

## GENERALLY RECOGNIZED AS SAFE NOTIFICATION

*Bacillus clausii* 088AE (MCC 0538)

Submitted September 2020



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## List of Abbreviations

%	Percentage
µg	Microgram
µm	Micrometer
ACLAME	A Classification of Mobile Genetic Elements
ADI	Acceptable Daily Intake
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
BSL-1	Biosafety Level 1
bw	Body Weight
°C	Degrees Celsius
CARD	Comprehensive Antibiotic Resistance Database
CFR	Code of Federal Regulations
CFU	Colony forming unit
cGMP	Current Good Manufacturing Practice
CLSI	Clinical and Laboratory Standards Institute
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CytK	Cytotoxin K
d	Day
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
FALCPA	Food Allergen Labelling and Consumer. Protection Act
FDA	U.S. Food and Drug Administration
FSSAI	Food Safety and Standards Authority of India
FSSR	Food Safety and Standards Regulations
g	Gram
GI	Gastrointestinal
GRAS	Generally Recognized As Safe
GRN	GRAS Notice
h	Hour
HACCP	Hazard Analysis and Critical Control Points
HBL	Haemolysin BL
kg	Kilogram
LD <sub>50</sub>	Median lethal dose
mg	Milligram
MIC	Minimum inhibitory concentration
mL	Milliliter
n	Number
NA	Not Applicable
NCBI	National Center for Biotechnology Information
NCMR	National Centre for Microbial Resources
NIH	National Institutes of Health
NLT	Not less than
NMT	Not more than

NOAEL	No Observed Adverse Effect Level
NR	Not Required
OECD	Organization for Economic Co-operation and Development
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
QPS	Qualified Presumption of Safety
R	Resistant
RH	Relative humidity
RNA	Ribonucleic Acid
S	Susceptible
USC	United States Code
USDA	United States Department of Agriculture
USP	United States Pharmacopeia
VFDB	Virulence Factor Database

## Part 1. 21 CFR 170.225: Signed Statements and Certification

### 1.1 Exemption Claim for *Bacillus clausii* 088AE

Advanced Enzyme Technologies Ltd. (herein after “Advanced Enzymes”) submits this Generally Recognized as Safe (GRAS) notice in accordance with 21 CFR part 170, subpart E. Advanced Enzymes has concluded that *Bacillus clausii* 088AE is GRAS by scientific procedures in accordance with both 21 CFR 170.30 (a) and (b) and is thereby exempt from pre-market approval requirements of the Food, Drug and Cosmetic Act.

#### Name and Address of Notifier

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#### Agent who is authorized to act on behalf of the Notifier:

Kevin O. Gillies  
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### 1.2 Name of Notified Microorganism

‘*Bacillus clausii* strain 088AE’. ‘088AE’ is the designation of the proprietary *Bacillus clausii* strain of Advanced Enzymes. The strain is deposited at National Centre for Microbial Resources (NCMR), India, under strain designation MCC 0538.

The product *Bacillus clausii* 088AE (MCC0538) is a spore preparation. Commercial preparations are known as SEBclausii, BioSEB CII.

In this GRAS notice, the *Bacillus clausii* strain 088AE is referred to as '*Bacillus clausii* 088AE'; '*B. clausii* 088AE' or *B. clausii* 088 AE (MCC0538).

### **1.3 Intended Conditions of Use**

*Bacillus clausii* 088AE is intended to be used in the following food categories:

Baked goods and baking mixes, breakfast cereals, beverages and beverage bases, coffee and tea, milk and milk products, dairy product analogs, fruit juices, condiments and relishes, confections and frostings, frozen dairy desserts and mixes, fruit and water ices, drinking water, sports drinks, gelatins, jams and jellies, puddings and fillings alcoholic beverages grain products and pastas, hard candy, soft candy, chewing gum, extracts, and flavorings, herbs, seeds, spices, seasonings, blends, nuts and nut products, plant protein products, processed fruits, processed vegetables and vegetable juices, snack foods, soups and soup mixes, sugar and sweet sauces, toppings, and syrups at a maximum level of approximately  $0.1 \times 10^9$  to  $2 \times 10^9$  colony forming units (cfu)/serving.

Based upon the estimated number of servings of food consumed per day, i.e. 18.2, in the US and the highest intended addition level of *B. clausii* 088AE per serving of  $2 \times 10^9$  cfu, the estimated daily intake (EDI) of the strain is  $3.6 \times 10^{10}$  cfu/day. (This EDI would be reached only if all target foods contained *B. clausii* at the maximum addition level and only if the targeted foods were the only foods consumed.)

*B. clausii* 088AE is not intended for use in foods that are targeted toward infants, such as infant formulas or foods formulated for infants, nor in meat and poultry products that come under USDA jurisdiction.

### **1.4 Statutory Basis for GRAS Status**

Advanced Enzymes has determined that the intended use of *Bacillus clausii* 088AE is GRAS through scientific procedures in accordance with 21 CFR §170.30(a) and (b).

### **1.5 Premarket Exempt Status**

Advanced Enzymes has determined that the intended use of *Bacillus clausii* 088AE is GRAS, therefore the use of the notified substance is exempt from pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act.

### **1.6 Data Availability**

Advanced Enzymes agrees to make the data and information that are the basis for the determination of GRAS status available to FDA upon request. Such data and information may be sent by Advanced Enzymes to FDA either in electronic format or on paper, or reviewed during customary business hours at 4880 Murrieta Street, Chino, CA 91710.

### **1.7 FOIA Statement**

None of the data and information in this GRAS notice is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. §552.

## **1.8 Certification**

To the best of our knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to Advanced Enzymes and pertinent to the evaluation of the safety and GRAS status of the intended use of *Bacillus clausii* 088AE.

## **1.9 FSIS Statement**

Not applicable.

## **1.10 Signature of Responsible Party or Agent**

Kevin O. Gillies



Kevin O. Gillies Consulting Services, LLC  
[info@kogilliesconsultingservices.com](mailto:info@kogilliesconsultingservices.com)  
September 9, 2020

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## Part 2. 21 CFR 170.230: Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

### 2.1 Identity/ Identification

The substance of this GRAS notification is a preparation of *B. clausii* 088AE spores. The diluents used in the manufacturing of *B. clausii* 088AE are approved as either food additives or GRAS substances.

#### 2.1.1 SCIENTIFIC NAME, TAXONOMY AND OTHER NAMES

Name of the food ingredient: *Bacillus clausii* 088AE

Synonyms: *Bacillus clausii* strain 088AE / *Bacillus clausii* (strain 088AE)/ *B. clausii* 088AE/ *B. clausii* 088AE (MCC0538)

##### Taxonomy:

Kingdom: Bacteria

Phylum: Firmicutes (Gram positive spore forming bacteria)

Class: Bacilli

Order: Bacillales

Family: Bacillaceae

Genus: *Bacillus*

Species: *clausii*

#### 2.1.2 DESCRIPTION/SOURCE INFORMATION AND GENOTYPIC, PHENOTYPIC CHARACTERIZATION OF THE ORGANISM

*B. clausii* 088AE is a nonpathogenic, non-toxicogenic naturally encapsulated spore-forming bacterium. *B. clausii* 088AE preparation is a light brown to brown coloured powder having total viable count not less than  $1.5 \times 10^9$  cfu/g. *Bacillus clausii* 088AE is deposited at National Centre for Microbial Resources (NCMR) India with deposit number MCC 0538.

##### 2.1.2.1 Genotypic Characterization

Genotypic characterization of *B. clausii* 088AE was carried out following 16S rRNA analysis and genomic sequencing. The *B. clausii* 088AE genome is sequenced for genome-based safety assessment. Whole-genome information was deposited in NCBI/GenBank database under the accession number CP031128. The parameters described below were assessed to establish the safety of *B. clausii* 088AE.

###### a) 16S rRNA

*B. clausii* 088AE was identified following 16S rRNA genes as phylogenetic markers. *B. clausii* 088AE can be clearly distinguished from the closely related species using 16S rRNA sequence



analysis. The 16S rRNA sequence showed 100% homology of *B. clausii* 088AE to *Bacillus clausii*.

#### **b) Genomic Sequencing**

Hybrid assembly was performed using MaSurCA Hybrid Assembler (Aleksey *et al*, 2013) between Illumina reads and nanopore reads. *Bacillus clausii* DSM 8716 strain was used as a reference. The final genome assembly was 4,598,457 bp in size with 44.74% G+C content. Gene prediction was done for assembled genome using *NCBI Prokaryotic Genome Annotation Pipeline* (PGAP) (Tatusova *et al*, 2016). The whole-genome project was deposited in NCBI/GenBank database under the accession number CP031128.

The assembled genome of *B. clausii* strain 088AE was compared with other bacterial genomes present in RefSeq genome database using NCBI-BLASTN (Altschul *et al*, 1990). *Bacillus clausii* (taxid:79880) was chosen as the reference organism for NCBI-BLASTN. The BLASTN results indicated ~99% sequence homology between the de-novo assembled genome with the genome of the reference strain *B. clausii* DSM 8716, further confirming the identity of *B. clausii* 088AE.

#### **c) Determination of mol G+C%**

The genomic DNA G+C content, defined as the proportion of guanines and cytosines within the overall number of nucleotides in the genome, is one of the features in taxonomic descriptions of micro-organisms (Meier-Kolthoff *et al.*, 2014). The mol % G+C content, based on the whole genome sequence of 4,598,457 bp, is 44.74%, which is in agreement to a value of 44.65 mol % G+C reported by Upadrasta *et al.* 2016 for *B. clausii*.

#### **d) Safety assessment in relation to antibiotic resistance genes**

A homology search between assembled genome of *B. clausii* strain 088AE and antibiotic resistance genes/proteins was performed using the Comprehensive Antibiotic Resistance Database (CARD) (Jia *et al*, 2017). In this case, BLASTX was used with the criteria (similarity >30%, coverage >70% and e-value < 1e-02) for the identification of significant hits. Critically important antimicrobials (CIAs) or highly important antimicrobials as per WHO (2016) and EFSA (2012) were screened in the data which was analyzed post homology alignment of the assembled genome of strain 088AE and CARD. Full coding genes for clinically relevant antibiotic resistance genes identified on the genome were: beta-lactamase (DUT88\_01930), bleomycin resistance gene (DUT88\_21580, vancomycin resistance gene (DUT88\_07250, erm34 gene (DUT88\_06975) which shows resistance to both erythromycin and clindamycin. These genes are inherent to the species and hence referred to as intrinsic resistance as no mobile elements were identified in the vicinity of these genes (Lakshmi *et al.* 2017). The percentage identity of three critical genes, viz. aminoglycoside o-nucleotidyl transferase (aadD2), aminoglycoside 6-adenylyltransferase ANT (6) and erm34 gene carried out by BLASTP using NCBI database, showed 100% identity to the respective protein from *B. clausii* DSM 8716.

The absence of mobile elements in the flanking regions of the above mentioned antibiotic resistance genes determined using ISfinder web-based software (Siguier *et al*, 2006) and using ACLAME database (Leplae *et al*, 2009), indicates high stability of the region. None of the genes coding for or contributing to resistance to antimicrobials relevant to their use in humans and

animals had mobile elements in its flanking region, which indicates, these genes are intrinsic and not transferable to any other organism. Thus, the strain *B. clausii* strain 088AE, does not pose any safety concerns with respect to the transmission of antibiotic resistance genes.

To support the genotypic analysis of antibiotic resistance genes, phenotypic analysis was carried out as per CLSI guidelines for its sensitivity/resistance against nine antibiotics, viz., ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol. The minimum inhibitory concentration (MIC) breakpoint values reported for *B. clausii* strain 088AE were below or equal to the break point as described by EFSA (2012) for all the antibiotics except for clindamycin and erythromycin. Clindamycin and erythromycin resistance was due to the presence of *erm34* gene (DUT88\_06975) which confers resistance to both erythromycin and clindamycin through methylation of their ribosomal target site (Weisblum, 1995). (Refer to section 2.1.3). Further, the GC content of the *erm34* gene is similar to the GC content of *B. clausii* genome, i.e. around 44%, suggesting that gene is structurally related to the total genome, and likely not “foreign DNA or horizontally transferred”, and is intrinsic to the species as it is chromosomally encoded (Lakshmi et al, 2017).

#### e) Analyses of risk associated with virulence factor genes

Virulence factor genes/proteins sequences were downloaded from Virulence Factor Database (VFDB) (Chen *et al*, 2004). The total number of sequences in the core database was 3072. A homology search between the assembled genome of *B. clausii* strain 088AE and virulence factor proteins was performed using BLASTX (similarity >30%, coverage >70% and e-value < 1e-02) to identify significant hits. A total of 706 virulence factor proteins were found to have significant homology with the assembled genome. According to the UniProt Cluster of Orthologous Groups (COG) database<sup>1</sup>, these genes were non-classical virulence factor genes and their determinants were related to inorganic ion transport and metabolism; coenzyme transport and metabolism; lipid transport and metabolism, secondary metabolites biosynthesis, transport and catabolism; nucleotide transport and metabolism (170); defense mechanisms (118); cell motility; intracellular trafficking, secretion, and vesicular transport (116); lipid transport and metabolism; secondary metabolites biosynthesis, transport and catabolism; general function prediction only (42); signal transduction mechanisms; transcription (125); amino acid transport and metabolism; signal transduction mechanisms (34); Posttranslational modification, protein turnover, chaperones (19); carbohydrate transport and metabolism; cell wall/membrane/envelope biogenesis (28); cell cycle control, cell division, chromosome partitioning (5); energy production and conversion (1); cell motility; signal transduction mechanisms (4); cell wall/membrane/envelope biogenesis; translation, ribosomal structure and biogenesis (30); replication, recombination and repair (3); cell motility; posttranslational modification, protein turnover, chaperones; intracellular trafficking, secretion, and vesicular transport (2); cell wall/membrane/envelope biogenesis; intracellular trafficking, secretion, and vesicular transport (1). Though multiple putative virulent factor genes were identified through the VFDB, they are likely not harmful since a majority of them were related to the transport

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<sup>1</sup> <https://www.uniprot.org/citations/10592175>; <https://dx.doi.org/10.1093/nar/28.1.33>

mechanism. Most of the genes identified were related to extracellular structure which could be correlated to the adhesion property.

To further confirm non-virulence of the strain 088AE, *in vitro* cytotoxicity testing against Vero cells was carried out as recommended by EFSA (2018) for *Bacillus* species that are not recommended on the QPS list. The fluorescence values, indicators of cell leakage and cytotoxicity, reported for samples from *B. clausii* strain 088AE were less than 20% of the positive control fluorescence indicating that the strain was not cytotoxic. (Refer to section 2.1.4).

#### **f) Identification of biogenic amine producing genes**

Protein sequences of the biogenic amine producing genes (amino acid decarboxylase) were downloaded from the Uniprot database. BLASTX was performed between the assembled genome and biogenic amine producing protein sequences. Only one amino acid decarboxylase, i.e. aspartate 1-decarboxylase decarboxylase (DUT88\_15095) was identified, which is known to produce beta-alanine from –aspartate. Unlike other products of amino acid decarboxylase, beta-alanine supplementation is known for its ergogenic effect on high intensity exercise performance in humans (Hobson *et al*, 2012). Hence, *B. clausii* strain 088AE does not possess biogenic amine producing genes of concern.

#### **g) Identification of mobile elements in assembled genome**

Mobile elements are DNA sequences that can move around the genome by changing their number of copies or simply by changing their location, often affecting the activity of nearby genes. These mobile elements are ubiquitous in bacteria and do not present safety risk factors in and of themselves. There is a perceived risk that the presence of such mobile elements may facilitate the horizontal transfer of genes, e.g. antibiotic resistance, from one bacterium to another. In the current study, mobile elements were predicted from the assembled genome by using ISfinder software web-based software (Siguier *et al*, 2006) and ACLAME database (version 0.4). A total of 337 insertion sites (IS element regions) were identified in the assembled genome. All the nucleotide sequences, which include plasmids, viruses and prophages, are downloaded from the ACLAME database (Leplae *et al*, 2009) (version 0.4). A homology search (BLASTN) was performed between the nucleotide sequences downloaded (1,25,190) from the above-mentioned database and the assembled genome. There were 186 regions in the assembled genome that had significant hits (coverage  $\geq 50\%$  and e-value  $\leq 1e-05$ ) against the mobile-element nucleotide sequences downloaded from the ACLAME database (Leplae *et al*, 2009). No region of concern, i.e. antibiotic resistance genes, virulence factor genes, and biogenic amine producing genes, were reported in the vicinity of the predicted mobile elements in the assembled genome thus ensuring the stability of the genome related to these potential risk factors.

#### **h) Analyses of toxin genes**

Gene mining was performed to find genes related to toxins known to be produced in the *Bacillus* genus, such as diarrheal enterotoxin bceT, haemolytic enterotoxin operon (hbl genes – hblA, hblC, hblD), non-haemolytic enterotoxin operon (nhe ABC genes – nheA, nheB, nheC),

cytotoxin K (cytK), enterotoxin FM (entFM) and emetic toxin cereulide (cesB). None of the above-mentioned toxin producing genes were identified in the genome, suggesting that of *B. clausii* strain 088AE does not produce these toxins and is safe for human consumption. The results obtained for the strain 088AE were on the same lines as for *B. clausii* UBBC07 when screened for the above-mentioned genes by Lakshmi et al, 2017.

### **i) Identification of CRISPR associated regions in assembled genome**

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) sequences were screened in the assembled genome of *B. clausii* strain 088AE using CRISPRC as Finder (Couvin *et al.*, 2018). CRISPRs are direct repeats found in the DNA of many bacteria (~40% of sequenced bacterial genomes). These CRISPRs are in range of 23-47 bp in length. Each of these repeats are separated by spacers of similar length. These spacers are unique in each of the genomes. These spacers indicate the non-coding region of genomic sequences between the genes. Eleven CRISPRs were identified from the assembled genome of *B. clausii* strain 088AE. The presence of a CRISPR system indicates an advantage in promoting genome stability by acting as a barrier to entry of foreign DNA elements.

### **Conclusion**

The *de novo* assembled genome of *B. clausii* strain 088AE generated without gaps resulted in a single scaffold. Full coding sequences conferring resistance to antibiotics such as beta-lactamase, bleomycin, vancomycin, erythromycin and clindamycin are present in the genome of *B. clausii* 088AE. These genes are inherent to the species, chromosomally-located and non-transferable as no mobile elements were found in the vicinity of these genes.

Multiple putative virulent factor genes, identified through the VFDB, have been analyzed and found not to be harmful as majority of them are related to transport mechanisms and to extracellular structures. *B. clausii* strain 088AE genome does not contain of any biogenic amine producing genes of concern. Absence of various enterotoxin and emetic toxin genes known to be present in some *Bacillus* species further ensures the non-toxigenic profile of the strain. There are no mobile elements identified with respect to the loci which have significant homology against antibiotic resistance genes, virulence factor genes, biogenic amine producing genes or enterotoxin genes. The presence of a CRISPR sequence in the assembled genome indicates an advantage in promoting genome stability by acting as a barrier to the entry of foreign DNA elements. The presence of anchoring related proteins increases their colonization and eventually reduce pathogenic adherence (Li *et al.* 2018).

In conclusion, *B. clausii* strain 088AE does not contain any sequences/genes in the genome that are health-risk associated, thus confirming the safety of the strain through the genome-based approach.

### **2.1.2.2 Phenotypic and Biochemical characterization**

The *B. clausii* strain 088AE is a Gram-positive, aerobic, alkalophilic, motile, rod shaped bacterium. Cell size ranges from 0.5  $\mu\text{m}$  to 0.7  $\mu\text{m}$  in width and 2.0  $\mu\text{m}$  to 4.0  $\mu\text{m}$  in length. Cells may grow in chains of 12-20 $\mu\text{m}$ . After 2 days of incubation on Nutrient Agar at 37°C, colonies are white to off-white and filamentous with filamentous margins having flat surfaces.

Spores were ellipsoidal which lie paracentrally-to-sub-terminally in sporangia which may be slightly swollen (Logan and Vos 2015).

Biochemical studies were carried out following [\*Bergey's Manual of Systematics of Archaea and Bacteria\*](#) for *Bacillus clausii*. *B. clausii* 088AE was positive for catalase, oxidase, gelatinase, protease (casein), amylase, and nitrate reductase enzymes. The strain was negative for indol, methyl red, Voges-Proskauer and citrate, urease, haemolysis and lecithinase. In the TSI test, the strain showed no gas production, including hydrogen sulphide, and the butt and slant turned yellow, indicating acid production. *B. clausii* was able to ferment D-glucose, sucrose, lactose, maltose, starch, dextrin, glycerol, mannitol, xylose, rhamnose, D-fructose, D-galactose, inulin, D-mannose, D-sorbitol, D-trehalose, and D-arabinose.

The results of biochemical tests of *B. clausii* 088AE were comparable to the reference strain of *Bacillus clausii* ATCC 700160 as presented below in Table 1. These analyses further confirm the identity of the strain *B. clausii* 088AE.

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**Table 1. Results of Morphological and Biochemical Tests (Harley and Prescott 2002)**

Test	Results	
	<i>Bacillus clausii</i> 088AE	<i>Bacillus clausii</i> ATCC 700160
Colony Characteristics	Colonies white and filamentous with filamentous margins	Colonies white and filamentous with filamentous margins
Gram Staining	Gram positive	Gram positive
Cell Morphology	Cells motile, rod shaped	Cells motile, rod shaped
Size	Cells 0.5 µm - 0.7 µm in width and 2.0 µm - 4.0 µm in length	Cells 0.5 µm - 0.7 µm in width and 2.0 µm - 4.0 µm in length
Arrangement	Single cells or in short chains	Single cells or in short chains
Catalase Test	Positive	Positive
Oxidase Test	Positive	Positive
Nitrate Reduction Test	Positive	Positive
Endospore stain	Spores ellipsoidal; lie paracentrally to subterminally in sporangia which may be slightly swollen	Spores ellipsoidal; lie paracentrally to subterminally in sporangia which may be slightly swollen
Indole Test	Negative	Negative
Methyl Red Test	Negative	Negative
Voges-Proskauer Test	Negative	Negative
Citrate Utilization Test	Negative	Negative
Urease Test	Negative	Negative
Triple Sugar Iron (H <sub>2</sub> S) Test	No production of hydrogen sulfide, Acidic Slant and Butt	No production of hydrogen sulfide, Acidic Slant and Butt
Gelatin hydrolysis Test	Positive	Positive
Casein hydrolysis Test	Positive	Positive
Starch hydrolysis Test	Positive	Positive
Haemolysis test	Negative	Negative
Lecithinase production	Negative	Negative
<b>Sugar Fermentation Tests</b>		
D-Glucose	Acid produced, No gas produced	Acid produced, No gas produced
Sucrose	Acid produced, No gas produced	Acid produced, No gas produced
Lactose	Acid produced, No gas produced	Acid produced, No gas produced
Maltose	Acid produced, No gas produced	Acid produced, No gas produced
Starch	Acid produced, No gas produced	Acid produced, No gas produced
Dextrin	Acid produced, No gas produced	Acid produced, No gas produced
Glycerol	Acid produced, No gas produced	Acid produced, No gas produced
Mannitol	Acid produced, No gas produced	Acid produced, No gas produced
Xylose	Acid produced, No gas produced	Acid produced, No gas produced
Rhamnose	Acid produced, No gas produced	Acid produced, No gas produced
D-Fructose	Acid produced, No gas produced	Acid produced, No gas produced
D-Galactose	Acid produced, No gas produced	Acid produced, No gas produced
D-Mannose	Acid produced, No gas produced	Acid produced, No gas produced
L-Arabinose	Acid produced, No gas produced	Acid produced, No gas produced
Inulin	Acid produced, No gas produced	Acid produced, No gas produced
D-Sorbitol	Acid produced, No gas produced	Acid produced, No gas produced
D-Trehalose	Acid produced, No gas produced	Acid produced, No gas produced

### 2.1.3 ANTIBIOTIC RESISTANCE (SUSCEPTIBILITY)

Three batches of the *B. clausii* 088AE strain were assessed for antibiotic susceptibility. The minimum inhibitory concentration (MIC: the lowest concentration of antibiotic that inhibits bacterial growth) of different antibiotics on *B. clausii* 088AE was evaluated following broth dilution assay method (CLSI, 2016). Results were interpreted as “Sensitive (S) / Resistant (R)” by comparing the MIC values with the breakpoint MIC value of each antibiotic following EFSA (2012) and CLSI (2012b) guidelines. Antibiotics tested included clindamycin, chloramphenicol, ampicillin, gentamicin, erythromycin, kanamycin, vancomycin, streptomycin and tetracycline using the broth dilution assay. The results are provided in Table 2.

**Table 2. Antibiotic Susceptibility of *B. clausii* 088AE**

Antibiotic	<i>Staphylococcus aureus</i> ATCC 29213			<i>Bacillus clausii</i> 088AE		
	MIC range <sup>1</sup> (µg/ml)	MIC (µg/ml)	Interpretation	MIC break-point <sup>4</sup> (µg/ml)	MIC (µg/ml)	Interpretation
Clindamycin	0.06 – 0.25	0.25	S <sup>3</sup>	4	≥8	R
Chloramphenicol	2 – 16	4	S	8	8	S
Ampicillin	0.5 – 2	2	S	NR <sup>5</sup>	NR	NR
Gentamicin	0.12 – 1	0.5	S	4	≤ 0.06	S
Tetracycline	0.12 – 1	0.5	S	8	1	S
Streptomycin	NA <sup>2</sup>	NA	NA	8	8	S
Kanamycin	1 – 4	2	S	8	2	S
Vancomycin	0.5 – 2	2	S	4	1	S
Erythromycin	0.25 - 1	0.5	S	4	≥8	R

1. Source: Clinical and Laboratory Standards Institute (CLSI) , 2016  
 2. NA = not available in CLSI (2012)  
 3. S = susceptible  
 4. Source: EFSA, 2012  
 5. NR = not required (EFSA, 2012)  
 6. R = resistant

The minimum inhibitory concentration (µg/ml) of chloramphenicol, ampicillin, gentamicin, kanamycin, vancomycin, streptomycin and tetracycline were within the recommended breakpoints (EFSA 2012). *B. clausii* 088AE strain showed resistance against clindamycin and erythromycin. Clindamycin and erythromycin resistance, as described in the section 2.1.2.1, were due to the presence of erm34 gene on chromosome, (DUT88\_06975), (Weisblum, 1995). No mobile elements were identified in the vicinity of the erm34 gene. The antibiotic resistance is inherent to the species (intrinsic resistance) and poses no risk of horizontal transfer. Laskshmi *et al.* (2017) reported clindamycin and erythromycin resistance in the strain *Bacillus clausii* UBBC07 and concluded that the resistance is due to chromosomal erm34 gene and is not horizontally transferable. Abbrescia *et al.* (2014) also reported clindamycin and erythromycin resistance in *B. clausii* strains.

The antibiotic sensitivity profile of *B. clausii* 088AE was also determined by the disk diffusion method. The antibiogram profile was compared with the reference strain, *B. clausii* DSM 8716 (*B. clausii* ATCC 700160). Both strains showed sensitivity to 31 antibiotics, including amoxicillin–clavulanic acid, cefaclor, cefoxitin, ceftizoxime, ceftriaxone, amikacin, cefazolin, cefprozil, doxycycline, gentamicin, imipenem, kanamycin, lomefloxacin, nafcillin, nalidixic acid, neomycin, nitrofurantoin, norfloxacin, streptomycin, tobramycin, azithromycin,

chloramphenicol, ciprofloxacin, ofloxacin, rifampicin, moxifloxacin, minocycline, meropenem, vancomycin, levofloxacin and tetracycline. Both strains were found resistant to aztreonam, cefepime, cefixime, cefotaxime, clindamycin, oxacillin, metronidazole, erythromycin and cefuroxime at the given concentrations. The test strain, *B. clausii* 088AE, was resistant to ceftazidime (CAZ, 30 µg) whereas, the reference strain, *B. clausii* DSM 8716 was sensitive. The antibiotic resistance profile of *B. clausii* DSM 8716 reported in this study is similar to the finding reported by Abbrescia *et al.* (2014).

The antibiotic sensitivity profile of various *Bacillus* strains, such as *Bacillus coagulans* and *Bacillus subtilis* is described in different GRAS Notices (GRNs).

GRN 597 describes antibiotic sensitivity of *Bacillus coagulans* strain SNZ 1969. The strain was susceptible to numerous antibiotics while resistance was noted for cefuroxime, metronidazole cefaclor, ceftiofur, colistin, novobiocin, and metronidazole.

Using the disc diffusion method, Sudha *et al.* (2010) reported that *Bacillus coagulans* Unique IS2 was sensitive to cefaclor, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, polymixin B, rifampicin, trimethoprim, and novobiocin; it displayed intermediate sensitivity to clindamycin, doxycycline, erythromycin, penicillin, and tetracycline; and resistance to bacitracin, colistin, methicillin, metronidazole, and streptomycin (GRN 526). Antibiotic susceptibility of *Bacillus coagulans* SANK 70285 spores preparation (GRN 691) was assessed using both the disc diffusion and the micro-dilution methods (Sakuma, 2016). *B. coagulans* SANK 70285 was found to be sensitive to streptomycin, gentamicin, bacitracin, novobiocin, polymixin, cefaclor, ciprofloxacin, rifampicin, chloramphenicol, tetracycline, erythromycin, kanamycin, colistin, nalidixic acid, clindamycin, ceftiofur, doxycycline, and penicillin. Antibiotics tested with the microdilution method were oxacillin, ampicillin, cefazolin, cefmetazole, flomoxef, imipenem, gentamicin, arbekacin, minocycline, ceftiofur, erythromycin, clindamycin, vancomycin, teicoplanin, linezolid, fosfomicin, sulfamethoxazole-trimethoprim, and levofloxacin. With the exception of flomoxef and linezolid, bacterial growth was inhibited with the lowest concentration of each tested antibiotics.

GRN 660 describes antibiotic sensitivity/resistance of *Bacillus coagulans* GBI-30, 6086. The strain was reported susceptible to ampicillin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, linezolid, neomycin, rifampicin, tetracycline, trimethoprim, vancomycin and virginiamycin, and resistant to kanamycin and streptomycin.

*Bacillus subtilis* DE111 (GRN 831) was assessed for antibiotic susceptibility following the zone of inhibition and minimal inhibitory concentration micro-dilution assays. The antibiotic susceptibility test (AST) results indicate that *Bacillus subtilis* DE111 was sensitive to gentamicin, kanamycin, neomycin, streptomycin, amoxicillin/ clavulanic acid, cefaclor, cephalothin, imipenem, ciprofloxacin, fosfomicin, erythromycin, clindamycin, quinupristin/dalfopristin, chloramphenicol, rifampicin, Sulfamethoxazole-trimethoprim, tetracycline, trimethoprim, vancomycin. Mixed results were reported, i.e. sensitive and resistance for ampicillin, penicillin, ceftriaxone and cefotaxime. Using the micro-dilution assay, *B. subtilis* DE111 was reported sensitive to all the aforementioned antibiotics (GRN 831).



It may be concluded that it is not unusual for *Bacillus* species that are safe and suitable for use in food to have a mixture of sensitivity and resistance to various antibiotics. The resistance to antibiotics reported for *Bacillus clausii* 088AE has been investigated and the genes responsible have been reported to be chromosomally encoded and unlikely to be transferrable.

#### 2.1.4 VIRULENCE ACTIVITY

Members of genus *Bacillus*, other than *Bacillus cereus* group species, have been reported to produce enterotoxins and emetic toxins. From *et al.* (2005) screened 333 *Bacillus* strains; eight strains belonging to *B. subtilis*, *B. mojavensis*, *B. pumilus* and *B. fusiformis* were found to produce cytotoxic and emetic toxins. The production of the *B. cereus*-like diarrhoeal enterotoxins by some strains of other *Bacillus* species was described in the SCAN opinion (EC, 2000). The current view is that the very few reports of *B. cereus*-like enterotoxins occurring in other species of *Bacillus* are likely to have resulted from a misidentification of the strain involved (From *et al.*, 2005). For other *Bacillus* species, concerns appear to be associated to the production of surfactin like-lipopeptides, although the relation between the presence of these compounds and/or other toxic factors and the risk of illness in human has not yet been established. In the absence of animal models shown to be able to distinguish hazardous from non-hazardous strains, the EFSA relies on the use of *in vitro* cell-based methods to detect evidence of a cytotoxic effect (EFSA 2014). A test for cytotoxicity using Vero cells was performed to demonstrate that *B. clausii* 088AE is not toxigenic (EFSA 2014).

The Vero cell test is based on the principle that the DNA intercalating agent propidium iodide will stain DNA of cells having leaky cell membranes, thereby enhancing the resulting intracellular fluorescent signal. Positive control contained Triton x 100 treated cells with leaky cell membranes (100% fluorescence). The DNA of intact cells would not show any uptake of propidium iodide, resulting in basal level, negligible fluorescence. The study showed that the sample of *B. clausii* 088AE did not elicit cytotoxicity on Vero cells (Table 3).

**Table 3. Test for Detection of Cytotoxicity Using Vero cells**

Test Article	Fluorescence Units in Live Cells	% Fluorescence with respect to positive control
Background	2.31	1.78
Positive control	129.85	100.00
Negative control	6.20	4.77
<i>B. clausii</i> 088AE – 10 µl	16.37	12.60
<i>B. clausii</i> 088AE – 50 µl	14.83	11.42
<i>B. clausii</i> 088AE – 100 µl	18.97	14.61

The fluorescence values for sample of *B. clausii* 088AE were less than 20% of the positive control fluorescence, indicating that the sample did not have any cytotoxic effect on Vero cells *in vitro* at 10-100 µl sample volume for the 2 hours incubation period.

### 2.1.5 **ANTIMICROBIAL ACTIVITY**

*B. clausii* 088AE was evaluated for its antimicrobial activity following CLSI (2012) guidelines as recommended by EFSA (2018) against five selected microorganisms (*Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212), the United States Pharmacopoeia (USP, 2008) against two microorganisms (*Escherichia coli* ATCC 8739, *Staphylococcus epidermis* ATCC 12228) and the Food and Agriculture Organization (2006) against one more microorganism (*Staphylococcus aureus* ATCC 6538). *B. clausii* 088AE showed an absence of antimicrobial activity against the tested standard reference microorganisms.

### 2.1.6 **ACID AND BILE SALT TOLERANCE**

The spore preparation of *B. clausii* 088AE was tested for its ability to survive under different simulated gastrointestinal conditions through an *in vitro* study. After 24 hours of exposure, *B. clausii* 088AE was stable in simulated saliva (92.3%), simulated intestinal fluid (100%) and simulated colonic fluid (97.22%). The preparation was fully stable in fasting-state simulated gastric juice (100%) and Fed-State Simulated Gastric Juice (97.11%) up to the stomach transit time (90 minutes). Ghelardi *et al.* (2015) also reported stability of *B. clausii* during transit time in human gastrointestinal tract.

The *in vitro* study concluded that *B. clausii* 088AE was stable and maintained its survivability under different simulated gastrointestinal conditions.

### 2.1.7 **ENTEROTOXINS**

*B. clausii* 088AE was screened for enterotoxin production by Duopath® Cereus Enterotoxins test kit (Merck KGaA, Darmstadt, Germany and D-cultural technique).

*B. clausii* 088AE was examined for absence of enterotoxins (hemolysin, hbl; nonhemolytic, nhe; cytotoxin, cytK) and emetic toxin (cereulide, ces) producing genes. The absence of PCR products for the toxin related genes in *B. clausii* strain 088AE indicates the absence of the above-mentioned toxin producing genes in *B. clausii* strain 088AE.

*B. clausii* 088AE was concluded to be negative for non-hemolytic enterotoxins and emetic toxin.

### **Conclusion**

*B. clausii* 088AE strain has been thoroughly analyzed for risk associated factors following genome-based analyses and phenotypic /biochemical studies. Various studies/analyses conducted on this strain showed no safety concern and concluded that the strain is safe for human consumption.

## **2.2 Manufacturing Process**

### **2.2.1 OVERVIEW**

*B. clausii* 088AE is produced as spores by batch and fed-batch type fermentation. Fermentation is in accordance with current Good Manufacturing Practices (cGMP) and the principles of Hazard Analysis and Critical Control Points (HACCP). The manufacturing facility is ISO 9001:2015, ISO 22000 and GMP certified.

*B. clausii* 088AE is produced by fermentation. Fermentation is a well-known process that occurs in food production and has been used for the cultivation of microorganisms for decades. Liquid state or submerged fermentation is used to produce the *B. clausii* 088AE. The typical fermentation batch size ranges from 100 L to 50,000 L, preferably 14,000 to 20,000 L.

The key steps for production of *B. clausii* 088AE are fermentation, recovery, formulation, and packaging. The process is illustrated in Figure 1.

### **2.2.2 FERMENTATION**

#### **2.2.2.1 Raw materials**

All materials used in the fermentation process (inoculum, seed, and main fermentation) are food-grade substances approved for this use. None of the top eight allergens (FALCPA, 2004) are used as a material in fermentation.

#### **2.2.2.2 Inoculum (Seed)**

A suspension of a pure culture of *B. clausii* 088AE is aseptically transferred to an inoculum flask containing fermentation medium.

The culture is grown in the flask under optimum conditions in order to obtain a sufficient amount of biomass, which can subsequently be used as inoculum for the seed fermentation.

#### **2.2.2.3 Seed Fermentation**

The inoculum is aseptically transferred to the seed fermenter containing seed fermentation medium. When a sufficient amount of biomass has developed (typically up to 15-20 hours), the content of the seed fermenter is used for inoculation of the main fermentation.

#### **2.2.2.4 Main fermentation**

During the main fermentation, the growth (cell-mass) of *B. clausii* 088AE takes place and the vegetative cells later converted to spores during late growth/stationary phase.

The fermentation in the main fermenter is operated as a batch and fed-batch fermentation. First, the content of the seed fermenter is aseptically transferred to the main fermenter containing fermentation medium. The fermentation process is continued for a predetermined time or until laboratory test data show that the desired biomass production has been obtained or that the rate of biomass production has decreased below a predetermined production rate. When the desired spore count is reached, the fermentation is complete.

### **2.2.3 RECOVERY**

The purpose of the recovery process is to separate the *B. clausii* 088AE spores from the fermentation media, concentrate the spores, and prepare dried powdered biomass.

The vegetative cells of *B. clausii* 088AE are converted to spores at the end of fermentation and are suspended in the fermentation media. During recovery, spores are separated from fermentation medium.

The steps of recovery include:

- Primary separation of spores (biomass) from the soluble media components
- Washing of concentrated spores (biomass)
- Spray drying

#### **2.2.3.1 Primary Separation**

The fermentation broth is passed through a high-speed centrifuge to separate the spores (biomass) from the soluble media components along with water. The spore biomass is collected as a thick slurry and subjected to further processing. Temperature and pH are controlled during this step.

#### **2.2.3.2 Washing**

Sterilized and demineralized water is added to the collected biomass slurry. Slurry is again passed through high-speed centrifuge and the washed biomass is collected. Temperature and pH are controlled during this step.

#### **2.2.3.3 Spray Drying**

The concentrated biomass suspension is spray-dried in presence of approved food-grade stabilizers (e.g. maltodextrin) to obtain the unformulated concentrate.

#### **2.2.4 FORMULATION AND PACKAGING**

*B. clausii* 088AE is sold as a powder preparation of different spore counts, depending on the final intended application.

For the manufacturing of the dry spore preparation, the spray-dried unformulated concentrate (not less than  $1.5 \times 10^{11}$  cfu/g) is further formulated with approved food grade formulating agents such as maltodextrin and adjusted to a declared spore count.

The *B. clausii* 088AE preparation is tested by Quality Control for all quality related aspects and released by Quality Assurance. The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for final preparations.

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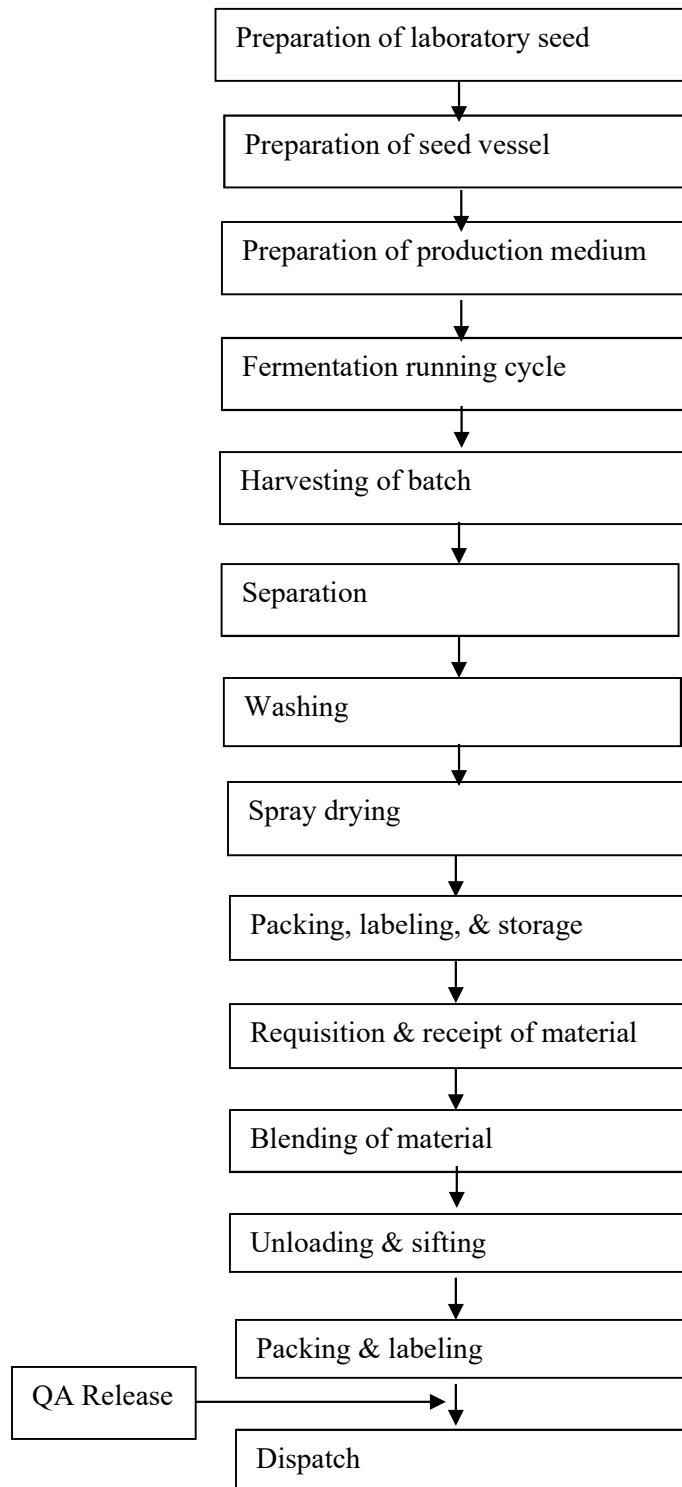


Figure 1. Manufacturing Process for *B. clausii 088AE*

## 2.3 Product Specifications and Compositional Variability

### 2.3.1 PRODUCT SPECIFICATIONS

Specifications for *B. clausii* 088AE preparation have been established by Advanced Enzymes and are summarized in Table 4. All methods have been validated for this purpose.

**Table 4. Product Specifications for *B. clausii* 088AE**

Product specification	Advanced Enzymes	
	Limits	Reference Method
Total viable count/ Assay (CFU/g)	Not less than 150 billion viable spore counts / g	SAP-QAD-Micro-039, internal method
Appearance/ Description	Light brown to brown colored powder	Visual
Microscopy/ Identity	Rod shaped cells containing round or oval shaped endospore either terminally or subterminally	Internal method
Moisture/ Loss on Drying	Not more than 7.0%	AOAC 926.08
Sieve test	100% through 40 mesh	Internal method
Arsenic	Not more than 2.0 ppm	AOAC 984.27
Cadmium	Not more than 1.0 ppm	AOAC 984.27
Lead	Not more than 3.0 ppm	AOAC 984.27
Mercury	Not more than 0.5 ppm	EPA 7471
Total yeast & mold count	Not more than 100 cfu/g	Harmonized method (IP,BP,EP and USP)
Total coliform	Not more than 100 cfu/g	FDA Bacteriological Analytical Manual
<i>E. coli</i>	Absent in 10 g	Harmonized Pharmacopoeial method (EP, BP, USP, and IP)
<i>Salmonella</i> spp.	Absent in 10 g	Harmonized Pharmacopoeial method (BP, USP and IP)
<i>P. aeruginosa</i>	Absent in 1 g	Harmonized method (IP,BP,EP and USP)
<i>Staphylococci</i> spp.	Absent in 1 g	Harmonized method (IP,BP,EP and USP)
<i>Listeria monocytogenes</i>	Absent in 25 g	Internal method

### 2.3.2 COMPLIANCE WITH SPECIFICATIONS

Three (3) non-consecutive production batches of *B. clausii* 088AE were analyzed and the results compared with food-grade specifications as presented in Table 4. As shown in Table 5, all tested batches were in compliance, demonstrating that the process is capable of producing product meeting the established specifications.

**Table 5. Analysis of Compositional Variability of *B. clausii* 088AE**

Parameter	Specification	Batch		
		101840	101842	101844
<i>B. clausii</i> viable spore count	Not less than 150 billion viable spore counts/g	159 billion viable spore count /g	166 billion viable spore count /g	168 billion viable spore count /g
Description	Light brown to brown colored powder	Light brown colored powder	Light brown colored powder	Light brown colored powder
Microscopy/ Identity	Rod shaped cells containing round or oval shaped endospore either terminally or subterminally	Complies	Complies	Complies
Sieve test	100% pass through 40 mesh.	Complies	Complies	Complies
Moisture/Loss on drying (%)	Not more than 7.0%	6.18%	6.12%	6.20%
Heavy Metal Analysis				
Arsenic	Not more than 2.0 ppm	Complies	Complies	Complies
Cadmium	Not more than 1.0 ppm	Complies	Complies	Complies
Lead	Not more than 3.0 ppm	Complies	Complies	Complies
Mercury	Not more than 0.5 ppm	Complies	Complies	Complies
Microbial Analysis				
Total yeast & mold count	Not more than 100 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g
Total Coliform	Not more than 100 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g
<i>E. coli</i>	Absent in 10g	Complies	Complies	Complies
<i>Salmonella</i> spp.	Absent in 10g	Complies	Complies	Complies
<i>P. aeruginosa</i>	Absent in 1g	Complies	Complies	Complies
<i>Staphylococci</i> spp.	Absent in 1g	Complies	Complies	Complies
<i>Listeria monocytogenes</i>	Absent in 25g	Complies	Complies	Complies

## 2.4 Shelf-Life Stability

The stability testing (shelf life) of *B. clausii* 088AE was assessed in a real-time stability study, in which the samples were stored in an environmental chamber at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  &  $60\% \text{ RH} \pm 5\%$ . In an accelerated stability study, samples were stored in an environmental chamber at accelerated storage conditions ( $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$  &  $75\% \text{ RH} \pm 5\%$ ) for a period of six months.

In the accelerated stability studies ( $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$  /  $75\% \pm 5\% \text{ RH}$ ) for six months, the activity drop of *B. clausii* 088AE was less than 15%. The real time stability studies ( $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  /  $60\% \pm 5\%$ ) showed less than 5% loss of viable count in 12 months study duration. Based on these findings and in accordance with ICH guideline Q1E<sup>2</sup>, the proposed shelf life of *B. clausii* 088AE is 2 years under real-time storage conditions, when stored in simulated market packing, e.g. double polybag bag in HDPE drum (powder).

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<sup>2</sup> European Medicines Agency, August 2003 CPMP/ICH/420/02; last accessed September 15, 2020



### Part 3: 21 CFR 170.235: Intended Use and Dietary Exposure

*B. clausii* 088AE is intended for addition at a level not exceeding  $2 \times 10^9$  cfu/serving to a wide variety of foods. The food categories, as defined in 21 CFR §170.3(n) to which *B. clausii* 088AE is to be added, are listed below:

- (1) Baked goods and baking mixes, including all ready-to-eat and ready-to-bake products, flours and mixes, requiring preparation before serving.
- (2) Beverages, alcoholic, including malt beverages, wines, distilled liquors, and cocktail mix.
- (3) Beverages and beverage bases, nonalcoholic, including only special or spiced teas, soft drinks, coffee substitutes, and fruit and vegetable flavored gelatin drinks, drinking water, sport drinks.
- (4) Breakfast cereals, including ready-to-eat and instant and regular hot cereals.
- (5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses.
- (6) Chewing gum, including all forms.
- (7) Coffee and tea, including regular, decaffeinated, and instant types.
- (8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs.
- (9) Confections and frostings, including candy and flavored frostings, marshmallows, baking chocolate, and brown, lump, rock, maple, powdered, and raw sugars.
- (10) Dairy product analogs, including nondairy milk, frozen or liquid creamers, coffee whiteners, toppings, and other nondairy products.
- (12) Fats and oils, including margarine, dressings for salads, butter, salad oils, shortenings and cooking oils.
- (16) Fresh fruit juices, including only raw fruits, citrus, melons, and berries, and home prepared "ades" and punches made therefrom.
- (20) Frozen dairy desserts and mixes, including ice cream, ice milks, sherbets, and other frozen dairy desserts and specialties.
- (21) Fruit and water ices, including all frozen fruit and water ices.
- (22) Gelatins, puddings, and fillings, including flavored gelatin desserts, puddings, custards, parfaits, pie fillings, and gelatin base salads.
- (23) Grain products and pastas, including macaroni and noodle products, rice dishes, and frozen multicourse meals, without meat or vegetables.
- (25) Hard candy and cough drops, including all hard type candies.
- (26) Herbs, seeds, spices, seasonings, blends, extracts, and flavorings, including all natural and artificial spices, blends, and flavors.
- (28) Jams and jellies, commercial, including only commercially processed jams, jellies, fruit butters, preserves, and sweet spreads.
- (30) Milk, whole and skim, including only whole, low-fat, and skim fluid milks.

(31) Milk products, including flavored milks and milk drinks, dry milks, toppings, snack dips, spreads, weight control milk beverages, and other milk origin products.

The intended addition of *B. clausii* 088AE and the food categories to which it will be added are identical to those stated for *Bacillus coagulans* as described in GRN 000399, 000526, 000597, 000691, to which FDA had no objection. *B. clausii* 088AE is intended to be added as a food ingredient in multiple food categories safely between  $0.1 \times 10^9$  to  $2 \times 10^9$  cfu/serving.

The intended usage of *B. clausii* 088 AE in food categories is similar to *Bacillus clausii* SNZ 1971 that Sanzyme Biologics reports to be self-affirmed GRAS.<sup>3</sup> *Bacillus clausii* SNZ 1971 is described as a food ingredient in bakery (biscuits, pastries, cookies, brownies, crackers), cereal bars, dairy products (yogurt, cottage cheese, hard cheeses, and milk drinks and substitute products) and vegetable and fruit juices.

The No Observed Adverse Effect Level (NOAEL) of *B. clausii* 088AE in Sprague Dawley rats, following oral administration for 90 days was 1000 mg/kg/day (Annex B-2, B-3). This dose corresponds to  $1.6 \times 10^{11}$  cfu/kg/day (as the strength of *B. clausii* 088AE provided for toxicity study was  $1.6 \times 10^{11}$  cfu/g of bacterial preparation) and  $1.1 \times 10^{13}$  cfu/day for healthy adult male person of 70 kg body weight. Therefore, the Acceptable Daily Intake (ADI) concluded from the NOAEL dose of 90-day toxicity study of *B. clausii* 088AE (adjusted with a 100x safety factor) is  $1.1 \times 10^{11}$  cfu/person/day.<sup>4</sup>

According to the USDA Nutrition Insights, a publication of the USDA Center for Nutrition Policy and Promotion (October 2000), males aged 51 or older consume the greatest servings of food/day which is about 18.2 servings of food/day from the following categories: grains, fruits, vegetables, milk, meat and others (fats, oils, sweets). Based upon the greatest estimate of servings of food consumed per day in the US (18.2) and the highest possible additional level of *B. clausii* 088AE per serving ( $2 \times 10^9$  cfu/serving), the maximum estimated daily intake (EDI) is  $3.6 \times 10^{10}$  cfu/day,<sup>5</sup> which is less than the ADI derived from the NOAEL from the 90-day chronic oral toxicity study i.e.  $1.1 \times 10^{11}$  cfu/per day.

As described above, the EDI value of *B. clausii* 088AE is highly exaggerated as it is unlikely that all products in the food categories that are listed above will contain *B. clausii* 088AE and unlikely that consumers will choose all of their food intake only from foods containing *B. clausii* 088AE.

In summary, even when the highly conservative EDI for *B. clausii* 088AE was used to compare with the ADI developed from the observed NOAL, the consumption of *B. clausii* 088AE was found to be well below the ADI and unlikely to present a risk to consumers.

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<sup>3</sup> <https://www.sanzymebiologics.com/blog/bacillus-clausii-snz-1971/>

<sup>4</sup> ADI = NOAL ( $1.1 \times 10^{13}$  cfu/day) / 100x safety factor =  $1.1 \times 10^{11}$  cfu/day

<sup>5</sup> Maximum Estimated Daily Intake of  $3.6 \times 10^{10}$  CFUs per day of *B. clausii* is calculated as follows:  $2.0 \times 10^9$  CFUs/serving (highest possible additional level of *B. clausii*) x 18.2 servings/day (greatest estimated serving of food).

**Part 4: 21 CFR 170.240: Self-Limiting Levels of Use**

There are no self-limiting levels of use of *Bacillus clausii* spores from *B. clausii* 088AE in food applications.

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**Part 5: 21 CFR 170.245: Experience Based on Common Use in Food before 1958**

While *Bacillus clausii* has a safe history of use in food, the statutory basis for our conclusion of GRAS status in this notice is scientific procedures, as described in Pariza *et al.* 2015, rather than common use in food prior to 1958.

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## Part 6: 21 CFR 170.250: Narrative

### 6.1. History of Consumption of *Bacillus clausii*

Bacteria of the *Bacillus* genus are among the most widespread microorganisms in the nature. Being ubiquitous in soil, air and water, they find their way easily into food products (Beleneva, 2008; Garbeva *et al.*, 2003). The *Bacillus* counts in wheat, grain and whole meal, are reported to be  $10^6$  CFU/g (Sorokulova, 2013). Beleneva (2008) reported isolation of 15 different species of *Bacillus* from invertebrates and Sea of Japan.

*Bacillus* species particularly the strains of *B. subtilis*, *B. subtilis* var. natto, *B. clausii*, *B. licheniformis*, and *B. coagulans* etc. are widely employed in the development of quality functional foods globally (Elshaghabe *et al.*, 2017). For example, Natto (Japan), Gari (Africa) Tapai Ubi (Malaysia), Douchi (China), Rabadi (India, Pakistan), Soibum (India), Ugba (Nigeria) are among the popular functional foods harboring the blend of *Bacillus* spp. (Elshaghabe *et al.*, 2017). Ahaotu *et al.*, 2013 reported presence of *B. clausii* in Ugba, a Nigerian traditional food, which is produced from the alkaline fermentation of seeds of the African oil bean tree. *B. clausii* strains have been isolated from numbers of traditional ethnic foods in India like Beetroot pickles, Toddy (Kerala, India ethnic alcoholic drink), Arishtam (Ayurvedic alcoholic drink) and Wine (Pal, 2013). *B. clausii* fermented whey is developed as functional dairy product, (Rochín-Medina *et al.*, 2018). In the aquaculture industry, *B. clausii* strain is used as functional feed preparation for Guppy fish (*Poeciliareticulata*) (Lakshmi *et al.*, 2017).

Strains of *B. clausii* are commercially explored for use in functional foods. *Bacillus clausii* SNZ1971 (self-affirmed GRAS, <https://www.sanzymebiologics.com/blog/bacillus-clausii-snz-1971/>) is intended to be used as food ingredient for consumers in food categories, like bakery (biscuits, pastries, cookies, brownies, crackers), cereal bars, dairy products (yogurt, cottage cheese, hard cheeses, and milk drinks and substitute products) and vegetable and fruit juices (Sanzyme Biologics, 2020).

In Italy, *B. clausii* has been widely used since 1960 as a bacterial therapy for viral diarrhea in children and for alleviating antibiotic related side effects (Jayanthi and Ratna Sudha, 2015). Another report suggests consumption of *B. clausii* by human beings since 1975 (Sensei *et al.* 2001). Enterogermina<sup>6</sup>, a two billion per five mL of *B. clausii* spore preparation (Sanofi-Aventis) is extensively studied across various populations of different geographical region. It was registered in 1958 in Europe and has had an over-the-counter medicinal status since 1999 (Green *et al.*, 1999; Cutting 2011, Lee *et al.* 2019).

### 6.2 Regulatory History of *Bacillus clausii*

*Bacillus clausii* strains have long been known to be safely consumed by the general human population. Reports suggest consumption of *B. clausii* by human beings for decades (Ghelardi *et al.*, 2015; Sensei *et al.* 2001). In Italy, *B. clausii* has been used since 1960 as a bacterial therapy for viral diarrhea in children and for alleviating antibiotic related side effects (Jayanthi and RatnaSudha, 2015). Enterogermina, a  $2 \times 10^9$  cfu/per five mL of *B. clausii* spore preparation (Sanofi-Aventis) is extensively studied across various populations of different geographical region. It is registered in 1958 in Europe and has an over-the-counter medicinal status since 1999

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<sup>6</sup> <https://www.enterogermina.in/product>

(Green *et al.*, 1999).<sup>7</sup> In Europe, strains of *B. clausii* are commercially available in products like Neoform BS 10 (CNCM MA23/3V and CNCM MA66/4M) (Scientific opinion, EC, 2003); Enterogermina® (*Bacillus clausii* strains O/C, N/R, SIN, T), MegaSporeBiotic (*B. clausii* SC-109), EnteroBacina (Bion Corporation, Elshaghabe *et al.*, 2017).

European Food Safety Authority has granted *B. clausii* Qualified Presumption of Safety (QPS) status since 2008 (EFSA, 2016) and has renewed its status annually since then. Further *B. clausii* does not appear on the list of pathogens in Annex III of Directive 2000/54/EC, as it is globally regarded as a safe microorganism

The American Type Culture Collection has classified different strains of *B. clausii* as Biosafety Level 1 (BSL-1), indicating that it is well-characterized agent which is not known to cause disease in healthy humans.

Food Safety and Standards Authority of India (FSSAI), has included *B. clausii* in the list of permitted components in food and health supplements (FSSR, 2018).

Sanzyme Biologic's *Bacillus clausii* SNZ 1971 has self-affirmed GRAS (generally recognized as safe) status and it is intended for use as a food ingredient for consumers in the following food categories: bakery (biscuits, pastries, cookies, brownies, crackers), cereal bars, dairy products (yogurt, cottage cheese, hard cheeses, and milk drinks and substitute products) and vegetable and fruit juices.<sup>8</sup>

### 6.3 Safety of *Bacillus clausii*—Oral Toxicity and Genotoxicity Studies

The safety of *B. clausii* 088AE and other strains have been evaluated in acute, subacute, sub-chronic, and chronic studies of oral toxicity and genetic toxicity assays.

#### 6.3.1. STUDIES OF BACILLUS CLAUSII 088AE

*B. clausii* 088AE, the notified strain, has been investigated in a series of toxicity studies complying with OECD Guidelines and conducted in accordance with the principles of Good Laboratory Practice (GLP) as published by the OECD (ENV/MC/CHEM(98)17) (Annex A, B-1).

**Acute oral toxicity test (OECD 423, 2001):** Using the step-wise method, 2 groups of n=3 female Sprague Dawley rats aged 9-10 weeks and weighing 163.28-178.47 g were dosed via gavage with 300 mg spore preparation  $4.77 \times 10^{10}$  cfu/kg bw and observed for 14 days. No indications of toxicity were reported. Two (2) similar groups of n=3 female Sprague Dawley rats were gavaged with 2000 mg/kg bw of the spore preparation, providing  $3.18 \times 10^{11}$  cfu/kg bw. Again, no indications of toxicity were reported (Annex A).

**Repeated-dose 90-day oral toxicity test (OECD 408, 2018):** Four groups of 10 male and 10 female Sprague Dawley rats, 6-7 weeks old and weighing 170-173 g (males, mean = 171.2 g) and 149-153 g (females, mean = 150.1 g) were assigned to receive daily gavage of doses of 0, 250, 500, and 1000 mg spore preparation/kg bw (providing 0, 0.40, 0.80, and  $1.59 \times 10^{11}$  cfu/kg bw) for 90 days. Groups of 5 rats/sex receiving 0 or 1000 mg spore preparation/kg bw/day were assigned to 28-day recovery groups. Rats were examined daily for signs of toxicity, morbidity, and mortality. They were subjected to detailed clinical examinations at day 0 and weekly

<sup>7</sup> Ibid

<sup>8</sup> <https://www.sanzymbiologics.com/blog/bacillus-clausii-snz-1971/>

thereafter during the treatment and recovery period. Ophthalmic examinations were performed on the control and high-dose rats at beginning and end of dosing. At week 13, all animals were assessed for sensory reactivity, grip strength, and motor activity. Feed consumption and body weight were recorded weekly. Blood and urine samples were taken at the end of dosing and after recovery. All animals were subjected to necropsy and weights of kidneys, liver, adrenals, testes, epididymis, uterus, thymus, spleen, brain, ovaries, and heart were recorded. Histological evaluations were performed on all tissues from control and high-dose rats (Annex B-1).

There were no mortality and no clinical abnormalities in rats treated at any dose. Ophthalmological examination revealed no abnormalities, nor did the neurotoxic assessment. There was no effect on feed intake or body weight gain, hematological or biochemical parameters. In males, statistically significant decrease in the absolute weight of thymus (G4), lungs (G3, G4); increase in absolute and relative weight of epididymis (G4); decrease in relative weight of lungs (G4) were noted. In females, a statistically significant decrease in absolute and relative weight of ovaries (G2), kidney (G2, G4) and brain (G2) was noted. However, there were no gross pathological changes noted in any of the organs that attained statistical significance. No treatment related gross pathological lesions were observed at all the tested doses.

The no observed adverse effect level (NOAEL) of *B. clausii* 088AE spore preparation in the Sprague Dawley rat, following oral administration for 90 days, was the highest dose tested, 1000 mg/kg bw/day providing  $1.59 \times 10^{11}$  spores/kg bw/day (Annex B-2, B-3). This dose corresponds to  $1.1 \times 10^{13}$  cfu/day for healthy adult male person of 70 kg body weight. Therefore, the Acceptable Daily Intake (ADI) concluded from the NOAEL dose of 90-day toxicity study of *B. clausii* 088AE (adjusted with a 100x safety factor) is  $1.1 \times 10^{11}$  cfu/person/day.

Based upon the greatest estimate of servings of food consumed per day in the US (18.2) and the highest possible additional level of *B. clausii* 088AE per serving ( $2 \times 10^9$  cfu/serving), the maximum Estimated Daily Intake (EDI) is  $3.6 \times 10^{10}$  cfu/day, which is significantly less than the ADI derived from the NOAEL from the 90-day chronic oral toxicity study i.e.  $1.1 \times 10^{11}$  cfu/per day.

As described above, the EDI value of *B. clausii* 088AE is highly exaggerated as it is unlikely that all products in the food categories that are listed above will contain *B. clausii* 088AE and unlikely that consumers will choose all of their food intake only from foods containing *B. clausii* 088AE.

In summary, even when the highly conservative EDI for *B. clausii* 088AE was employed to compare with the ADI developed from the NOAEL (90 day study), the estimated consumption of *B. clausii* 088AE was found to be significantly below the calculated ADI, thus unlikely to present a risk to consumers.

**Bacterial reverse mutation test—Ames assay (OECD 471,1997):** The test was conducted using *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2 uvrA (pKM101) in the presence and absence of S9 metabolic activation. The test was conducted in triplicate at concentrations of 0.05, 0.16, 0.5, 1.6 and 5  $\mu$ L/plate. No significant increase in the number of histidine revertant colonies was reported, and it is concluded that, under the conditions of this study, *B. clausii* 088AE spore preparation is non-mutagenic (Annex C).

**In vitro mammalian chromosomal aberration test in human lymphocytes (OECD 473, 2016):** Cultures of human peripheral blood lymphocytes were exposed to *B. clausii* 088AE spore preparation at concentrations of 0, 0.125, 0.25 and 0.5 mg/mL in the presence and absence of metabolic activation for 3 or 24 hours. No significant concentration related increase was reported

in the incidence of structural chromosome aberrations at any tested concentration, and it was concluded that *B. clausii* 088AE is non-clastogenic in the presence and absence of microsomal enzymes (Annex D).

***In vivo* micronucleus test in mice (OECD 474, 2016):** Four groups of 5 male mice were gavaged with *B. clausii* 088AE spore preparation at doses of 0, 500, 1000, and 2000 mg/kg bw on two consecutive days, after which bone marrow was aspirated and examined microscopically. A total of 4000 polychromatic erythrocytes per mouse were examined for the presence of micronucleated cells. No evidence of toxicity was reported in treated mice or in their bone marrow with no increase in the incidence of micronucleated polychromatic erythrocytes. Based on the results obtained, it was concluded that *B. clausii* 088AE is non-mutagenic under the conditions tested (Annex E).

### 6.3.2. **STUDIES OF OTHER STRAINS OF BACILLUS CLAUSII**

The safety of *B. clausii* UBBC07 in animal models was reported by Lakshmi *et al.* 2017. In the acute toxicity study, the oral LD<sub>50</sub> for *B. clausii* UBBC07 was found to be >5000 mg/kg (630 billion cfu/kg) body weight. In the subacute toxicity study, no mortality was reported and all rats appeared normal, without showing any signs or symptoms of abnormality at doses up to 1000 mg/kg/day (1.3 x 10<sup>11</sup> cfu/kg/day) by the oral route of administration for 28 days. No significant effect on general health, body weight, food consumption, hematological or clinical chemistry profile or urine parameters was found. Relative organ weight and histological observations of vital organs in all treated group were unaffected. The NOAEL (No Observed Adverse Effect Level) for *B. clausii* UBBC07 was found to be 1000 (1.3. x 10<sup>11</sup> cfu) mg/kg body weight/day in subacute toxicity study (Lakshmi *et al.* 2017). The results are consistent with the results presented herein for *B. clausii* 088AE.

## 6.4 **Safety of *Bacillus clausii*—Human Studies**

Several researchers carried out studies with different *Bacillus clausii* strains on human subjects, including children and adults, and evaluated the safety aspects. These studies are summarized in Table 6. In all studies with dosages ranging from 2-6 x 10<sup>9</sup> cfu/day, *Bacillus clausii* was reported to be effective and well tolerated.

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**Table 6. Human Studies of *Bacillus clausii***

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Adults					
Sudha MR <i>et al.</i> (2013)	Phase II, randomized, multiple arm trial of <i>B. clausii</i> strain UBBC-07 for treatment of acute diarrhea	27 patients (average age of 35.44±8.08 years) with acute diarrhea	<i>B. clausii</i> strain UBBC-07 Dose: one capsule containing 2×10 <sup>9</sup> cfu two times a day	10 days	Safety was evaluated by assessing the incidence and type of adverse effects such as increase in blood pressure and pulse rate, physical examination and clinical laboratory tests, i.e. complete blood count, serum glutamic pyruvic transaminase, serum creatinine, and stool examination and microscopy, on day 1 and day 10. No significant changes in safety parameters were observed during treatment.

**Table 6. Human Studies of *Bacillus clausii***

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Nista <i>et al.</i> (2004)	Double-blind, randomized, placebo-controlled trial to study the effect of <i>B. clausii</i> therapy to reduce the side-effects of <i>H. pylori</i> treatment	120 Patients: 60 patients (male/female 33/27, mean age 46.2± 12.83) 60 patients (male/female 25/35, mean age 43.1± 13.36)	<i>Enterogermina</i> containing <i>B. clausii</i> spores Dose: 1 vial containing 2×10 <sup>9</sup> spores, three times a day	14 days Side-effects were studied for 4 weeks	The side-effects were assessed using a validated questionnaire and were recorded for 4 weeks from the start of therapy. The study showed lower incidence of self-reported side-effects and better tolerability to multiple antibiotic treatment during and after a standard seven-day anti- <i>H. pylori</i> regimen when compared with placebo.
Infants & Children					
Marseglia G. L. <i>et al.</i> (2007)	Randomized, single-blind, multi-centre, two arm parallel-group trial of <i>B. clausii</i> spores in the prevention of recurrent respiratory infections	80 Children (39 males and 41 females, mean age 4.3 ± 1.5 years) with recurrent respiratory infections	Enterogermina- Preparation of <i>B. clausii</i> spores Dose: One vial of <i>B. clausii</i> (2 billion spores per 5 ml) two times a day	90 Days	Safety and tolerability of the probiotic were evaluated on the basis of the number and type of adverse events recorded according to

**Table 6. Human Studies of *Bacillus clausii***

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
					<p>the principles of good clinical practice</p> <p>None of the children were withdrawn from the study because of adverse events and very few mild adverse events occurred (3 diarrhoea episodes). Indeed, such events were either related to the underlying disease (RI) or not considered treatment-related.</p> <p>The tolerability profile exhibited in the <i>B. clausii</i> group was similar to that of the control group. The proportion of patients who experienced adverse events was similar in the two groups both during the treatment phase and the follow-up. <i>B. clausii</i> is concluded as safe and well tolerated.</p>

**Table 6. Human Studies of *Bacillus clausii***

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
de Castro, J <i>et al.</i> (2019)	Open-label, multicenter, observational study of <i>B. clausii</i> in the treatment of acute community-acquired diarrhea among Filipino children	3178 patients of acute community-acquired diarrhea lasting for less than 48 hours (median age of 2 years) Age Range: 1 month and 6 years of age	Erceflora® (Sanofi, Philippines) containing <i>Bacillus clausii</i> in the following four bacterial stains: O/C, SIN, N/R, and T. Dose: One to two vials of <i>Bacillus clausii</i> , each 5-mL vial containing an aqueous suspension for oral administration of 2 billion spores	5 to 7 days	Therapy with <i>Bacillus clausii</i> was well-tolerated, and the adverse event rate was very low (0.09%). All reported adverse events, which included vomiting, erythematous rashes and stool color change, were mild to moderate.
Kiran M <i>et al.</i> (2017)	Phase IV clinical study of <i>B. clausii</i> in the treatment of diarrhea	259 patients of diarrhea were recruited for the study out of which 215 patients completed trial and 44 patients were lost to follow-up. Age Group: Patients of either sex having age more than 1 year and less than 12 years	Suspension of <i>Bacillus clausii</i> (2 billion spores per 5 ml) Dose: 2 vials per day containing 2 billion spores per 5 ml, in the interval of 12 hours	5 days	Side effects were evaluated using a list of questions and were recorded for 4 weeks from the start of therapy. The incidences of diarrhea, epigastric pain and nausea in patients treated with <i>B. clausii</i> were significantly lower compared to placebo group. Intensity of diarrhea and nausea in patients treated with <i>B. clausii</i> was significantly lower

**Table 6. Human Studies of *Bacillus clausii***

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
					compared to placebo group.

## 6.5 Decision Tree

Pariza *et al.* 2015, proposed a ‘decision tree’ process to determine the safety of microorganisms for human and animal consumption. The decision tree is a step-wise approach addressing various aspects of safety including identity, history of safe use, genomic and phenotypic safety evaluation. The decision tree process considers scenario as substantially increased exposure to a culture that has an established record of safety in a more limited application; a new strain without a history of safe use that was isolated from a food or feed; or a new strain isolated from a non-food or non-feed source. It is modeled on previous decision trees that are used worldwide to evaluate the safety of microbial enzymes for use in human food or animal feed (Pariza and Cook, 2010; Pariza and Johnson, 2001; Pariza and Foster, 1983). The safety of *B. clausii* 088AE has been established using the Pariza *et al.* 2015 decision tree and the scientific procedures for determining safety of microbial cultures to be consumed by Humans or Animals.

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? **YES**
2. Has the strain genome been sequenced? **YES**
3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? **YES**
4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? **YES**
5. Does the strain produce antimicrobial substances? **NO**
6. Has the strain been genetically modified using rDNA techniques? **NO**
7. 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')? **NO**
8. Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? **NO**

Conclusion: The strain is “deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption” (Pariza *et al.*, 2015).

## 6.6 Safety Assessment and GRAS Determination

This section presents an assessment that demonstrates that the intended use of *B. clausii* 088AE spore preparation is safe and is GRAS based on scientific procedures.

This safety assessment and GRAS determination entails two steps. In the first step, the safety of the intended use of *B. clausii* 088AE is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of consumers to *B. clausii* 088AE under its intended conditions of use is not harmful. In the second step, the intended use of *B. clausii* 088AE is determined to be GRAS by demonstrating that the safety of this spore preparation under its intended conditions of use is based on publicly available and accepted information and is generally recognized as safe by qualified scientific experts.

The regulatory framework for establishing whether the intended use of a substance (or microorganism) is GRAS, in accordance with Section 201(s) of the Federal Food Drug and Cosmetic Act, is set forth under 21 CFR §170.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: 1) through scientific procedures under §170.30(b); or 2) through experience based on common use in food, in the case of a substance used in food prior to January 1, 1958, under §170.30(c). This GRAS determination employs scientific procedures established under §170.30(b) as described by Pariza *et al.* 2015.

A scientific procedure for GRAS determination requires the same quantity and quality of scientific evidence as is needed to obtain approval of the substance as a food additive. In addition to requiring scientific evidence of safety, a GRAS determination also requires that this scientific evidence of safety be generally known and accepted among qualified scientific experts. This “common knowledge” element of a GRAS determination consists of two components:

1. Data and information relied upon to establish the scientific element of safety must be generally available; and
2. There must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific-procedures GRAS determination and the scientific procedures recommended by Pariza *et al.* 2015 are applied below in an analysis of whether the intended use of *B. clausii* 088AE spore preparation is safe and GRAS for the intended uses.

#### **6.6.1 EVIDENCE OF SAFETY**

The food ingredient *B. clausii* 088AE has been studied in detail to establish its safety for human consumption. Studies included a polyphasic approach for strain identification; genome analyses to evaluate the concerns of antibiotic resistance, virulence factors, biogenic amines, various toxins; safety of production process; toxicological studies in animals including acute oral toxicity and 90 days repeated dose oral toxicity. Safety concerns with the antibiotic resistance to clindamycin and erythromycin were also investigated. Antibiotic sensitivity/resistance of various *Bacillus* strains are described in GRN 526,597,660,691,831, demonstrating both intrinsic antibiotic resistance and sensitivity to an array of antibiotics in common use. The antibiotic resistance is a strain-

dependent phenomenon and intrinsic and non-transferable antibiotic resistance is not a safety concern.

Identification of a microorganism is of paramount importance in determining its safety. *B. clausii* 088AE was analyzed for 16S rRNA gene sequence, mol G +C % and phenotypic and biochemical characteristics to establish its identity. Phenotypic and biochemical characteristics of *B. clausii* 088AE were also compared and found similar to a reference strain *B. clausii* ATCC 700160. These studies unambiguously confirm the identity of the strain as *Bacillus clausii*.

*B. clausii* 088AE showed resistance to clindamycin and erythromycin. Genome analysis confirmed that the genes responsible for the antibiotic resistance are not flanked by mobile elements and the resistance is intrinsic and non-transferable horizontally.

A homology search between the assembled genome of *B. clausii* 088AE and virulence factor genes/proteins was performed using BLASTX. The analyses showed no safety concern with respect to virulence factors genes. Further, to confirm the non-virulence of the strain, an in vitro cytotoxicity test against Vero cells was conducted. *B. clausii* 088AE did not show any cytotoxicity. Genome analyses showed absence of genes related to diarrheal enterotoxin bceT, haemolytic enterotoxin operon (hbl genes – hblA, hblC, hblD), non-haemolytic enterotoxin operon (nhe ABC genes – nheA, nheB, nheC), cytotoxin K (cytK), enterotoxin FM (entFM) and emetic toxin cereulide (cesB), suggesting the strain does not produce these toxins.

A BLASTX analysis was performed between the assembled genome and biogenic amine producing proteins. *B. clausii* 088AE does not contain any biogenic amine-producing gene of concern. Laboratory studies confirmed that the strain does not produce biogenic amines. None of the regions of concern, i.e., antibiotic resistance genes, virulence factor genes, and biogenic amine producing genes were reported in the vicinity of the predicted mobile elements in the assembled genome thus ensuring the stability of the genome and constant safe use of the strain. Eleven CRISPRs were identified from the assembled genome of *B. clausii* strain 088AE. The presence of a CRISPR system indicates an advantage in promoting genome stability by acting as a barrier to entry of foreign DNA elements.

No indications of toxicity were reported in 14-day acute and 90-day oral toxicity studies or in genotoxicity assays in strain 088AE or other strains of *B. clausii*. No adverse effects were reported when the spores of various *B. clausii* strains were administered to humans in controlled clinical trials. Finally, *B. clausii* vegetative cells and spore forms have a safe history of use in foods, dietary supplements and registered drugs. Therefore, the weight of the scientific evidence clearly supports the safety of the intended use of *B. clausii* 088AE spore preparation for human consumption.

## **6.6.2 CONCLUSION OF THE EXPERT PANEL**

The expert panel, qualified by their training and experience, has unanimously concluded that Advanced Enzymes' *B. clausii* 088AE spores, manufactured consistent with cGMP



and meeting food grade specifications, is Generally Recognized As Safe (GRAS) based on scientific procedures for addition to baked goods and baking mixes, breakfast cereals, beverages and beverage bases, coffee and tea; milk and milk products, dairy product analogs, fruit juices, condiments and relishes, confections and frostings, frozen dairy desserts and mixes, fruit and water ices, drinking water, sports drinks, gelatins, jams and jellies, puddings and fillings alcoholic beverages grain products and pastas, hard candy, soft candy, chewing gum, extracts, and flavorings, herbs, seeds, spices, seasonings, blends, nuts and nut products, plant protein products, processed fruits, processed vegetables and vegetable juices, snack foods, soups and soup mixes, sugar and sweet sauces, toppings, and syrups, at a maximum level of  $2 \times 10^9$  colony forming units (cfu)/serving.

Further, it is the opinion of the expert panel that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusions (Annex F).

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## Part 7: 21 CFR 170.255: Bibliography-Regulations / References

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## **Part 8: Annex Index**

GRN\_Annex A\_B. clausii 088AE\_Acute oral tox\_BIO-ATX379

GRN\_Annex B-1\_B. clausii 088AE\_90 days tox\_BIO-CTX043

GRN\_Annex B-2\_B. clausii 088AE\_90 days tox\_BIO-CTX043

GRN\_Annex B-3\_B. clausii 088AE\_90 days tox\_BIO-CTX043

GRN\_Annex C\_B. clausii 088AE\_AMES study\_BIO-GNT656

GRN\_Annex D\_B. clausii 088AE\_CA study\_BIO-GNT192

GRN\_Annex E\_B. clausii 088AE\_MNT studyBIO-GNT193

GRN\_Annex F\_B. clausii 088AE\_GRASPanelReport\_AdvancedEnzymeTechnologies

**[DOCUMENT END]**



**FDA USE ONLY**

GRN NUMBER 000971	DATE OF RECEIPT Sep 24, 2020
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

DEPARTMENT OF HEALTH AND HUMAN SERVICES  
Food and Drug Administration  
**GENERALLY RECOGNIZED AS SAFE  
(GRAS) NOTICE** (Subpart E of Part 170)

Transmit completed form and attachments electronically via the Electronic Submission Gateway (*see Instructions*); OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety (*HFS-200*), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5001 Campus Drive, College Park, MD 20740-3835.

**SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION**

1. Type of Submission (*Check one*)  
 New       Amendment to GRN No. \_\_\_\_\_       Supplement to GRN No. \_\_\_\_\_

2.  All electronic files included in this submission have been checked and found to be virus free. (*Check box to verify*)

3. Most recent presubmission meeting (*if any*) with FDA on the subject substance (*yyyy/mm/dd*): \_\_\_\_\_

4. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? (*Check one*)  
 Yes If yes, enter the date of communication (*yyyy/mm/dd*): \_\_\_\_\_  
 No

**SECTION B – INFORMATION ABOUT THE NOTIFIER**

<b>1a. Notifier</b>	Name of Contact Person Anil Kumar Gupta, PhD	Position or Title VP - Research and Development	
	Organization ( <i>if applicable</i> ) Advanced Enzyme Technologies, Ltd		
	Mailing Address ( <i>number and street</i> ) Magnetica LIC Service Road, Louiswadi		
City Thane (W)	State or Province Maharashtra	Zip Code/Postal Code 400604	Country India
Telephone Number +91 22 25830284	Fax Number	E-Mail Address anil@advancedenzymes.com	
<b>1b. Agent or Attorney (if applicable)</b>	Name of Contact Person KEVIN O. GILLIES	Position or Title MEMBER	
	Organization ( <i>if applicable</i> ) KEVIN O. GILLIES CONSULTING SERVICES, LLC		
	Mailing Address ( <i>number and street</i> ) 1759 GRAPE ST.		
City Denver	State or Province CO	Zip Code/Postal Code 80220	Country US
Telephone Number +1-816-590-9836	Fax Number	E-Mail Address INFO@KOGILLIESCONSULTING.COM	

## SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Bacillus clausii 088AE (MCC 0538)

2. Submission Format: *(Check appropriate box(es))*

- Electronic Submission Gateway  Electronic files on physical media  
 Paper  
If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes \_\_\_\_\_

Total number of pages \_\_\_\_\_

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

- Yes *(Proceed to Item 5)*  No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

- a) GRAS Notice No. GRN \_\_\_\_\_  
 b) GRAS Affirmation Petition No. GRP \_\_\_\_\_  
 c) Food Additive Petition No. FAP \_\_\_\_\_  
 d) Food Master File No. FMF \_\_\_\_\_  
 e) Other or Additional *(describe or enter information as above)* \_\_\_\_\_

6. Statutory basis for conclusions of GRAS status *(Check one)*

- Scientific procedures *(21 CFR 170.30(a) and (b))*  Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? *(see 21 CFR 170.225(c)(8))*

- Yes *(Proceed to Item 8)*  
 No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

- Yes, information is designated at the place where it occurs in the submission  
 No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

- Yes, a redacted copy of the complete submission  
 Yes, a redacted copy of part(s) of the submission  
 No

## SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Bacillus clausii 088AE is intended to be used in the following food categories:

Baked goods and baking mixes, breakfast cereals, beverages and beverage bases, coffee and tea, milk and milk products, dairy product analogs, fruit juices, condiments and relishes, confections and frostings, frozen dairy desserts and mixes, fruit and water ices, drinking water, sports drinks, gelatins, jams and jellies, puddings and fillings alcoholic beverages grain products and pastas, hard candy, soft candy, chewing gum, extracts, and flavorings, herbs, seeds, spices, seasonings, blends, nuts and nut products, plant protein products, processed fruits, processed vegetables and vegetable juices, snack foods, soups and soup mixes, sugar and sweet

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

*(Check one)*

- Yes  No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

*(Check one)*

- Yes  No, you ask us to exclude trade secrets from the information FDA will send to FSIS.

**SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE**

*(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)*

- PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).
- PART 3 of a GRAS notice: Dietary exposure (170.235).
- PART 4 of a GRAS notice: Self-limiting levels of use (170.240).
- PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).
- PART 6 of a GRAS notice: Narrative (170.250).
- PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

**Other Information**

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

Yes       No

Did you include this other information in the list of attachments?

Yes       No

**SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS**

1. The undersigned is informing FDA that Advanced Enzyme Technologies, Ltd.  
*(name of notifier)*

has concluded that the intended use(s) of Bacillus clausii 088AE (MCC 0538)  
*(name of notified substance)*

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Advanced Enzyme Technologies, Ltd. *(name of notifier)* agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

Advanced Enzyme Technologies, Ltd, 4880 Murrieta Street, Chino, CA 91710  
*(address of notifier or other location)*

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance. The notifying party certifies that the information provided herein is accurate and complete to the best of his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

**3. Signature of Responsible Official,  
Agent, or Attorney**

**Kevin O. Gillies**

Digitally signed by Kevin O. Gillies  
Date: 2020.09.23 14:55:53 -06'00'

**Printed Name and Title**

Kevin O. Gillies, Agent

**Date (mm/dd/yyyy)**

09/23/2020

## SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667.pdf	Administrative
	GRN_AnnexA_B.clausii088AE_Acuteoraltox_BIO-ATX379.pdf	Administrative
	GRN_AnnexB-1_B.clausii088AE_90daystox_BIO-CTX043.pdf	Administrative
	GRN_AnnexB-2_B.clausii088AE_90daystox_BIO-CTX043.pdf	Administrative
	GRN_AnnexB-3_B.clausii088AE_90daystox_BIO-CTX043.pdf	Administrative
	GRN_AnnexC_B.clausii088AE_AMESstudy_BIO-GNT656.pdf	Administrative
	GRN_AnnexD_B.clausii088AE_CAstudy_BIO-GNT192.pdf	Administrative
	GRN_AnnexE_B.clausii088AE_MNTstudy_BIO-GNT193.pdf	Administrative
	GRN_AnnexF_B.clausii088AE_GRASPanelReport_AdvancedEnzymeTechnologiesLtd.pdf	Administrative

**OMB Statement:** Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, [PRASStaff@fda.hhs.gov](mailto:PRASStaff@fda.hhs.gov). (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

**SECTION G – LIST OF ATTACHMENTS**

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	GRASNOTIFICATION_B.clausii088AE-09232020-AdvancedEnzymeTechnologiesLtd.pdf	Administrative
	GRASNOTIFICATION_B.clausii088AE-09222020-AdvancedEnzymeTechnologiesLtd.pdf	GRAS Notice

**OMB Statement:** Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, [PRASStaff@fda.hhs.gov](mailto:PRASStaff@fda.hhs.gov). (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

**From:** [Kevin O. Gillies](#)  
**To:** [Hall, Karen](#)  
**Cc:** [Ankit Rath;](#) [Rasika Rath;](#) [Anil Gupta](#)  
**Subject:** [EXTERNAL] Re: Regarding GRAS Notice 000971  
**Date:** Monday, June 14, 2021 10:33:32 AM  
**Attachments:** [B. clausii GRAS FDA Query Response 11june.pdf](#)  
[Annexure I.pdf](#)  
[Annexure II \(1\).pdf](#)  
[Annexure III \(1\).pdf](#)  
[Annexure IV \(1\).pdf](#)  
[Annexure V.pdf](#)

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**CAUTION:** This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Good morning Karen. I hope this email finds you well. Please the Advanced Enzymes responses to the FDA technical review questions that you provided in your email of June 4, 2021.

Please contact me if there should be further questions related to GRN000971.

Best,  
Kevin

On Fri, Jun 4, 2021 at 7:15 AM Hall, Karen <[Karen.Hall@fda.hhs.gov](mailto:Karen.Hall@fda.hhs.gov)> wrote:

Dear Kevin,

After reviewing Advanced Enzyme Technologies Ltd. GRAS Notice 000971 for the intended use of *Bacillus clausii* strain 088AE spore preparation, we noted some concerns attached to this email that need to be addressed. Responses may be sent in an email or in a separate document. Please do not send a revised copy of the notice. We respectively request a response within 10 business days. If you are unable to complete the response within that time frame or have questions, please contact me to discuss further options at 240-402-9195 or via email.

Kind Regards,

Karen

**Karen Hall**

*Regulatory Review Scientist*

Division of Food Ingredients  
Office of Food Additive Safety

Center for Food Safety and Applied Nutrition  
U.S. Food and Drug Administration

[Karen.Hall@fda.hhs.gov](mailto:Karen.Hall@fda.hhs.gov)



## GRN 000971: Query Response

### Chemistry

1) On page 22, product specifications Table 4, the notifier states that they used AOAC method 984.27 for the analyses of arsenic, cadmium and lead (a method validated for the quantification of nutrient elements in infant formula using acid decomposition followed by ICP-OES analyses). The method is not validated for the quantification of arsenic, cadmium, and lead and thus it is not appropriate. Please provide results from an appropriate method such as FDA EAM 4.7 or AOAC 2015.01.

#### **Notifier's response:**

The heavy metal analysis was also carried out following AOAC 2015.01 method. Please refer to Annexure I for analysis results.

### Toxicology

The notifier describes multiple unpublished animal toxicity studies to corroborate the safety of their *A. clausii* MCC0538 spore preparation. Please address the following questions regarding interpretations and conclusions from these studies.

- 1) On page 31, the notifier states that no treatment-related gross pathological alterations were observed under the conditions of the study.
  - a. Histopathological results from the study are not described in the safety narrative. Please clarify if treatment-related histopathological (microscopic) lesions were identified in the described study.

#### **Notifier's response:**

Please refer to Annexure II for the response.

- 2) Upon review of the attached Annexes (B1-B3) containing the OECD 408-compliant study report, significantly increased absolute and relative spleen and uterus weights were reported in treated male rats (G2, G3, G4, M) and female rats (G2, G3, G4, F), respectively. Notably, splenomegaly may be an indirect indicator of immune stimulation or infection.
  - a. Please address these effects associated with *A. clausii* MCC0538 spore treatment and discuss why such effects are not considered treatment related or adverse.

#### **Notifier's response:**

A detailed response from the contract toxicology lab (M/s Bioneds) is presented in the Annexure II.



## **Microbiology**

As *Bacillus clausii* was recently reclassified<sup>1</sup>, we note that we will use the new nomenclature for the notified strain (i.e., *Alkalihalobacillus clausii* strain MCC0538 spore preparation) in our correspondence, but will provide the strain's previous name in a footnote, linking the old nomenclature with the new nomenclature. In a future GRAS notice, we recommend that the notifier use the new nomenclature for strains affected by taxonomic reorganization, but should also provide the strain's previous name, clearly linking the old nomenclature with the new nomenclature, and include a reference to the publication.

1. Please state that this ingredient is a spore preparation and provide an approximate ratio of spores to vegetative cells.

### **Notifier's Response:**

*A. clausii* (*B. clausii*) strain MCC 0538 preparation contains only spores. During manufacturing, most of the vegetative cells get converted into the spores at the end of the fermentation. Further holding of fermentation broth at 60°C for 30 min before harvest, completes sporulation process and effectively inactivates/kills vegetative cells. The final preparation contains approx. 100% spore population. The viable cell count of the final product, with and without heat treatment, has shown almost the same cell count, which further confirms that the ingredient contains almost 100% spores.

2. For the administrative record, please describe whether *A. clausii* (previously classified as *B. clausii*) strain MCC 0538 produces antibiotics.

### **Notifier's Response:**

*A. clausii* (*B. clausii*) strain MCC 0538 doesn't produce antibiotics.

The strain was tested for its antibiotic activity (antimicrobial activity) against 8 test bacteria as described in the section 2.1.5 of the dossier. The ability of the *A. clausii* (*B. clausii*) strain MCC 0538 to produce antimicrobial compounds was assessed according to the Clinical and Laboratory Standards Institute (CLSI, 2012) guidelines as recommended by the European Food Safety Authority (EFSA, 2012), the United States Pharmacopoeia (USP, 2008) and as per the Food and Agriculture Organization (2006). The strain did not show any antimicrobial activity. These results suggest lack of antibiotic production by *A. clausii* (*B. clausii*) strain MCC 0538.

3. Please state whether *A. clausii* (previously classified as *B. clausii*) strain MCC 0538 is genetically engineered.

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<sup>1</sup> Patel, S., and Gupta, R. S. (2020). A phylogenomic and comparative genomic framework for resolving the polyphyly of the genus *Bacillus*: Proposal for six new genera of *Bacillus* species, *Peribacillus* gen. nov., *Cytobacillus* gen. nov., *Mesobacillus* gen. nov., *Neobacillus* gen. nov., *Metabacillus* gen. nov. and *Alkalihalobacillus* gen. nov. *International Journal of Systematic and Evolutionary Microbiology*, 70(1), 406-438. doi: 10.1099/ijsem.0.003775

**Notifier's Response:**

*A. clausii* (*B. clausii*) strain MCC 0538 is not a genetically engineered strain.

4. On page 10, the notifier states "A total of 706 virulence factor proteins were found to have significant homology with the assembled genome", however, only lists a total of 698 non-classical virulence factor genes and their determinants. Please clarify this discrepancy.

**Notifier's Response:**

Analysis of *A. clausii* (*B. clausii*) strain MCC 0538 genome resulted in 706 hits as virulence factor proteins based on the sequence similarity, out of which 698 were identified as non-classical virulence factors proteins. Other 8 sequences could not be characterized as virulence factor. Therefore, we change virulence factor proteins numbers to 698 instead of 706 as mentioned in the dossier.

5. On page 11, the notifier states, "Unlike other products of amino acid decarboxylase, beta-alanine supplementation is known for its ergogenic effect on high intensity exercise performance in humans (Hobson *et al*, 2012). Hence, *B. clausii* strain 088AE does not possess biogenic amine producing genes of concern". We note that the population of beta-alanine supplement-users referenced by the notifier represents a subset of the general population. As such, please describe whether production beta-alanine by *A. clausii* (previously classified as *B. clausii*) strain MCC 0538 poses a safety concern to the general population.

**Notifier's Response:**

The genome of the *A. clausii* (*B. clausii*) strain MCC 0538 contains the gene aspartate decarboxylase responsible for the production of beta-alanine. Aspartate decarboxylase gene is known to be present in different bacterial species such as *Lactobacillus plantarum* (Evanovich *et al*, 2019), *Bacillus clausii* (<https://www.uniprot.org/uniprot/A0A268RZ17>), *Bacillus subtilis* (Deng *et al*, 2015). Bacteria of these species are known to be consumed by human beings.

Evanovich *et al*. (2019) indicated that the gene aspartate decarboxylase in *Lactobacillus plantarum* did not produce any deleterious amount of  $\beta$ -alanine when analyzed in the culture media. The low level of beta-alanine could be attributed to limited availability of the substrate and metabolic status of the microbial cells.

A systematic risk assessment on the oral consumption of beta-alanine was carried out by Dolan *et al*, 2019. In total, 101 human and 50 animal studies were included. The main effect of  $\beta$ -alanine supplementation on taurine concentration was reported for murine models, but only when the daily dose was  $\geq 3\%$   $\beta$ -alanine in drinking water. The results of this review indicated that  $\beta$ -alanine supplementation within the doses used in the available research designs, does not adversely affect those consuming it. The presence of the gene aspartic decarboxylase in the strain *A. clausii* (*B. clausii*) MCC 0538, is unlikely to produce such high concentration of beta-alanine,

therefore the presence of this gene in *A. clausii* (*B. clausii*) MCC 0538 doesn't pose a safety concern.

6. On page 16, the notifier states, "Both strains were found resistant to aztreonam, cefepime, cefixime, cefotaxime, clindamycin, oxacillin, metronidazole, erythromycin and cefuroxime at the given concentrations. The test strain, *B. clausii* 088AE, was resistant to ceftazidime (CAZ, 30 µg) whereas, the reference strain, *B. clausii* DSM 8716 was sensitive". Please describe whether this poses a potential safety concern.

**Notifier's Response:**

The resistance mechanism for ceftazidime is known to be mediated through beta-lactamase mechanism and also associated with its level of expression (Alanbar *et al*, 2020). The resistance to ceftazidime (CAZ, 30 ug) does not have any safety concern since the identified beta-lactamase gene in the genome of *A. clausii* (*B. clausii* 088AE) is intrinsic and do not pose a risk of horizontal gene transfer.

7. For the administrative record, please briefly specify how the purity of the initial inoculum is ensured, and state whether the fermentation process is conducted in a contained, sterile environment.

**Notifier's Response:**

The procedure of inoculum preparation is carried out by the trained personnel in the biosafety cabinets, installed in class 100000 cleanroom, subsequently growth of the inoculum on laboratory shaker is also carried out in the similar cleanroom environment.

Initial inoculum purity of *A. clausii* (*B. clausii*) strain MCC 0538 is ensured by following its specific growth pattern, direct microscopic evaluation, and characteristic viable growth on agar medium.

The fermentation process for *A. clausii* (*B. clausii*) strain MCC 0538 is carried out in a contained, sterile environment in a closed vessel.

8. In Table 4 (page 22), the notifier does not list an aerobic plate count among the microbial specifications. Does the notifier perform an aerobic plate count? If not, please explain why this specification is not included.

**Notifier's Response:**

The spore count analysis for *A. clausii* (*B. clausii*) strain MCC 0538 is carried out on the 'nutrient agar' medium, which is similar to the method of aerobic plate count. Therefore, the total aerobic plate count is not separately mentioned in the specifications. However, the presence of other microbes (pathogens) are analysed separately following the harmonized pharmacopeial method (see point 9).

Moreover, the aerobic plate count method is not applicable to products containing viable microorganisms as active ingredients (USP 42, Microbiology tests, Chapter 61,

page 6387 (Please refer to Annexure III), therefore the product *A. clausii* (*B. clausii*) does not require a separate testing for aerobic microbial count.

9. In Table 4 (page 22), the notifier lists the reference method for total yeast and mold count, *Escherichia coli*, *Salmonella* serovars, *Pseudomonas aeruginosa*, and *Staphylococcus* spp. as “harmonized method (IP, BP, EP, and USP)” or “harmonized pharmacopeial method (EP, BP, USP, and IP)” or “harmonized pharmacopeial method (BP, USP, IP)”. Please explain what this refers to.

**Notifier’s Response:**

The harmonised pharmaceutical method refers to the method of analysis adopted for “2.6.12 Microbiological examination of non-sterile products: Microbiological enumeration test” and “2.6.13 Microbiological examination of non-sterile products: Test for specified microorganism” ref European pharmacopoeia 8.0 chapter 5.8”, which is adopted by EP (European Pharmacopoeia), JP (Japanese Pharmacopoeia) and USP (United States Pharmacopoeia). As the IP (Indian Pharmacopoeia) method is same as USP, it is considered as a part of the harmonised method. (Annexure IV)

10. The notifier states that the method used to detect total coliforms is “FDA Bacteriological Analytical Manual” (page 22). For the administrative record, please provide the chapter number from the FDA Bacteriological Analytical Manual used for this referenced method.

**Notifier’s Response:**

The method used to detect total coliforms is adapted from FDA Bacteriological Analytical Manual”, reference used is “Section G: Solid medium method- Coliforms” under “Bacteriological Analytical Manual Chapter 4: Enumeration of *Escherichia coli* and the Coliform Bacteria I FDA”. (Annexure V)

11. References to “*Salmonella typhimurium*” on page 31 and in Appendix C should read *Salmonella* Typhimurium, as serovars are not italicized. Please make a statement that corrects this reference.

**Notifier’s Response:**

*Salmonella enterica* subsp. *enterica* serovar Typhimurium is mentioned as *Salmonella* Typhimurium.

12. Please provide an updated literature search that discusses the safety of *A. clausii* (previously classified as *B. clausii*), including the date (month and year) the literature search was performed and discuss whether there are any study results that may be contradictory to a GRAS conclusion. Please discuss how these studies pertain to the safety of the intended uses of the ingredient. Examples include, but are not limited to, the following:

- a. Khatri, A. M., Rai, S., Shank, C., McInerney, A., Kaplan, B., Hagmann, S. H. F., and Kainth, M. K. (2021). A tale of caution: prolonged *Bacillus clausii* bacteraemia after probiotic use in an immunocompetent child. *Access Microbiology*, 3(3), 1-5. doi: 10.1099/acmi.0.000205
- b. Princess, I., Natarajan, T., Ghosh, S. (2020). When good bacteria behave badly: a case report of *Bacillus clausii* sepsis in an immunocompetent adult. *Access Microbiology*, 2(4), 1-3. doi: 10.1099/acmi.0.000097

**Notifier's Response:**

An updated list of scientific publications on *A. clausii* (*B. clausii*) is presented in Table 1, which also includes earlier publications establishing the safety of *A. clausii* (*B. clausii*), The safety of *A. clausii* is demonstrated in these studies following preclinical and clinical trials, genome analysis, and other scientific experiments.

**Table 1. Scientific publications on *A. clausii* (*B. clausii*)**

Safety reports of <i>Bacillus clausii</i>	Search date/Publication year
Navarra, P., Milleri, S., III Perez, M., Uboldi, M. C., Pellegrino, P., De Fer, B. B., and Morelli, L. (2021). Kinetics of intestinal presence of spores following oral administration of <i>Bacillus clausii</i> formulations: three single-centre, crossover, randomised, open-label studies. <i>European Journal of Drug Metabolism and Pharmacokinetics</i> , 46, 375–384. <a href="https://doi.org/10.1007/s13318-021-00676-2">https://doi.org/10.1007/s13318-021-00676-2</a>	May 2021
García, J. P., Alzate, J. A., Hoyos, J. A., and Cristancho, E. (2021). Bacteremia after <i>Bacillus clausii</i> administration for the treatment of acute diarrhea: a case report. <i>Biomédica</i> , 41(2), 1-22. <a href="https://doi.org/10.7705/biomedica.5662">https://doi.org/10.7705/biomedica.5662</a>	May 2021
Khatri, A. M., Rai, S., Shank, C., McInerney, A., Kaplan, B., Hagmann, S. H. F., and Kainth, M. K. (2021). A tale of caution: prolonged <i>Bacillus clausii</i> bacteraemia after probiotic use in an immunocompetent child. <i>Access Microbiology</i> , 3(3), 1-5. doi: 10.1099/acmi.0.000205	February 2021
De Castro, J. A., Kesavelu, D., Lahiri, K. R., Chaijitraruch, N., Chongsrisawat, V., Jog, P. P., Liaw, Y. H., Nguyen, G. K., Nguyen, T. V. H., Pai, U. A., Phan, H. N. D., Quak, S. H., Tanpowpong, P., and Guno, M. J. (2020). Recommendations for the adjuvant use of the poly-antibiotic-resistant probiotic <i>Bacillus clausii</i> (O/C, SIN, N/R, T) in acute, chronic, and antibiotic-associated diarrhea in children: consensus from Asian experts. <i>Tropical Diseases, Travel Medicine and Vaccines</i> , 6, 21. <a href="https://doi.org/10.1186/s40794-020-00120-4">https://doi.org/10.1186/s40794-020-00120-4</a>	January 2021
Plomer, M., III Perez, M., and Greifenberg, D. M. (2020). Effect of <i>Bacillus clausii</i> capsules in reducing adverse effects associated with helicobacter pylori eradication therapy: a randomized, double-blind, controlled trial. <i>Infectious Diseases and Therapy</i> , 9, 867–878. <a href="https://doi.org/10.1007/s40121-020-00333-2">https://doi.org/10.1007/s40121-020-00333-2</a>	December 2020
Patel, C., Patel, P., and Acharya, S. (2020). Therapeutic prospective of a spore-forming probiotic— <i>Bacillus clausii</i> UBBC07 against acetaminophen-induced uremia in rats. <i>Probiotics and Antimicrobial Proteins</i> , 12, 253–258. <a href="https://doi.org/10.1007/s12602-019-09540-x">https://doi.org/10.1007/s12602-019-09540-x</a>	May 2020
Princess, I., Natarajan, T., and Ghosh, S. (2020). When good bacteria behave badly: a case report of <i>Bacillus clausii</i> sepsis in an immunocompetent adult. <i>Access Microbiology</i> , 2(4), 1-3. doi: 10.1099/acmi.0.000097	February 2020

Joshi, S., Udani, S., Sen, S., Kirolikar, S., and Shetty, A. (2019). <i>Bacillus clausii</i> septicemia in a pediatric patient after treatment with probiotics. <i>The Pediatric Infectious Disease Journal</i> , 38(9), e228-e230. doi:10.1097/INF.0000000000002350	September 2019
Ratna, M. S., Jayanthi, N., Pandey, D. C., and Verma, A. K. (2019). <i>Bacillus clausii</i> UBBC-07 reduces severity of diarrhoea in children under 5 years of age: a double blind placebo controlled study. <i>Beneficial Microbes</i> , 10(2), 149-154. <a href="https://doi.org/10.3920/BM2018.0094">https://doi.org/10.3920/BM2018.0094</a>	May 2019
Kong, X. X., Jiang, J. L., Qiao, B., Liu, H., Cheng, J. S., and Yuan, Y. J. (2019). The biodegradation of cefuroxime, cefotaxime and cefpirome by the synthetic consortium with probiotic <i>Bacillus clausii</i> and investigation of their potential biodegradation pathways. <i>Science of The Total Environment</i> , 651(1), 271-280. <a href="https://doi.org/10.1016/j.scitotenv.2018.09.187">https://doi.org/10.1016/j.scitotenv.2018.09.187</a>	May 2019
Gargar, J. D., and Divinagracia R. M. (2019). When good things go bad: a case series of bacteremia from probiotics. <i>Chest</i> , 155(4), 92A. <a href="https://doi.org/10.1016/j.chest.2019.02.091">https://doi.org/10.1016/j.chest.2019.02.091</a>	April 2019
Nacinovich, F., Fernández Osés, P., Sucari, A., Gentiluomo, J., Merkt, M., Castillo, S., Zanella, E., Ramirez, S., Montaña, S., Zitto, T., and Pennini, M. (2019). Probiotics in the critically ill: friends or foes? Persistent bacteriemia due to <i>Bacillus clausii</i> . 29 <sup>th</sup> European Congress of Clinical Microbiology and Infectious Diseases, 13-16, Amsterdam, Netherlands.	April 2019
Kapse, N., Engineer, A., Gowdaman, V., Wagh, S., and Dhakephalkar, P. (2018). Genome profiling for health promoting and disease preventing traits unraveled probiotic potential of <i>Bacillus clausii</i> B106. <i>Microbiology and Biotechnology Letters</i> , 46(4), 334–345. <a href="https://doi.org/10.4014/mbl.1804.04001">https://doi.org/10.4014/mbl.1804.04001</a>	February 2018
Ianiro, G., Rizzatti, G., Plomer, M., Lopetuso, L., Scaldaferri, F., Franceschi, F., Cammarota, G., and Gasbarrini, A. (2018). <i>Bacillus clausii</i> for the treatment of acute diarrhea in children: a systematic review and meta-analysis of randomized controlled trials. <i>Nutrients</i> , 10, 1074. <a href="https://doi.org/10.3390/nu10081074">https://doi.org/10.3390/nu10081074</a>	October 2018
Lakshmi, S. G., Jayanthi, N., Saravanan, M., and Ratna, M. S. (2017). Safety assesment of <i>Bacillus clausii</i> UBBC07, a spore forming probiotic. <i>Toxicology Reports</i> , 4, 62-71. <a href="https://doi.org/10.1016/j.toxrep.2016.12.004">https://doi.org/10.1016/j.toxrep.2016.12.004</a>	February 2017
Kiran, M., and Pawaskar, L. (2017). Efficacy and safety for suspension of <i>Bacillus clausii</i> while treating the patient of diarrhoea. <i>Indian Journal of Basic and Applied Medical Research</i> , 7(1), 251-257.	January 2017
Lopetuso, L. R., Scaldaferri, F., Franceschi, F., and Gasbarrini, A. (2016). <i>Bacillus clausii</i> and gut homeostasis: state of the art and future perspectives. <i>Expert Review of Gastroenterology and Hepatology</i> , 10(8), 943-948. DOI:10.1080/17474124.2016.1200465	January 2016
Upadrasta, A., Pitta, S., and Ratna, M. S. (2016). Draft genome sequence of <i>Bacillus clausii</i> UBBC07, a spore-forming probiotic strain. <i>Genome Announcement</i> , 4(2), e00235-16. doi:10.1128/genomeA.00235-16	January 2016
Tewari, V. V., Dubey, S. K., and Gupta, G. (2015). <i>Bacillus clausii</i> for prevention of late-onset sepsis in preterm infants: a randomized controlled trial. <i>Journal of Tropical Pediatrics</i> , 61(5), 377–385. <a href="https://doi.org/10.1093/tropej/fmv050">https://doi.org/10.1093/tropej/fmv050</a>	February 2015
Jayanthi, N., and Ratna, M. S. (2015). <i>Bacillus clausii</i> -the probiotic of choice in the treatment of diarrhea. <i>Journal of Yoga and Physical Therapy</i> , 5(4), 211. <a href="http://dx.doi.org/10.4172/2157-7595.1000211">http://dx.doi.org/10.4172/2157-7595.1000211</a>	January 2015
Marseglia, G. L., Tosca, M., Cirillo, I., Licari, A., Leone, M., Marseglia, A., Castellazzi, A. M., and Ciprandi, G. (2007). Efficacy of <i>Bacillus clausii</i> spores in the prevention of recurrent respiratory infections in children: a pilot study. <i>Therapeutics and clinical risk management</i> , 3(1), 13–17. <a href="https://doi.org/10.2147/tcrm.2007.3.1.13">https://doi.org/10.2147/tcrm.2007.3.1.13</a>	January 2007
Ciprandi, G., Vizzaccaro, A., Cirillo, I., and Tosca, M. A. (2005). <i>Bacillus clausii</i> exerts immuno-modulatory activity in allergic subjects: a pilot study. <i>European Annals of Allergy and Clinical Immunology</i> , 37(4), 129-134.	June 2005

Ciprandi, G., Tosca, M.A., Milanese, M., Caligo, G., and Ricca, V. (2004). Cytokines evaluation in nasal lavage of allergic children after <i>Bacillus clausii</i> administration: a pilot study. <i>Pediatric Allergy and Immunology</i> , 15(2), 148-151. <a href="https://doi.org/10.1046/j.1399-3038.2003.00102.x">https://doi.org/10.1046/j.1399-3038.2003.00102.x</a>	June 2004
Nista, E. C., Candelli, M., Cremonini, F., Cazzato, I. A., Zocco, M. A., Franceschi, F., Cammarota, G., Gasbarrini, G., and Gasbarrini, A. (2004). <i>Bacillus clausii</i> therapy to reduce side-effects of anti- <i>Helicobacter pylori</i> treatment: randomized, double-blind, placebo-controlled trial. <i>Alimentary Pharmacology and Therapeutics</i> , 20(10), 1181-1188. <a href="https://doi.org/10.1111/j.1365-2036.2004.02274.x">https://doi.org/10.1111/j.1365-2036.2004.02274.x</a>	January 2004

*A. clausii* (*B. clausii*) has been subject to several systematic clinical, preclinical, and randomized controlled trials. The results of these trials demonstrated safety and health benefits of *A. clausii* (Ciprandi *et al.*, 2004, Nista *et al.*, 2004, Ciprandi *et al.*, 2005, Sudha *et al.*, 2013, Tewari *et al.*, 2015, De Castro *et al.*, 2019, Patel *et al.*, 2019, Paparo *et al.*, 2020, Plomer *et al.*, 2020). These studies have established the safety of *A. clausii* (*B. clausii*) with no incidence of adverse effects and serious adverse effects in both sex of infant, children, adult and aged population. Genomic data also demonstrated the safety of *A. clausii* (*B. clausii*) strains (Lakshmi *et al.*, 2017; Kapse *et al.*, 2018). Lopetuso *et al.* (2016) reviewed the link between clinical benefits and multi-modal gut homeostasis following *A. clausii* (*B. clausii*) administration. The colonization of *A. clausii* (*B. clausii*) appears to be transient in the human gastrointestinal tract (Navarra *et al.*, 2021). Based on the safety findings, *A. clausii* (*B. clausii*) is recommended as an adjunct treatment with oral rehydration solution for acute viral diarrhea, antibiotic-associated diarrhea, *Clostridium difficile*-induced diarrhea, and as adjunct treatment of *Helicobacter pylori* by an Asian expert panel of pediatricians, pediatric gastroenterologists and a pediatric infectious disease specialist (De Castro *et al.*, 2020).

While several systematic clinical trials and other studies establish the safety of *A. clausii* (*B. clausii*), a few case reports describe its adverse effects in high-risk population consist of patients with immune-compromised complexity, post-surgery and critically illness. The studies included in the table 2 describe association of *A. clausii* (*B. clausii*) with possible bacteremia infection in a total nine patients without a clear clinical site of infection. Eight out of 9 patients were immunocompromised and had co-morbidities like type II diabetes mellitus, congenital heart disease, chronic obstructive lung disease, cancer, ischemic colitis, underwent surgery, whereas one patient had a single episode of non-bloody, non-bilious emesis and non-bloody diarrhea, respiratory viral infections (Table 2).

*A. clausii* (*B. clausii*) is known to be safely consumed by human being for decades and it is not uncommon that the strains are occasionally isolated from diseased/immunocompromised persons. Such cases have been sporadically reported for several beneficial microorganisms consumed by general human population, like *Bacillus coagulans* (Banerjee *et al.*, 1998; GRN 691, 2017), *Bacillus subtilis* (Edberg, 1991; Logan, 2004; GRN 831, 2018; GRN 905, 2019), *Lactobacillus rhamnosus* GG (Kunz *et al.*, 2004; Land *et al.*, 2005), *Lactobacillus fermentum* CECT5716 (GRN 820, 2018) and *Sachcharomyces boulardii* (Lungarotti *et al.*, 2003; Lolis *et al.*, 2008; GRN 841, 2019) in critically ill and immunosuppressed patients.

Therefore, a few reports on association of *A. clausii* (*B. clausii*) with diseased conditions appears more coincidental. A systematic analysis of reports indicates that the growth of *A. clausii* (*B. clausii*) in such cases may only be opportunistic in immunocompromised population, and would not be defined as virulent. Importantly, no cases of bacteremia have been linked to the ingested strain of *A. clausii* (*B. clausii*).

Based on the above review of the scientific reports, and history of the safe consumption of the *A. clausii* (*B. clausii*), it can be concluded that the consumption of *A. clausii* (*B. clausii*) is safe for the general human population and the intended use is generally recognized as safe (GRAS) based on scientific procedures.

**Table 2. Case reports of bacteremia associated to *A. clausii* (*B. clausii*) in patients with chronic medical conditions.**

Reference	Search date	Patient/medical history/conditions	Symptoms
García, J. P., Alzate, J. A., Hoyos, J. A., and Cristancho, E. (2021). Bacteremia after <i>Bacillus clausii</i> administration for the treatment of acute diarrhea: a case report. <i>Biomédica</i> , 41(2), 1-22. <a href="https://doi.org/10.7705/biomedica.5662">https://doi.org/10.7705/biomedica.5662</a>	May 2021	Pt#1: An 87-year-old woman with arterial hypertension, chronic obstructive lung disease, and a cholecystectomy	Fever, elevated WBC count
Khatri, A. M., Rai, S., Shank, C., McInerney, A., Kaplan, B., Hagmann, S. H. F., and Kainth, M. K. (2021). A tale of caution: prolonged <i>Bacillus clausii</i> bacteraemia after probiotic use in an immunocompetent child. <i>Access Microbiology</i> , 3(3), 1-5. doi: 10.1099/acmi.0.000205	February 2021	Pt#1: A 17-month-old girl with non-bloody, non-bilious emesis and non-bloody diarrhea, respiratory viral infections	Fever, elevated WBC count
Princess, I., Natarajan, T., Ghosh, S. (2020). When good bacteria behave badly: a case report of <i>Bacillus clausii</i> sepsis in an immunocompetent adult. <i>Access Microbiology</i> , 2(4), 1-3. doi: 10.1099/acmi.0.000097	February 2020	Pt#1: A middle-age female type II diabetes mellitus	Fever
Joshi, S., Udani, S., Sen, S., Kirolikar, S., Shetty, A. (2019) <i>Bacillus clausii</i> septicemia in a pediatric patient after treatment with probiotics. <i>The Pediatric Infectious Disease Journal</i> , 38(9), e228-e230. doi:10.1097/INF.0000000000002350	September 2019	Pt#1: A 4-month-old male infant with congenitally corrected transposition of great arteries, mild pulmonary stenosis and dextrocardia	Fever, respiratory distress
Gargar, J. D., Divinagracia R. M. (2019) When good things go bad: a case series of bacteremia from probiotics. <i>Chest</i> , 155(4), 92A. <a href="https://doi.org/10.1016/j.chest.2019.02.091">https://doi.org/10.1016/j.chest.2019.02.091</a>	April 2019	Pt#1&2: Stage IV lung cancer Pt#3: Ischemic colitis	Fever, elevated WBC count
Nacinovich, F., Fernández Osés, P., Sucari, A., Gentiluomo, J., Merkt, M., Castillo, S., Zanella, E., Ramirez, S., Montaña, S., Zitto, T., Pennini, M. (2019) Probiotics in the critically ill: friends or foes? Persistent bacteremia due to <i>Bacillus clausii</i> . 29 <sup>th</sup> European Congress of Clinical	April 2019	Pt#1: Male, 69 years old; underwent aortic valve replacement Pt#2: Male, 71 years old; underwent mitral	Fever



Microbiology and Infectious Diseases, 13-16, Amsterdam, Netherlands.		valve replacement and Tr valve repair	
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## References:

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**TEST REPORT**

Report No : TUV(I)/10231/18-19/0101803793

Date : 01 Nov 2018

 Name & Address of Customer : Advanced Enzymes Technologies Ltd.  
 Plot No A 61/62, Malegaon MIDC, Sinnar,,  
 Nashik  
 Pin Code: 422103

 Reg No. : 10231/18-19  
 CA No. : 0101803793  
 Date of sample receipt : 22 Oct 2018  
 Date(s) of analysis : 29 Oct 2018 - 01 Nov 2018  
 Sample Drawn by : Customer

SINo	Test Name	Results	Unit	LOQ	Test Method
<b>Sample Name : Bacillus Clausii</b>		<b>CA No : 0101803793</b>			
Batch No: 101841					
<b>Aflatoxins</b>					
1	Aflatoxin B2	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
2	Aflatoxin G1	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
3	Aflatoxin G2	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
4	Total Aflatoxin	< LOQ	µg/kg	--	TUV/03/SOP/016 Based on AOAC 2005.08
5	Aflatoxin B1	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
<b>Heavy Metals</b>					
6	Cadmium	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
7	Lead	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
8	Mercury	< LOQ	mg/kg	0.025	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
9	Arsenic	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
<b>Mycotoxins</b>					
10	Ochratoxin A	< LOQ	ug/kg	1.0	TUV/03/SOP/084



## TEST REPORT


Report No : TUV(I)/10231/18-19/0101803793

Date : 01 Nov 2018

LOQ-Limit of Quantification

Verified & Authorized by



  
Atulkumar Rajage  
Head - Instrumentation Laboratory

-- End of Report --

1. Test Results are based on & related only to the particular sample(s) tested.
2. This Report cannot be re-produced, except when in full, without the written permission from TUV India Pvt. Ltd.
3. This Certificate reflects our findings at the time and place of testing.
4. Sample(s) will be retained by us for a period of one month for non-perishable items only. Perishable items will be destroyed after completion of tests.
5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes.
6. All terms and conditions of our quotation on the basis of which this testing service has been provided are deemed to be fully accepted by the customer and are deemed to be in full force and effect.
7. This Report is exclusively for the use of the customer whose name and address is indicated above. No third party can derive rights against the company on the basis of this report. No third party has any right to raise any claims on the company.
8. For Biological Analysis : Our analytical findings reflect the quality of the sample at the time of testing. No responsibility can be accepted for the possible consequences of further development of micro-organisms which may depend upon storage, handling & weather conditions which may influence the results at a later date/time.

## TEST REPORT

Report No : TUV(I)/10231/18-19/0101803794

Date : 01 Nov 2018

Name & Address of Customer : Advanced Enzymes Technologies Ltd.  
Plot No A 61/62, Malegaon MIDC, Sinnar,,  
Nashik  
Pin Code: 422103

Reg No. : 10231/18-19  
CA No. : 0101803794  
Date of sample receipt : 22 Oct 2018  
Date(s) of analysis : 29 Oct 2018 - 01 Nov 2018  
Sample Drawn by : Customer

SINo	Test Name	Results	Unit	LOQ	Test Method
<b>Sample Name : Bacillus Clausii</b>		<b>CA No : 0101803794</b>			
Batch No: 101842					
<b>Aflatoxins</b>					
1	Aflatoxin B2	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
2	Aflatoxin G1	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
3	Aflatoxin G2	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
4	Total Aflatoxin	< LOQ	µg/kg	--	TUV/03/SOP/016 Based on AOAC 2005.08
5	Aflatoxin B1	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
<b>Heavy Metals</b>					
6	Cadmium	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
7	Lead	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
8	Mercury	< LOQ	mg/kg	0.025	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
9	Arsenic	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
<b>Mycotoxins</b>					
10	Ochratoxin A	< LOQ	ug/kg	1.0	TUV/03/SOP/084



## TEST REPORT


Report No : TUV(I)/10231/18-19/0101803794

Date : 01 Nov 2018

LOQ-Limit of Quantification

Verified & Authorized by



  
Atulkumar Rajage  
Head - Instrumentation Laboratory

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5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes.
6. All terms and conditions of our quotation on the basis of which this testing service has been provided are deemed to be fully accepted by the customer and are deemed to be in full force and effect.
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8. For Biological Analysis : Our analytical findings reflect the quality of the sample at the time of testing. No responsibility can be accepted for the possible consequences of further development of micro-organisms which may depend upon storage, handling & weather conditions which may influence the results at a later date/time.





## TEST REPORT

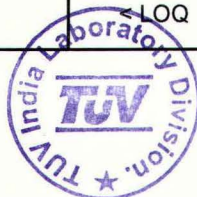
Report No : TUV(I)/10231/18-19/0101803792

Date : 01 Nov 2018

Name & Address of Customer : Advanced Enzymes Technologies Ltd.  
Plot No A 61/62, Malegaon MIDC, Sinnar,,  
Nashik  
Pin Code: 422103

Reg No. : 10231/18-19  
CA No. : 0101803792  
Date of sample receipt : 22 Oct 2018  
Date(s) of analysis : 29 Oct 2018 - 01 Nov 2018  
Sample Drawn by : Customer

SINo	Test Name	Results	Unit	LOQ	Test Method
<b>Sample Name : Bacillus Clausii</b>		<b>CA No : 0101803792</b>			
Batch No: 101840					
<b>Aflatoxins</b>					
1	Aflatoxin B2	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
2	Aflatoxin G1	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
3	Aflatoxin G2	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
4	Total Aflatoxin	< LOQ	µg/kg	--	TUV/03/SOP/016 Based on AOAC 2005.08
5	Aflatoxin B1	< LOQ	µg/kg	0.5	TUV/03/SOP/016 Based on AOAC 2005.08
<b>Heavy Metals</b>					
6	Cadmium	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
7	Lead	0.15	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
8	Mercury	< LOQ	mg/kg	0.025	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
9	Arsenic	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 20th Edition
<b>Mycotoxins</b>					
10	Ochratoxin A	< LOQ	ug/kg	1.0	TUV/03/SOP/084



## TEST REPORT


Report No : TUV(I)/10231/18-19/0101803792

Date : 01 Nov 2018

LOQ-Limit of Quantification

Verified & Authorized by



  
Atulkumar Rajage  
Head - Instrumentation Laboratory

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1. Test Results are based on & related only to the particular sample(s) tested.
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5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes.
6. All terms and conditions of our quotation on the basis of which this testing service has been provided are deemed to be fully accepted by the customer and are deemed to be in full force and effect.
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8. For Biological Analysis : Our analytical findings reflect the quality of the sample at the time of testing. No responsibility can be accepted for the possible consequences of further development of micro-organisms which may depend upon storage, handling & weather conditions which may influence the results at a later date/time.

No.: SL 4171/21

Date: 11/06/2021

**TO WHOMSOEVER IT MAY CONCERN**

**Study No.:** BIO-CTX 043**Study Code:** 90-OR**Subject:** Response to Queries

**1. Histopathological results from the study are not described in the safety narrative. Please clarify if treatment-related histopathological (microscopic) lesions were identified in the described study.**

**Bioneeeds response:**

There were no test item-related microscopic changes in the study. Few random incidences like ectopic thymic tissue in thyroid gland, squamous cyst in glandular stomach were considered to be background findings and not related to test item administration as similar findings were noted both in control and/or test item treated groups. Kindly note that all the mentioned findings (incidental) are well documented in the raw data and also in the report (Please refer Appendix 1 and 2 of BIO-CTX 043 pathology report).

**2. Upon review of the attached Annexes (B1-B3) containing the OECD 408-compliant study report, significantly increased absolute and relative spleen and uterus weights were reported in treated male rats (G2, G3, G4, M) and female rats (G2, G3, G4, F), respectively. Notably, splenomegaly may be an indirect indicator of immune stimulation or infection.**

**a. Please address these effects associated with *A. clausii* MCC0538 spore treatment and discuss why such effects are not considered treatment related or adverse.**

**Bioneeeds response:** Statistically significant higher absolute and relative weight of spleen in all males and uterus in all females was noted when compared to vehicle control group.

The percent difference is as below:

<b>Group</b>	<b>Spleen (Percent difference when compared to control)</b>	<b>Group</b>	<b>Uterus (Percent difference when compared to control)</b>
G2M	34.3%	G2F	17.3%
G3M	31.7%	G3F	20.4%
G4M	29.5%	G4F	20.6%

**Spleen:** The percent increase of spleen weight in male rats ranged from 29 to 34%. Although, these weights were statistically significant, the magnitude of change was minimal, lacked dose correlation and any gross pathological observations. At microscopy, spleen of high dose animals were within normal histological limits and comparable to concurrent control. Also, similar change

**No.:** SL 4171/21

**Date:** 11/06/2021

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in weights were not observed in female rats. Hence, in the absence of dose-correlation, gross and histological changes, the minimal variation in splenic weights were not considered as test item-related, and hence considered an incidental finding and not an adverse finding and cannot be attributed to test item.

**Uterus:** The increase in uterus weight in all test item treated females ranged between 17 to 20% when compared to control females. Considering the minimal variations and absence of associated gross or histopathology changes in uterus, the observed significance is considered incidental and normal biological variation.

Representative Histopathology images of Spleen in males and Uterus in females is included in this document.

The test item *Bacillus clausii*, which is a probiotic, has not resulted in any adverse effects in all the parameters evaluated in the 90-day repeated dose toxicity study in rats. There were no test item related clinical signs, changes in body weights or feed consumption. No toxicologically significant changes were noted in clinical pathology parameters, organ weights and its ratios. No test item related gross or histopathology changes were noted.

Hence, the increase in spleen weight in male rats and increase in uterus weight in female rats are considered an incidental finding and not an adverse finding and cannot be attributed to test item.

For Bionees India Private Limited,



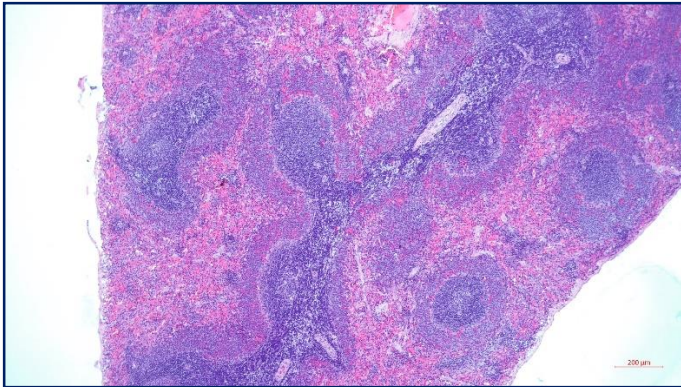
Dr. NITIN M. SHETTY

Chief Technical Office

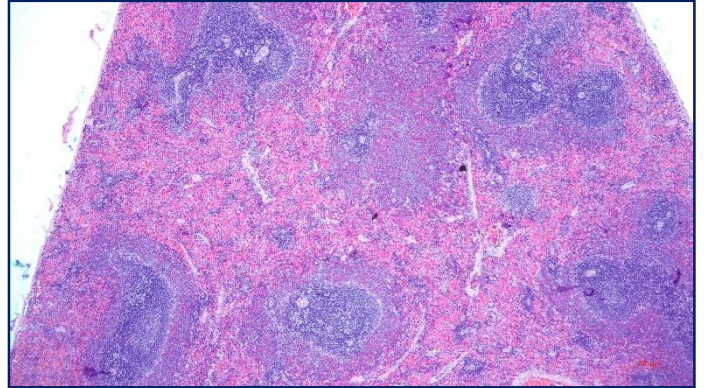
**REPRESENTATIVE HISTOPATHOLOGY IMAGES**

**BIO-CTX 043**

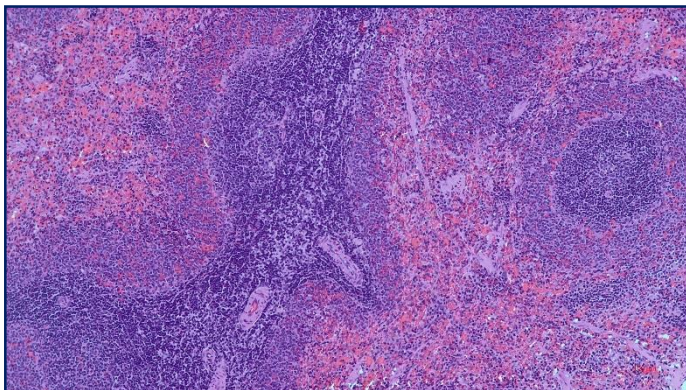
**Representative Histopathology Images: Spleen**



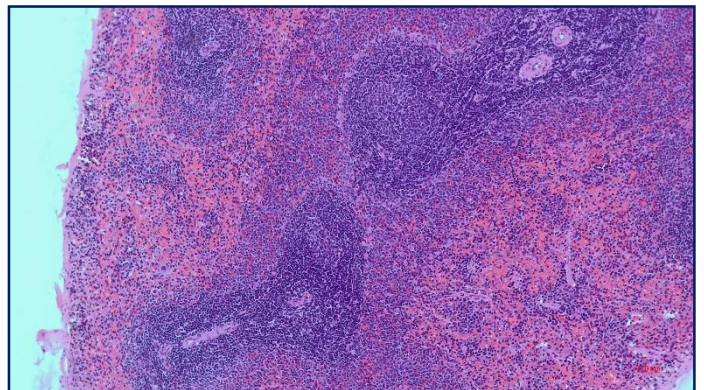
**Control, Male, H&E, 5X**



**High dose, Male , H&E, 5x**

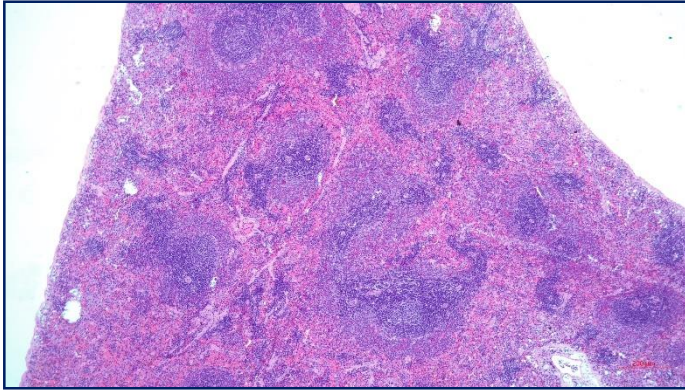


**Control, Male, H&E, 10X**

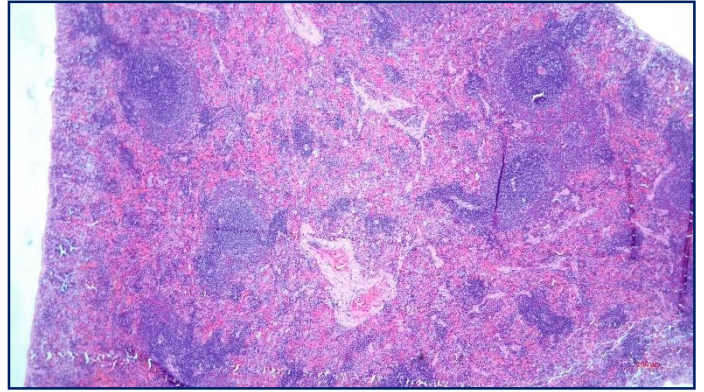


**High dose, Male , H&E, 10x**

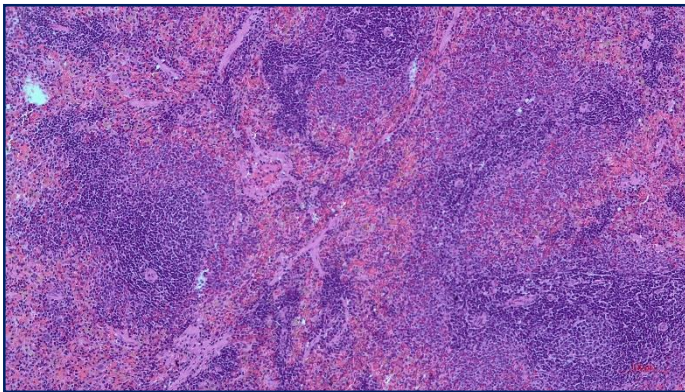
**BIO-CTX 043**  
**Representative Histopathology Images: Spleen**



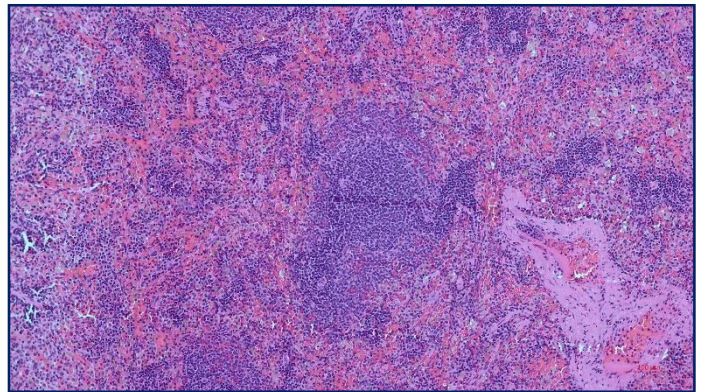
**Control, Female, H&E, 5X**



**High dose, Female , H&E, 5x**

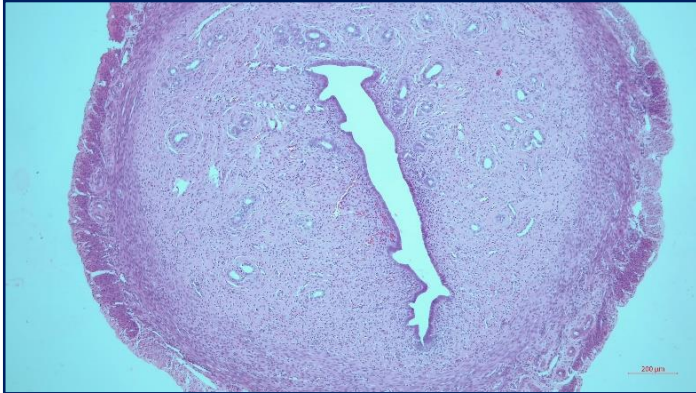


**Control, Female, H&E, 10X**

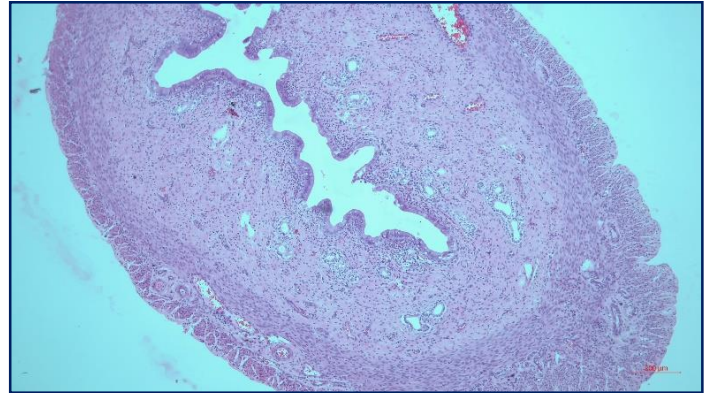


**High dose, Female , H&E, 10x**

**BIO-CTX 043**  
**Representative Histopathology Images: Uterus**



**Control, Female, H&E, 5X**



**High dose, Female , H&E, 5x**



Twenty-five pages have been removed in accordance with copyright laws. The removed reference citations are:

United States Pharmacopeia (USP) 42, Microbiological Tests, (61) 6387

European Pharmacopoeia 8.0, 5.8. Pharmacopoeial harmonisation p677-682.

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