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APPENDIX B: Safety

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Appendices B

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1 Toxicity of the enzyme

1.1 Toxin homology study

The mature *Bifidobacterium bifidum* lactase (CB108 lactase) sequence is given below in FASTA format (**Confidential Commercial Information**).

Please refer to Appendix B Confidential Attachment

The UniProt annotated Protein Knowledge database[1] (http://www.uniprot.org), release 2018_02 of 28 Feb 2018, contains 556,825 reviewed proteins, of which 5865 sequences are manually annotated as toxins and 6313 as venom proteins (http://www.uniprot.org/biocuration_project/Toxins/statistics). These toxin and venom sequences are grouped in the animal toxin database subset (http://www.uniprot.org/program/Toxins).

A BLAST search for homology of the lactase sequence against the complete Uniprot database was performed, with a threshold E-value of 0.1. The majority of matches were beta-galactosidases, with none of the top 1000 database matches being annotated as either toxin or venom.

In addition, a specific BLAST search for homology of the mature lactase sequence was performed against the Uniprot animal toxin database. This yielded no matches. Therefore, the lactase sequence does not share homology with a known toxin or venom sequence.

Please refer to Appendix B1 Toxin Homology Search Results (**Confidential Commercial Information**) submitted separately as in the excel file for detailed analysis results.

1.2 Safe Strain Lineage concept

The Safe Strain Lineage concept has been discussed by Pariza and Johnson (2001) in their publication on the safety of food enzymes and is commonly utilized by enzyme companies in the determination of the safety of their products for specific uses, as appropriate.

The primary issue in evaluating the safety of a production strain is its toxigenic potential, specifically the possible synthesis by the production strain of toxins that are active via the oral route. The toxigenic potential of the production organism is confined to the TOS originating from the fermentation.

As the toxicological evaluation is based on the TOS originating from fermentation of the production organism, studies conducted on strains from the Safe Strain Lineage can support other production strains pertaining to this same Safe Strain Lineage.

DuPont has determined by scientific procedures that production organism *Bacillus subtilis* CB108 is safe as a production organism as it pertains to the DuPont *Bacillus subtilis* Safe Strain Lineage (see Appendix B2, B3). A review of toxicology studies conducted with enzyme preparations produced by *Bacillus subtilis* strains indicates that, regardless of the *Bacillus subtilis* production strain, all enzyme preparations are not mutagenic, clastogenic or aneugenic in genotoxicity assays and do not adversely affect any specific target organ. Due to the consistency of the findings from enzyme preparations derived from different

[1] The Uniprot Consortium (2017) Uniprot: The universal protein knowledgebase. Nucleic Cids Res. 45: D158-D169.

Bacillus subtilis strains within the lineage, it is expected that any new enzyme preparation produced from *Bacillus subtilis* strains in the lineage would have a similar toxicological profile. The position of the food enzyme object of the current dossier as well as the position of the strain providing the supportive toxicological studies is presented in the DuPont *Bacillus subtilis* Safe Strain Lineage.

1.3 Toxicological testing

Toxicology studies with CB108 lactase produced by *B. subtilis* have not been conducted. Instead, the safety of CB108 Lactase from *B. subtilis* has been assessed using toxicology studies conducted on earlier strains of the DuPont *B. subtilis* Safe Strain Lineage (Appendix B2).

For the determination of the safety of CB108 lactase, we use the results of toxicology studies conducted on a practically identical lactase from *B. bifidum* produced in *B. subtilis* strain (Referred to as BIF917). *B. subtilis* CB108 lactase has essentially the same expression system as used for *B. subtilis* BIF917 lactase, both of which have the same IUBMB No. 3.2.1.23. BIF917 lactase produced in *B. subtilis* received GRAS status (GRN 579) on Nov. 5th, 2015 (Please refer to Appendix C for the FDA no question letter) for use in the production of galacto-oligosaccharide for infant formula and in the production of fresh dairy products.

To assess the safety of CB108 lactase produced by *B. subtilis,* we will refer the studies conducted from BIF917 lactase. The lactase has been subjected to the following toxicology tests:

- 13-week oral (gavage) toxicity in CD rats
- Acute Oral LD_{50} (limit test) in rats
- *In vitro* Chromosomal Aberration Study with human peripheral blood lymphocytes
- Bacterial reverse mutation assay (Ames assay)

All safety studies were conducted in accordance with internationally accepted guidelines (OECD) and are in compliance with the principles of Good Laboratory Practices ("GLP") according to the FDA/OECD.

Toxicology studies summaries are included below.

1.3.1. Test article description

The test material, Ultra-Filtered Concentrate (UFC), used in all toxicology investigations had the following characteristic (**Confidential Commercial Information**):

Please refer to Appendix B Confidential Attachment.

1.3.2. Toxicological tests

A. Acute Oral Toxicity in Rats – Up and Down Procedure

a. Procedure:

The objective of this study is to determine the oral toxicity of BIF917 lactase given by a single administration using the Up and Down procedure. The study was conducted according to OECD Guideline No. 425 (2008). A single dose of 5000 mg/kg was administered by oral gavage to fasted female rats. Since the first animal survived, two additional animals were dosed simultaneously after a minimum of 48 hours. All rats were observed for mortality, body weight, and clinical signs for 14 days after dosing. All rats were necropsied at the end of the 14 days.

b. Results:

No incidents of mortality, body weight loss, or clinical signs were observed. No gross lesions were noted at necropsy. Under the conditions of this assay, the oral LD_{50} for BIF917 lactase in female rats was greater than 5000 mg/kg bw/day.

c. Evaluation:

According to the provisions of Directive 67/548/EEC amended by Commission Directive 2001/59/EC of August 6, 2001, Annex VI, classification is not required.

According to the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS), under the conditions of this study, classification is not required

B. Bacterial Reverse Mutation Assay – Ames assay

a. Procedure:

The objective of this assay was to assess the potential of BIF917 lactase to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and *Escherichia coli* strain WP2 *uvr*A. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). The assay was performed in two phases using the plate incorporation methodology for the positive control, 2-aminoanthracene, with *E. coli* and the treat and plate methodology for the all remaining strains and assays.

A screening (dose range) test was performed first to select the dose levels for the confirmatory assay. Vehicle control, positive control and 8 doses of the test article were plated, two plates per dose, with overnight cultures of all four strains of *Salmonella typhimurium* and *E. coli* WP2 *uvr*A in the presence and absence of S-9 mix. In the confirmatory assay, 6 doses of the test article along with appropriate vehicle and positive controls were plated in triplicate in the presence and absence of S-9 mix. All dose levels were expressed in terms of total protein (TP). The highest dose level tested was 5000 µg TP/plate, which is the maximum dose required by the OECD guideline. The positive controls used for assays without S-9 mix were 2-nitrofluorene, N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and ICR-191. For assays with S-9 mix, the positive control was 2-aminoanthracene. Vehicle control plates were treated by the addition of sterile deionized water. This assay was conducted in accordance with OECD guideline No. 471 (1997).

b. Results:
In the screening assay, BIF917 lactase was not toxic to the test bacteria up to and including the highest dose level tested (5000 µg TP/plate) in both the absence and presence of S-9 mix. No positive mutagenic responses were observed with any of the tester strains in the presence of S-9 mix or with tester strains TA1535, TA1537 and WP2 *uvr*A in the absence of S-9 mix. Toxicity was observed beginning at 1500 µg TP/plate with strains TA98 and TA 1537 in the absence of S-9 mix. Based on the findings of the screening assay, 5000 µg TP/plate was selected as the highest dose level for the confirmatory assay. In the confirmatory assay, six dose levels (15, 50, 150, 500, 1500, and 5000 µg TP/plate) were tested. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of metabolic activation.

Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

c. Evaluation:

Under the conditions of this assay, BIF917 lactase has not shown any evidence of mutagenic activity in the Ames assay in both presence and absence of metabolic activation.

C. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes

a. Procedure:
The objective of this assay was to investigate the potential of BIF917 lactase to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). In this assay, human lymphocytes were stimulated to divide by the addition of a mitogen (e.g., phytohemagglutinin, PHA). Mitotic activity began at about 40 hours after PHA stimulation and reached a maximum at approximately 3 days.

BIF917 lactase was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and two main assays. Ten concentrations of BIF917 lactase were used in the preliminary assay and at least 4 dose levels were then selected for the definitive assay with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index). Cytotoxicity is characterized by the percentage of mitotic suppression in comparison to the controls. In the absence of cytotoxicity, the highest dose selected would be 5000 µg TP/ml, as recommended by the OECD guideline. All dose levels were expressed in terms of total protein.

In the preliminary assay, all cultures with or without S-9 mix were treated for 4 hours and continuously for 20 hours in the absence of S-9 mix. In the definitive assay, cultures with and without S-9 mix were exposed to the test article for 4 hours, and continuously for 20 hours in the absence of S-9 mix. For the preliminary and the definitive assays, cells were collected 20 hours (1.5 normal cell cycles) after initiation of treatment. Two hours prior to harvest, Colcemid was added to the cultures at a final concentration of 0.1 µg/ml to arrest mitosis.

Cells were collected by centrifugation, treated with 0.075 M KCl, washed with fixative, capped and stored overnight or longer. To prepare slides, the cells were re-suspended in fixative and then collected by centrifugation. The suspension of fixed cells was applied to glass microscope slides and air-dried. The slides were stained with Giemsa, permanently mounted and scored.

i. The mitotic index was recorded as the percentage of cells in mitosis per 500 cells counted. From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main assays.
ii. Metaphase analysis (i.e., evaluation of chromosomal aberration) was conducted on at least 200 metaphases for each dose level (100 per duplicate treatment).
iii. Cells were scored for both chromatid-type and chromosome-type aberrations.
iv. Mitomycin C and cyclophosphamide were used as positive controls for cultures without S9 and cultures with S9, respectively.

This assay was conducted in accordance with OECD guideline No. 473 (1977).

b. Results:
In the preliminary assay, the dose levels ranged from 0.5 to 5000 µg TP/ml. Exposure period was 4 hours for both cultures with and without S9 mix, and continuously for 20 hours in the absence of S-9 mix. All cells were harvested after 20 hours after treatment initiation. Visible precipitation of the test material in the culture medium was observed at 5000 µg TP/mL. At the conclusion of the treatment period, hemolysis was observed at dose levels $\geq$ 1500 µg TP/mL in the S-9 mix activated 4-hour and the non-activated 20-hour treatment groups. Substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was observed at any dose levels $\geq$ 1500 µg/mL in the non-activated 4-hour exposure group and at dose levels $\geq$ 150 µg TP/mL in the non-activated 20-hour exposure group. Based on those findings, dose levels ranging from 100 to 1500 µg/ml were used in the definitive assays for the non-activated 4-hour treatment group, from 2 to 550 µg TP/mL for the activated 4-hour treatment group, and from 10 to 200 µg TP/mL for the non-activated 20-hour treatment group.

In the definitive assay, at the conclusion of the treatment period, precipitate was observed in the S-9 mix activated 4-hour exposure group at dose levels ≥ 450 µg TP/mL and at dose levels ≥ 100 µg TP/mL in the non-activated 20-hour treatment group. Based on these findings, the doses chosen for microscopic analysis ranged from 100 to 500 µg TP/mL for the S-9 mix activated 4-hour exposure group, from 200 to 850 µg TP/mL for the non-activated 4-hour exposure group and from 25 to 100 µg TP/mL for the non-activated 20-hour exposure group.

The test article did not induce any statistically significant increases in the frequency of cells with aberrations in either the presence or absence of S-9 mix. No increase in polyploidy metaphases was noticed. Significant increases in aberrant metaphases were demonstrated with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

c. Evaluation:
Under the conditions of this test, BIF917 lactase did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation. All of the vehicle control cultures had frequencies of cells with chromosomal aberrations within the expected range. The positive control items inducted statistically significant increases in the frequency of cells with aberrations. BIF917 lactase is not considered to be clastogenic.

D. A 90-days Oral Toxicity (Gavage) Study in Wistar Rats (DuPont Haskell, 2014b).

a. Procedure:
The objective of this study was to investigate the potential of BIF917 lactase to induce systemic toxicity after repeated daily oral administration to Crl:CD(SD) rats of both sexes for 90 continuous days. Groups of 10 animals per sex were treated by oral gavage with 0 (deionized water), 100, 300 or 1000 mg total protein (TP)/kg bw/day. The dosing volume was 10 ml/kg bw/day.

Animals of the same sex were housed in groups of two to three in solid floor polypropylene cages with stainless steel mesh lids and softwood bedding (non-aromatic) with access to water via an automatic system and feed *ad libitum*. For environmental enrichment, the animals were provided a supply of wooden chew blocks and cardboard fun tunnels. All groups were housed under controlled temperature, humidity and lighting conditions.

All animals were observed daily for mortality and signs of morbidity. Body weight and feed consumption were recorded weekly. Water consumption was recorded twice weekly for each cage. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study termination. Urinalysis, clinical chemistry and hematology were conducted at study termination. A functional observation

battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 12 for the control and all treated groups. All animals were sacrificed at the end of the 13-week study. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination was initially conducted on selected organs from control and high dose animals.

This study was conducted in accordance with OECD guideline No. 408 (September 1998).

b. Results:

No treatment-related deaths were noted during the 13-week period. One control and one mid dose male died following sublingual bleeding for clinical pathology parameters. One mid dose male was found dead on day 55 but was not considered as treatment related. There were no biological or statistical differences between the control and treated groups with respect to clinical observation, feed consumption, water consumption, ophthalmologic examinations, body weights, and body weight gains. There were no treatment-related changes in hematology and clinical chemistry at the end of week 13. There were no differences in the functional observation battery, grip strength and locomotor activity assays between treated and control animals.

At necropsy, there were no treatment related findings on organ weights, macroscopic findings and histopathologic examinations. All microscopic findings were considered to be within the background incidence of findings reported in this age and strain of laboratory animals.

c. Evaluation and conclusion:

Daily administration of BIF917 lactase by oral gavage to rats at doses of 0, 100, 300 or 1000 mg TP/kg bw/day for 90 consecutive days did not result in treatment-related effects on clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength or locomotor activities. No macroscopic or microscopic changes could be attributed to treatment. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 1000 mg TP/kg bw/day equivalent to 1416.4 mg TOS (total organic solid)/kg bw/day in male and female rats.

CONCLUSION

The safety of BIF917 lactase is assessed in a battery of toxicology studies investigating its genotoxicity and systemic toxicity potential. Under the conditions of the mutagenicity assays BIF917 lactase is not a mutagen or clastogen. Daily administration of BIF917 lactase by gavage for 90 continuous days did not result in overt signs of systemic toxicity.

A NOAEL is established at 1000 mg TP/kg bw/day corresponding to 1416.4 mg TOS/kg bw/day.

For safety evaluation of CB108 lactase from *B. subtilis*, the data obtained with BIF917 lactase from *B. subtilis* with a NOAEL of 1416.4 mg TOS/kg bw/day is used as bridging data.

2 Information on the source micro-organism

The function of the genetic modification is to produce the CB108 lactase enzyme of the donor strain *Bifidobacterium bifidum*, using a known safe *B. subtilis* host strain.

2.1 The production strain

B. subtilis, including genetically engineered strains, is listed as a permitted source for several enzymes, including alpha-amylase, glucanase, protease and xylanase, in Table V of the Food Additive Table of Division 16 of the Canadian Food and Drug Regulations (http://www.hc-sc.gc.ca/fn-an/securit/addit/list/5-enzymes-eng.php). The species *Bacillus subtilis* is an accepted source of enzymes in the literature and pathogenic strains are not described in the Bergey Manual or in the ATCC and other catalogs. The species *Bacillus subtilis* does not appear on the Proposal for a Council Directive amending the "Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agent at work" and is also not present on the European Guideline 93/88/EEG, the list of pathogens from the Dutch Guidelines for Genetically Modified Organisms (COGEM), the German "*Berufsgenossenschaft der chemischen Industrie*", or the Belgian "VLAREM II". *B. subtilis* is accepted as a safe host for the construction of Risk Group I GMMs in several countries, like Germany, The Netherlands, and accepted as a host of certified host-vector systems under the NIH Guidelines in the USA.

2.2 The host

The host strain is *B. subtilis* BG125, a previously described laboratory strain (Dedonder *et al*., 1977) which was obtained as *Bacillus subtilis* strain 1A10 from the Bacillus Genetic Stock Center, Ohio State University, Columbus, Ohio. This strain was developed into a host strain by Genencor International, nowadays part of DuPont Industrial Biosciences. It is derived from the well known *B. subtilis* type strain 168.

2.3 The donor organism

The donor is *Bifidobacterium bifidum.* The strain was not used as such but only the published DNA sequence of the lactase gene was used to synthesize the coding region of interest.

2.4 The vector

The genetic modification of the *B. subtilis* host involved recombinant DNA techniques to introduce multiple copies of the gene, encoding a truncated wild type *Bifidobacterium bifidum*

lactase, into the *B. subtilis* host. The modification employed a method by which a plasmid pUB110 derived expression plasmid, containing the gene and the endogenous alanine racemase gene used as marker gene, is introduced into the host strain. Several genomic genes have been deleted from the host strain as well.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the final construct was verified by Southern blot analysis to confirm that only the intended genetic modifications to the *B. subtilis* strain had been made.

3 Pathogenicity and toxicity of the modified micro-organism

3.1 The production strain

B. subtilis occurs ubiquitous in the environment (soil, water, plants and animals) and as a result can be also found in food (de Boer and Diderichsen, 1991). The bacterium has already been used for decades for the production of food enzymes with no known reports of adverse effects to human health or the environment (de Boer and Diderichsen, 1991). Alpha-amylase enzyme preparation from *B. subtilis* has been used commercially since 1929, when it was used in the manufacture of chocolate syrup to reduce its viscosity (Reed, 1966).

Recently the US Food and Drug Administration reviewed the safe use of food-processing enzymes from recombinant microorganisms, including *B. subtilis* (Olempska-Beer et al. 2006). An extensive risk assessment of *B. subtilis*, including its history of commercial use has been published by the US Environmental Protection Agency (1997). It was concluded that *B. subtilis* is not a human pathogen nor is it toxigenic.

Bacillus subtilis is exempted as a host of certified host-vector systems under the NIH Guidelines in the USA since 1994 (NIH, 2016). The US EPA has added *Bacillus subtilis* to the list of exempted organisms in 1997 (US EPA, 1997). *Bacillus subtilis* is a low-risk-class microorganism, i.e., category 1 of the European Federation of Biotechnology (Frommer *et al.*, 1989), and it can be used under the lowest containment level at large scale, GILSP, as defined by OECD (1992).

JECFA has evaluated food enzymes derived from *B. subtilis*, including some genetically engineered strains, and concluded that these food enzymes do not constitute a toxicological hazard (e.g. JECFA 1972, 1992, 1993, 1998a, 1998b, 1999b, 2004a, 2004b, 2009), as well as other substances like Riboflavin (vitamin B2) (JECFA 1999a). The European Food Safety Agency (EFSA) maintains a list of the biological agents to which the Qualified Presumption of Safety (QPS) assessment can be applied. In 2007, the Scientific Committee set out the overall approach to be followed and established the first list of the biological agents. The QPS list is reviewed and updated annually by the Panel on Biological Hazards (BIOHAZ). If a defined taxonomic unit does not raise safety concerns or if any possible concerns can be excluded, the QPS approach can be applied and the taxonomic unit can be recommended to be included in the QPS list. The safety of *B. subtilis* as a production organism has been assessed by EFSA and was accorded QPS status in 2007

(http://www.efsa.europa.eu/en/topics/topic/qps.htm?wtrl=01) provided the qualification requirements are met (EFSA, 2007). For *Bacillus* strains the specific requirement is absence of toxigenic activity.

Numerous oral toxicity, mutagenicity and carcinogenicity studies using enzyme products from *B. subtilis* 168-derived strains have been performed, and no evidence of a toxic or mutagenic effect has been observed.

3.2 The donor

The donor is *Bifidobacterium bifidum.*

No pathogenic strains of *Bifidobacterium bifidum* are described in the Bergey Manual or in the ATCC and other catalogues. The species *B. bifidum* does not appear on the Proposal for a Council Directive amending the "Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agent at work" and is also not present on the European Guideline 93/88/EEG, the list of pathogens from the Dutch Guidelines for Genetically Modified Organisms (COGEM), the German "Berufsgenossenschaft der chemischen Industrie", or the Belgian "VLAREM II". *B. bifidum* is accepted as a safe host for the construction of Risk Group I GMMs in several countries, like Germany.

Bifidobacterium spp. are classified as Risk Group 1 microorganisms, with the exeption of *B. dentium* (previously called *Actinomyces eriksonii*) which is classified as a human pathogen of Risk Group 2. The safety of *B. bifidum* has been assessed by EFSA and been accorded QPS status without any qualification requirements (see http://www.efsa.europa.eu/en/topics/topic/qps.htm?wtrl=01).

A limited search in PubMed using "bifidum pathogenicity safety" as keywords yielded only one result, an article demonstrating that a particular *Bifidobacterium bifidum* strain can be considered a probiotic able to inhibit the pathogenic bacterium *Helicobacter pylori*.

Indeed searching for "bifidum" alone mainly yields results on Bifidobacteria due to their promising health-promoting properties.

3.3 The host

Bacillus subtilis has a long history of safe use in industrial-scale enzyme production. The long industrial use and wide distribution of *Bacillus subtilis* in nature has never led to any pathogenic symptoms. Moreover, no case demonstrating invasive properties of the species has been found in the literature.

During recent years, genetic engineering techniques have been used to improve the industrial production strains of *Bacillus subtilis* and considerable experience on the safe use of recombinant *Bacillus subtilis* strains at industrial scale has accumulated.

Secondary metabolites are of no safety concern in fermentation products derived from industrial *Bacillus subtilis*. In addition, food enzymes from *Bacillus subtilis* have been subjected to significant number of toxicological tests (including 90-days toxicological tests), as part of their safety assessment for the use in food products manufacturing processes. These studies show that there is no need for concern with fermentation products as produced by use of *Bacillus subtilis.*

Therefore, *Bacillus subtilis* can be considered generally safe not only as a production organism of its natural enzymes, but also as a safe host for other safe gene products.

3.4 Allergenicity of CB108 lactase

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (Amfep) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that the enzyme residues in bread and other foods do not represent any unacceptable risk to consumers.

Despite this lack of general concern, the potential that CB108 lactase could be a food allergen was assessed by comparison with sequences of known allergens and is summarized here. The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.* (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics *et al.* (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores ($<10^{-7}$) may suggest a biologically relevant similarity (i.e., in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

> The Codex Commission states:
>
> "A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

The mature *Bifidobacterium bifidum* lactase sequence is given above in Section 1.1 above.

A full-length sequence alignment against known allergens in the Food Allergy Research and Resource Program (FARRP) AllergenOnline database[2], February 1, 2018 V18A, containing 2093 peer-reviewed allergen sequences listed in the database[3] (using E-value <0.1) yielded no matches.

The search for 80-amino acid stretches within the sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database (February 1, 2018 V18A[4]), identified one match, a pollen sequence from the Sycamore tree *Platanus orientalis*. However, the full alignment generated a large E-value of 1.2×10^3, rendering the match insignificant. The insignificance of the match is further discussed in Appendix B5. supported by the following weight-of-evidence data:

- The Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of food enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residues in bread and other foods do not represent any unacceptable risk to consumers (Dauvrin *et al*., 1998). The allergenicity potential of food enzymes was further studied by Bindslev-Jensen *et al.* (2006) and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*". The investigation comprised enzymes produced by wild-type and GE strains as well as wild-type enzymes and protein engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern regarding food allergy.
- Enzymes, including lactase, when used as digestive aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more). Wüthrich (1996) published a list of enzymes used as digestive aids and concluded that they are not potent allergens by ingestion.
- Food enzymes are used in small amounts (i.e., at ppm levels) during food processing, resulting in very small amounts of the enzyme protein in the final food. Additionally, any residual enzyme proteins still present in the final food will be subjected to denaturation due to heating and subsequent digestion in the gastro-intestinal tract, further reducing the levels of enzyme. A high concentration of

[2] http://www.allergenonline.org/index.shtml
[3] http://www.allergenonline.org/databasebrowse.shtml
[4] http://www.allergenonline.org/index.shtml

protein generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman *et al.*, 2008).

- Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions (Pariza and Foster, 1983; Pariza and Johnson, 2001; Pariza and Cook, 2010). Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are typically present in a narrow range of foods.
- The source of the gene for the lactase, Bifidobacterium bifidum, has not been associated with incidences of allergy.

Taken together, these data indicate a lack of allergenic concern for food enzymes in general and the lactase enzyme specifically based on the weight-of-evidence approach. Although cautioned against in Codex (2009), researched by Herman *et al*. (2009) and further elaborated by Ladics *et al.* (2011) and on AllergenOnline.org, there is no evidence that a short identical contiguous amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (>35%). This database allows isolated identity matches of 8 contiguous identical amino acids to satisfy demands by some regulatory authorities for this precautionary search. Performing the 8 contiguous identical amino acid search produced no sequence matches with known allergens.

In conclusion, bioinformatic analyses based on sequence homology and the above weight-of evidence information indicate that the *Bifidobacterium bifidum* lactase is unlikely to pose a risk of food allergenicity. As for all enzyme products, an MSDS for the lactase product would include a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

4 Genetic stability of the source organism

The production strain proved to be 100% stable after at least 60 generations of fermentation, judged by CB108 lactase production. Stability of the source organism is also included in Appendix E.

5 Pariza-Johnson Decision tree

Pariza and Johnson (2001) have published guidelines for the safety assessment of microbial enzyme preparations. These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations.

DuPont IB has evaluated CB108 lactaseaccording to the safety scheme of Pariza and Johnson (2001) (Appendix B6) and determined that this enzyme preparation is safe for use in food as a processing aid. This determination employed an extensive review of published

and unpublished safety data available on the enzyme, the production organism, the enzyme manufacturing process, and the enzyme product (Pariza and Johnson, 2001).

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Appendix B1: Toxin homology search results (Confidential Commercial Information)

Please refer to Excel file submitted separately (**Confidential Commercial Information)**

Appendix B2: Safe Strain Lineage (Confidential Commercial Information)

Please refer to Appendix B Confidential Attachment

Appendix B3 Toxicology Test Summaries (Confidential Commercial information)

Please refer to Appendix B Confidential Attachment

Appendix B4: Certificate of analysis of test items (Confidential Commercial Information)

Please refer to Appendix B Confidential Attachment

Appendix B5: Risk assessment for potential food allergenicity

Sequence Based Analysis Risk Assessment for Potential Food Allergenicity of the *Bifidobacterium bifidum* Lactase Expressed in *Bacillus subtilis*

The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.* (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics *et al.* (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores ($<10^{-7}$) may suggest a biologically relevant similarity (i.e., in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:

> "A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

The mature *Bifidobacterium bifidum* lactase sequence is given above in Appendix B Confidential Attachment.

A full-length sequence alignment against known allergens in the Food Allergy Research and Resource Program (FARRP) AllergenOnline database[5], February 1, 2018 V18A, containing 2093 peer-reviewed allergen sequences listed in the database[6] (using E-value <0.1) yielded no matches (See Supplementary 1).

The search for 80-amino acid stretches within the sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database (February 1, 2018 V18A[7]), identified one match, a pollen sequence from the Sycamore tree *Platanus orientalis* (See Supplementary 1). However, the full alignment generated a large E-value of 1.2×10^{3}, rendering the match insignificant. The insignificance of the match is further supported by the following weight-of-evidence data:

- The Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of food enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residues in bread and other foods do not represent any unacceptable risk to consumers (Dauvrin *et al*., 1998). The allergenicity potential of food

[5] http://www.allergenonline.org/index.shtml
[6] http://www.allergenonline.org/databasebrowse.shtml
[7] http://www.allergenonline.org/index.shtml

enzymes was further studied by Bindslev-Jensen *et al.* (2006) and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*". The investigation comprised enzymes produced by wild-type and GE strains as well as wild-type enzymes and protein engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern regarding food allergy.

- Enzymes, including lactase, when used as digestive aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more). Wüthrich (1996) published a list of enzymes used as digestive aids and concluded that they are not potent allergens by ingestion.
- Food enzymes are used in small amounts (i.e., at ppm levels) during food processing, resulting in very small amounts of the enzyme protein in the final food. Additionally, any residual enzyme proteins still present in the final food will be subjected to denaturation due to heating and subsequent digestion in the gastro-intestinal tract, further reducing the levels of enzyme. A high concentration of protein generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman *et al.*, 2008).
- Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions (Pariza and Foster, 1983; Pariza and Johnson, 2001; Pariza and Cook, 2010). Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are typically present in a narrow range of foods.
- The source of the gene for the lactase, Bifidobacterium bifidum, has not been associated with incidences of allergy.

Taken together, these data indicate a lack of allergenic concern for food enzymes in general and the lactase enzyme specifically based on the weight-of-evidence approach. Although cautioned against in Codex (2009), researched by Herman *et al*. (2009) and further elaborated by Ladics *et al.* (2011) and on AllergenOnline.org, there is no evidence that a short identical contiguous amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (>35%). This database allows isolated identity matches of 8 contiguous identical amino acids to satisfy demands by some regulatory authorities for this precautionary search. Performing the 8 contiguous identical amino acid search produced no sequence matches with known allergens (See Supplementary 1).

In conclusion, bioinformatic analyses based on sequence homology and the above weight-of evidence information indicate that the *Bifidobacterium bifidum* lactase is unlikely to pose a risk of food allergenicity. As for all enzyme products, an MSDS for the lactase product would include a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

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Appendix B5 Supplement 1 **(Confidential Commercial Information)** .

Please refer to Appendix B Confidential Attachment

Appendix B6: Analysis of safety based on Pariza/Johnson Decision tree

Pariza and Johnson (2001), and Pariza and Cook (2010) have published guidelines for the safety assessment of microbial enzyme preparations. These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree. The outcome of this inquiry is that the CB108 lactase enzyme product is "ACCEPTED" as safe for its intended use.

1. **Is the production strain[8] genetically modified[9,10]?** Yes. *Go to 2.*

2. **Is the production strain modified using rDNA techniques?** Yes. *Go to 3a.*

3a. **Does the expressed enzyme product which is encoded by the introduced DNA[11,12] have a history of safe use in food[13]?** Yes, lactase from safe species has history of safe use in foods. Although lactase isolated from *Bifidobacterium bifidum* does not have extensive history of safe use, this species is extensively used as probiotic in human nutrition, with no known safety concerns. In addition, the enzyme will be inactivated in the food manufacture process. Also, the US FDA reviewed the GRAS status of BIF917 lactase enzyme preparation from *B. subtilis* for use as a processing aid to produce lactose-reduced fresh dairy products, and galacto-oligosaccharide (GOS) and responded with a "No Questions Letter" (GRN 579). Finally, the safety of CB108 lactase is further supported by its lack of sequence similarity with known food allergens and oral toxins. Furthermore, the amino acid sequence of CB108 and BIF917 are 100% identical. *Go to 3c.*

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[8] Production strain refers to the microbial strain that will be used in enzyme manufacture. It is assumed that the production strain is nonpathogenic, nontoxigenic, and thoroughly characterized;steps 6–11 are intended to ensure this

[9] The term "genetically modified" refers to any modification of the strain's DNA, including the use of traditional methods (e.g., UV or chemically-induced mutagenesis) or rDNA technologies.

[10] If the answer to this or any other question in the decision tree is unknown, or not determined, the answer is then considered to be NO.

[11] Introduced DNA refers to all DNA sequences introduced into the production organism, including vector and other sequences incorporated during genetic construction, DNA encoding any antibiotic resistance gene, and DNA encoding the desired enzyme product. The vector and other sequences may include selectable marker genes other than antibiotic resistance, noncoding regulatory sequences for the controlled expression of the desired enzyme product, restriction enzyme sites and/or linker sequences, intermediate host sequences, and sequences required for vector maintenance, integration, replication, and/or manipulation. These sequences may be derived wholly from naturally occurring organisms or incorporate specific nucleotide changes introduced by *in vitro* techniques, or they may be entirely synthetic.

[12] If the genetic modification served only to delete host DNA, and if no heterologous DNA remains within the organism, then proceed to step 5.

[13] Engineered enzymes are considered *not* to have a history of safe use in food, unless they are derived from a safe lineage of previously tested engineered enzymes expressed in the same host using the same modification system.

3c. Is the test article free of transferable antibiotic resistance gene DNA[14]? Yes.

No antibiotic resistance genes were used in the construction. *Go to 3e.*

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products? Yes, inserted DNA is well characterized and free of unsafe attributes. *Go to 4.*

4. Is the introduced DNA randomly integrated into the chromosome? No. *Go to 6.*

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[15]? Yes. The *B. subtilis* BG125 safe lineage is well established (Appendix B2). Its safety as a production host and methods of modification are well documented and their safety have been confirmed through repeated toxicology testing (see Appendix B3).

Conclusion: Article is accepted.

[14] Antibiotic resistance genes are commonly used in the genetic construction of enzyme production strains to identify, select, and stabilize cells carrying introduced DNA. Principles for the safe use of antibiotic resistance genes in the manufacture of food and feed products have been developed (IFBC, 1990; "FDA Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096135.htm)

[15] In determining safe strain lineage one should consider the host organism, all of the introduced DNA, and the methods used to genetically modify the host (see text). In some instances the procedures described by Pariza and Foster (1983) and IFBC (1990) may be considered comparable to this evaluation procedure in establishing a safe strain lineage