions (anaerobic). Aminoglycosides such as gentamycin, kanamycin, streptomycin and neomycin mainly act on Gram negative aerobic bacteria.
An important aspect of intrinsic antibiotic resistance is the presence of efflux pumps in the genome of CH106 that pump out the antibiotics. These pumps are a-specific and are required for growth under natural conditions.

13. On page 11, the notifier states that contamination of the ingredient is tested for in quality control step 3 and quality control step 4 during the manufacturing process. Please describe what steps are taken if contamination is observed during these steps of the manufacturing process.

Response:
Any contaminations that are found in QC step 3 (freezing of cell suspensions) and QC step 4 (lyophilization) and are outside of the specifications will lead to disqualification of that particular batch which will then be destroyed. This will typically involve heat sterilization before disposal as waste.

14. Please confirm the last date on which a literature search pertaining to the safety of *A. soehngenii* "CH106" was performed. On page 23, the notifier states that a comprehensive literature search was done "inclusive to 23 February 2021". If this date is correct, please discuss whether any publications relevant to the safety of the article of commerce have been published in the past 19 months.

Response:
An additional survey was conducted inclusive to October 23, 2022 to search for relevant publications related to the safety of *Anaerobutyricum soehngenii* CH106 and *Anaerobutyricum soehngenii* in general, published after February 2021. Since then, two relevant papers were published. The first paper by Seegers et al. (2021) describes the safety studies that were done to establish the safe use of *Anaerobutyricum soehngenii* CH106 for human consumption. These are the results that are also presented in GRN 1065 (See Section 6.3.1). The second paper by Koopen et al. (2021) describes the effect of a single high dose of *A. soehngenii* L2-7 to the duodenum in metabolic syndrome subjects. This study is also discussed in

GRN 1065 (See Section 6.4.1). Both papers present a positive safety profile for the use of *A. soehngenii*.

- Seegers et al., *Toxicological safety evaluation of live Anaerobutyricum soehngenii strain CH106.* J Appl Toxicol., 2021. 42:244-257 DOI: 10.1002/jat.4207
- Koopen et al., *Duodenal Anaerobutyricum soehngenii infusion stimulates GLP-1 production, ameliorates glycaemic control and beneficially shapes the duodenal transcriptome in metabolic syndrome subjects : a randomised double-blind placebo-controlled cross-over study*. Gut, 2021. DOI: 10.1136/gutjnl-2020-323297

15. In the description of the results of the hematological analysis done in the 90-day oral toxicity study, the notifier states: "Animals in the high-dose group exhibited significantly decreased white blood cell counts and animals in all dose groups had significantly increased red blood cell counts, hematocrit, and hemoglobin content compared to control" (see page 26 of the notice). Please confirm whether these findings were observed in both males and females. FDA notes that the authors of Seegers et. al (2022)[1] show an *increase* in white blood cell counts in high-dose males, but no changes in red blood cell counts, hematocrit and hemoglobin content in any of the male test groups.

[1] Seegers J., et al., *Toxicological safety evaluation of live Anaerobutyricum soehngenii strain CH106.* J Appl Toxicol., 2022. 42:244-257.

Response:
Indeed the observation that the finding that "Animals in the high-dose group exhibited significantly decreased white blood cell counts and animals in all dose groups had significantly increased red blood cell counts, hematocrit, and hemoglobin content compared to control "is not entirely accurate since this relates to the female population only. In Seegers et. al it states, "A statistically significant lower white blood cell count was observed in the female HD group, while females in all groups showed slight but significantly increased red blood cell count, hemoglobin content and hematocrit values". This is in line with the statement in GRN 001065, but is indeed restricted to female animals. It further states that "All values, however, were within historical control range (historical data not shown) and no histopathological observations were made that would raise suspicion in related adverse events."
No significant changes were observed for white and red blood cell counts, nor hematocrit and hemoglobulin for male animals in any dose group.

Ecam Ricert

Mérieux NutriSciences

CAELUS HEALTH

PAASHEUVELWEG N.25

AMSTERDAM 1105 NL

Monte di Malo, November 02nd 2022

We hereby declare that all the analytical methods listed below, that are performed at our facilities have been validated and are suitable for their intended use.

Water activity MI_009_2011_Rev1
Humidity MI_578_2020_Rev0

Total Aerobic mesophilic bacteria count EU PHARMA 01/2021:20612

Enumeration of Yeasts and molds EU PHARMA 01/2021:20612

Detection of Salmonella spp EU PHARMA 01/2021: 20612 +01/2021: 20613 + 01/2014: 20631

Detection of Listeria monocytogenes UNI EN ISO 11290-1:2017

Enumeration of presumptive Bacillus cereus AFNOR BKR 23/06-02/10

Enumeration of Enterobacteriaceae UNI EN ISO 21528-2:2017/EC1:2018

Detection of Staphylococcus aureus EU PHARMA 01/2021: 20612 +01/2021: 20613

Sulfite reducing anaerobes count MI_137_2013_Rev0

Heavy metals (Arsenic, Cadmium, Lead, Mercury) UNI EN 13805:2014 + UNI EN 15763:2010

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Reg. Soc. Trib. di VI 01650050246 - REA di VI-175400

Best regards,

[redacted]

Twelve pages have been removed in accordance with copyright laws. The removed reference citation is:

C. Engels, H. Ruscheweyh, N. Beerenwinkel, et al. " the Common Gut Microbe Eubacterium halli also Contributes to Intestinal Propionate Formation", Frontiers in Microbiology, vol. 7, Article 713, pp. 1-12, 2016.

Eleven pages have been removed in accordance with copyright laws. The removed reference citation is:

A. Koopen, et al., " Duodenal Anaerobutyricum soehngenii infusion stimulates GLP-1 production, ameliorates glycaemic control and beneficially shapes the duodenal transcriptome in metabolic syndrome subjects: a randomised double-blind placebo-controlled cross-over study", Gut, 0, pg 1-11, 2021. doi:10.1136/gutjnl-2020-323297

Thirteen pages have been removed in accordance with copyright laws. The removed reference citation is:

Seegers, J.F.M.L., Bui, T.P.N., de Vos, W.M. (2021). Remarkable Metabolic Versatility of the Commensal Bacteria Eubacterium hallii and Intestinimonas butyriciproducens: Potential Next-Generation Therapeutic Microbes. In: Mojgani, N., Dadar, M. (eds) Probiotic Bacteria and Postbiotic Metabolites: Role in Animal and Human Health. Microorganisms for Sustainability, vol 2. Springer, Singapore. https://doi.org/10.1007/978-981-16-0223-8_5

Fourteen pages have been removed in accordance with copyright laws. The removed reference citation is:

Seegers JFML, Gül IS, Hofkens S, Brosel S, Schreib G, Brenke J, Donath C and de Vos WM, 2022. Toxicological safety evaluation of live Anaerobutyricum soehngenii strain CH106. Journal of Applied Toxicology, 42, 244–257. https://doi.org/10.1002/jat.4207

Two pages have been removed in accordance with copyright laws. The removed reference citation is:

Shetty SA, Ritari J, Paulin L, Smidt H, De Vos WM. 2017. Complete genome sequence of Eubacterium hallii strain L2-7. Genome Announc 5:e01167-17. https://doi.org/10.1128/genomeA.01167-17.

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CAELUS
HEALTH

January 30, 2023

Dear Sir/Madam

In a communication received from the Division of Food Ingredients on the GRAS Notification, registered under number GRN 001065, dated January 23, 2023 Caelus was requested to provide a statement for clarification purposes. These statements are hereby provided.

1. The notifier acknowledges that the authorization under 21 CFR 172.320 of the addition of proline and arginine as nutrients added to food does not apply to their use as cryoprotectant. Therefore, the composition of cryoprotectants will be limited to the use of GRAS ingredients that are commonly used for this purpose such as sucrose, maltodextrin and sodium chloride as mentioned in the notification.
2. This notice specifically refers to the use of the powder form of the ingredient in conventional foods, not for the encapsulated form of the product, intended for use as a dietary supplement.
3. In table 1.3-1 in the amendment of November 2, 2022, the average serving size is listed as "g/ml" (gram per milliliter). For the record we confirm that the unit should be g for solid foods and mL for liquid foods.

Yours sincerely,

Luc Sterkman, MD
CEO Caelus Pharmaceuticals BV

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CAELUS
HEALTH

April 4, 2023

Dear Sir/Madam

Following a previous statement, submitted in January this year, we provide you with an additional statement.

As can be read in the GRAS dossier in the first paragraph of section 6.9, GRAS panel evaluation, where reference is made to a GRAS panel evaluation that was convened by Caelus, it was concluded that "*A. soehngenii CH106 is GRAS for use in non-exempt term infant formula and specified conventional food products, [as described in Part 1.3,] on the basis of scientific procedures*".
Nonetheless we do not intend to use *A. soehngenii* CH106 in infant formula or infant foods.

Therefore, we hereby state that the ingredient is not intended for use in infant formula and infant foods.

Yours sincerely,

Luc Sterkman, MD
CEO Caelus Pharmaceuticals BV