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SCIENTIFIC OPINION

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Safety of *Clostridium butyricum* TO-A as a novel food pursuant to Regulation (EU) 2015/2283

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

Following a request from the European Commission, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) was asked to deliver an opinion on *Clostridium butyricum* TO-A as a novel food (NF) pursuant to Regulation (EU) 2015/2283. The NF, which is proposed by the applicant to be used as a food supplement, is sufficiently characterised. The information provided on the production process, composition, stability and specifications of the NF is sufficient and does not raise safety concerns. Based on the findings of a repeated dose 90-day oral toxicity study in rats, and considering an uncertainty factor of 200, the Panel estimated a safe dose in humans of 4.5×10^6 colony forming unit (CFU)/kg body weight (bw) per day. However, considering that appropriate initial bacteria colonisation of the gastrointestinal tract in humans, in particular during the first 3 years of life, profoundly affects health during infancy and childhood, that disruptions to the microbiota early in life can have lasting health effects into adulthood, and taking into account that the 90-day oral toxicity study was conducted in adult rats, the Panel considers that the target population for the NF should be restricted to children above 3 years of age, adolescents and adults, excluding pregnant and lactating women. The Panel concludes that the NF, *C. butyricum* TO-A, is safe at 1.0×10^8 CFU/day for other children (3 to < 10 years), 2.0×10^8 CFU/day for adolescents from 10 to < 14 years, 2.8×10^8 CFU/day for adolescents from 14 to < 18 years and 3.2×10^8 CFU/day for adults, excluding pregnant and lactating women.

KEYWORDS

Clostridium butyricum, food supplement, microorganism, novel foods, safety

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1 | INTRODUCTION

1.1 | Background and Terms of Reference as provided by the requestor

On 10 November 2021, the company 'TOA Biopharma Co. Ltd.' submitted an application to the European Commission in accordance with Article 10 of Regulation (EU) 2015/2283 to authorise the placing on the Union market of *Clostridium butyricum* TO-A as a novel food (NF).

The applicant requests to authorise the use of *Clostridium butyricum* TO-A as a NF in food supplements as defined in Directive 2002/46/EC, excluding infants below 3 months of age.

The applicant has requested data protection under Article 26 of Regulation (EU) 2015/2283.

In accordance with Article 10(3) of Regulation (EU) 2015/2283, the European Commission asks EFSA to provide a scientific opinion on *Clostridium butyricum* TO-A.

In this opinion on *Clostridium butyricum* TO-A, EFSA should also document whether and to what extent the requirements of Article 26(2)(c) of Regulation (EU) 2015/2283 are fulfilled regarding the data for which the applicant is requesting data protection.

1.2 | Additional information

In 2020, *Clostridium* (*C.*) *butyricum* was notified to the EFSA Panel on Biological Hazards (BIOHAZ) for its assessment of qualified presumption of safety (QPS). The BIOHAZ Panel did not recommend *C. butyricum* for QPS status because some strains contain pathogenicity factors. Thus, the BIOHAZ Panel excluded this species for further QPS evaluation (EFSA BIOHAZ Panel, 2020). This conclusion was confirmed by the BIOHAZ Panel in 2022 (EFSA BIOHAZ Panel, 2022).

In 2022, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) assessed the feed additive BIO-THREE®, which is based on viable cells/spores of a combination of three bacteria including the NF, i.e. *Clostridium butyricum* TO-A (FERM BP-10866), plus *Bacillus subtilis* FERM BP-07462 and *Enterococcus lactis* FERM BP-10867. The FEEDAP Panel concluded that BIO-THREE® is safe for the target species (chickens, turkeys, all avian species) under the proposed conditions of use. The Panel also concluded that the additive is safe for the consumers of products derived from animals receiving the additive (EFSA FEEDAP Panel, 2022). Following that assessment, the feed additive was authorised by Commission Implementing Regulation (EU) 2024/2185.[1]

In 2014, another *C. butyricum* strain, i.e. *C. butyricum* (CBM 588) (also denominated as *C. butyricum* MIYAIRI 588 (CBM 588)), was authorised to be placed on the market in food supplements at 1.35×10^8 CFU/day in the Union as a NF by Commission Implementing Decision 2014/907/EU.[2] Consequently, *C. butyricum* (CBM 588) was also included in the Union list of novel foods.[3]

2 | DATA AND METHODOLOGIES

2.1 | Data

The safety assessment of this NF is based on data supplied in the application and information submitted by the applicant following EFSA requests for supplementary information.[4]

Administrative and scientific requirements for NF applications referred to in Article 10 of Regulation (EU) 2015/2283 are listed in the Commission Implementing Regulation (EU) 2017/2469.[5]

A common and structured format on the presentation of NF applications is described in the EFSA guidance on the preparation and presentation of a NF application (EFSA NDA Panel, 2021). As indicated in this guidance, it is the duty of the applicant to provide all the available (proprietary, confidential and published) scientific data (including both data in favour and not in favour) that are pertinent to the safety of the NF.

[1]Commission Implementing Regulation (EU) 2024/2185 of 3 September 2024 concerning the authorisation of a preparation of *Bacillus subtilis* FERM BP-07462, *Enterococcus lactis* FERM BP-10867 and *Clostridium butyricum* FERM BP-10866 as a feed additive for all poultry species for fattening, all poultry species reared for laying or breeding and ornamental birds (holder of authorisation: Toa Biopharma Co., Ltd.). C/2024/5963. OJ L, 2024/2185.

[2]Commission Implementing Decision of 11 December 2014 authorising the placing on the market of *Clostridium butyricum* (CBM 588) as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council (notified under document C(2014) 9345). OJ L 359, 16.12.2014, pp. 153–154.

[3]Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, pp. 72–201.

[4]https://open.efsa.europa.eu/questions/EFSA-Q-2022-00010.

[5]Commission Implementing Regulation (EU) 2017/2469 of 20 December 2017 laying down administrative and scientific requirements for applications referred to in Article 10 of Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, pp. 64–71.

The applicant submitted a confidential and a non-confidential version of a dossier following the EFSA guidance on the preparation and presentation of a NF application (EFSA NDA Panel, 2021) and the administrative guidance for the preparation of applications on novel foods pursuant to Article 10 of Regulation (EU) 2015/2283 (EFSA, 2021).

In accordance with Art. 38 of Regulation (EC) No 178/2002[6] and taking into account the protection of confidential information and of personal data in accordance with Articles 39 to 39e of the same Regulation, and of the Decision of EFSA's Executive Director laying down practical arrangements concerning transparency and confidentiality,[7] the non-confidential version of the dossier has been published on Open.EFSA.[8]

According to Art. 32c(2) of Regulation (EC) No 178/2002 and to the Decision of EFSA's Executive Director laying down the practical arrangements on pre-submission phase and public consultations, EFSA carried out a public consultation (PC-0430)[9] on the non-confidential version of the technical dossier from 05/04/2023 to 26/04/2023 for which no comments were received.

This NF application includes a request for protection of proprietary data, in accordance with Article 26 of Regulation (EU) 2015/2283. The data requested by the applicant to be protected comprise:

- Annex_I_2_1_1_Manufacturing_process.
- Annex_I_3_1_1_Nutritional_analysis (part 1–5).
- Annex_I_3_1_2_*C butyricum*_analysis (part 1–5).
- Annex_I_3_1_3_Purity (part 1–5).
- Annex_I_9_2_1_AMES.
- Annex_I_9_2_2_Micronucleous.
- Annex_I_9_2_3_Ames_supernatant.
- Annex_I_9_2_4_Micronucleus_supernatant.
- Annex_I_9_2_5_in_vivo_micronucleus_comet_supernatant.
- Annex_I_9_2_6_Lysis_efficiency_French_Press.
- Annex_I_9_2_6_Appendix1_SEM_images.
- Annex_I_9_2_7_Ames_Lysate.
- Annex_I_9 _2_8_Micronucleus_Lysate.
- Annex_I_9_3_Subchronic_toxicity.

2.2 | Methodologies

The assessment follows the methodology set out in the EFSA guidance on NF applications (EFSA NDA Panel, 2021) and the principles described in the relevant existing guidance documents from the EFSA Scientific Committee. The legal provisions for the assessment are laid down in Article 11 of Regulation (EU) 2015/2283 and in Article 7 of Commission Implementing Regulation (EU) 2017/2469.

This assessment concerns only the risks that might be associated with the consumption of the NF under the proposed conditions of use, and it is not an assessment of the efficacy of the NF with regard to any claimed benefit.

3 | ASSESSMENT

3.1 | Introduction

The NF which is the subject of the application is *Clostridium butyricum* TO-A. The NF is produced by anaerobic fermentation. The biomass is separated by centrifugation, dried and mixed with potato starch. The NF is proposed to be used in food supplements (as defined in Directive 2002/46/EC). The target population proposed by the applicant is the general population excluding infants below 3 months of age.

The NF falls under Article 3(2)(a) (ii) food consisting of, isolated from or produced from microorganisms, fungi or algae, as defined in Regulation (EU) 2015/2283.

3.2 | Identity of the NF

The NF is dried *C. butyricum* TO-A, which is a spore-former, strictly anaerobic and belongs to the Gram-positive *Clostridium* genus.

[6]Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, pp. 1–48.

[7]Decision available at: https://www.efsa.europa.eu/en/corporate-pubs/transparency-regulation-practical-arrangements.

[8]The non-confidential version of the dossier has been published on Open.EFSA and is available at the following link: https://open.efsa.europa.eu/dossier/NF-2021-1068.

[9]https://connect.efsa.europa.eu/RM/s/consultations/publicconsultation2/a0l09000006r2l7/pc0430.

C. butyricum TO-A was originally isolated from the gut of healthy humans. When grown anaerobically at 37°C in iron sulfite agar for 24 h, circular black colonies of *C. butyricum* TO-A appear. Gram-staining reveals straight or slightly curved rod-shaped cells and oval spores.

The applicant was requested to clarify the nature of the NF, and in particular, the respective percentages of cells and spores. In reply, the applicant provided analyses of cells and spores for five batches of the NF, which indicated that the NF is a mixture of spores and vegetative cells with about 85% (range: 74%–89%) of spores.

The full taxonomic classification of the employed strain is the following: Domain: Bacteria; Phylum: Firmicutes; Class: Clostridia; Order: Clostridiales; Family: Clostridialaceae; Genus: *Clostridium*; Species: *Clostridium butyricum*; Strain: *Clostridium butyricum* TO-A. The applicant indicated as in-house identifiers of *C. butyricum* TO-A the following ones: Clostridium, Clostridium TO-A, CB TO-A, *Clostridium butyricum*, CB, CBT.

The strain is deposited under number FERM BP-10866 at the Japanese National Institute of Technology and Evaluation, which follows the Budapest Treaty on the International Recognition of the Deposit of Microorganisms. The certificate of deposit was provided by the applicant.

The complete genome of *C. butyricum* TO-A and its two plasmids pCBTOA1 and pCBTOA2 were sequenced, and the whole genome sequence (WGS) was provided. The overall organisation of the genome was determined based on the WGS alignment with assembled *C. butyricum* genomes in the NCBI GenBank. The provided sequence produced a nearly complete genome that belonged to this bacterial species.

The taxonomic identification of the strain TO-A as *C. butyricum* was established by alignment and non-alignment tools for the WGS data. The 16S rRNA gene analysis and the alignment-based calculation of average nucleotide identity (ANI) assigned the strain to the *Clostridium butyricum* species, with an ANI of 99.99 with the type strain *C. butyricum* KNU-L09^T. Likewise, the phylogenetic tree based on the core gene alignment grouped the test strain together with *C. butyricum* KNU-L09^T. Based on these results, the taxonomic identification of the strain was confirmed as *Clostridium butyricum*.

The WGS data allowed the assessment of the presence of antimicrobial resistance genes and potential toxin and virulence factors. The screening of the strain genome revealed that no putative antimicrobial resistance genes above 80% identity and 70% coverage were present (NCBI Bacterial Antimicrobial Resistance Reference Gene database and ResFinder database). The phenotypic resistance of the test strain was assessed according to the EFSA cut-off values for Gram-positive bacteria (EFSA FEEDAP Panel, 2018). The phenotypic testing of the antimicrobial susceptibility indicated that the strain is resistant to the aminoglycosides gentamicin, kanamycin and streptomycin. The minimum inhibitory concentration for kanamycin and streptomycin was 128 mg/L as compared to the cut-off values defined by the EFSA FEEDAP Panel in 2018 (16 mg/L for kanamycin and 8 mg/L for streptomycin, respectively). However, this phenotypic resistance is intrinsic to these species and anaerobes in general (EFSA FEEDAP Panel, 2009, 2014; Isa et al., 2016) and no horizontal gene transfer is expected.

The major virulence factors of pathogenic *Clostridium* spp. (i.e. *C. botulinum, C. difficile, C. tetani and C. perfringens*) include α, β and ε toxins and highly potent neurotoxins (BONT/A-G) and they could be found in other *Clostridium* species. However, according to interrogations performed with the WGS of *C. butyricum* TO-A against the virulence factor database (VFDB; full set, accessed on 14/07/2021), genes for these specific toxins or other (neuro)toxins are not present. Three factors were found in the database (with different percentages of identity), i.e. chaperonin GroEL (VFG012102: WP_011967678), RNA methyltransferase (VFG012183: WP_011969031.1) and urease accessory protein UreG (VFG019533: WP_000238754.1), which are all genes that are related to normal cellular functions in bacteria.

The Panel considers that the NF is sufficiently characterised and does not raise safety concerns.

3.3 | Production process

According to the information provided, the NF is produced in line with good manufacturing practice (GMP) and Hazard Analysis Critical Control Points (HACCP) principles.

Flow charts of the production process and detailed information of the culture conditions and media together with the quality control methods involved at each step were provided (confidential information).

The first step of the process consists in the cultivation and harvesting of *C. butyricum*. Initially, the *C. butyricum* TO-A inoculum is prepared from a culture aliquot stored at −80°C. Successive subculture steps in liquid media of increasing volumes are performed in order to prepare the inoculation of the main fermenter. The main fermentation is conducted under strict anaerobic conditions at a certain temperature and pH (confidential information) and its microscopic morphology is confirmed. Biomass separation is achieved by centrifugation at a certain speed (confidential information).

The cells are spray-dried to bulk powder and mixed with potato starch to achieve the desired concentration of *C. butyricum* TO-A. Cell counting, microbiological and purity tests as well as qualitative visual inspection of the powder are performed. The final product is packed to scale and stored under certain conditions (confidential information).

The applicant was requested to provide more information on the potato starch, e.g. if there is some kind of particular potato starch used (e.g. resistant starch), which would favour survival of the *C. butyricum* TO-A spores/cells in the gastrointestinal tract, or if some kind of encapsulation is applied. In reply, the applicant stated that neither resistant starch nor any form of encapsulation is used. According to the applicant, the main purpose of the potato starch is 'to stabilise the spores by keeping a dry environment'.

The Panel considers that the production process is sufficiently described and does not raise safety concerns.

3.4 | Compositional data

The NF, dried *C. butyricum* TO-A, including the potato starch, consists of carbohydrates (~96%), moisture (~1.7%), dietary fibre (~1.5%), proteins (~0.6%) and minor amounts of fat (< 0.2%).

In order to confirm that the manufacturing process is reproducible and adequate to produce on a commercial scale a product with certain characteristics, the applicant provided analytical information for five independent lots of the NF (Table 1).

TABLE 1 Batch to batch analysis of the NF.

	Batch number					
Parameter (unit)	**#1**	**#2**	**#3**	**#4**	**#5**	**Method of analysis**
Species identification	Conforms	Conforms	Conforms	Conforms	Conforms	WGS and 16S rRNA; Gram-staining
Sulphite-reducing (*Clostridium*) counts (CFU/g)	5.2×10^9	4.0×10^9	3.9×10^9	4.9×10^9	5.3×10^9	ISO 15213: 2003
Moisture & volatile matters (%)	1.81	1.64	1.59	2.09	1.52	Gravimetry
Crude protein (%)	0.60	0.57	0.63	0.72	0.63	Kjeldahl, Regulation (EC) n° 152/2009
Crude fat (%)	< 0.20	< 0.20	< 0.20	< 0.20	< 0.20	Gravimetry Regulation (EC) n° 152/2009
'Dietary fibre' (%)[a]	1.91	1.22	1.80	0.96	1.51	Gravimetry Regulation (EC) n° 152/2009
Ash (%)	0.43	0.48	0.46	0.42	0.34	Gravimetry Regulation (EC) n° 152/2009
Sodium (Na) (%)	0.078	0.076	0.076	0.076	0.077	PNT-MF-860/PNT-MF-859 (ICP-OES)
Carbohydrates (%)	95.25	96.09	95.52	95.81	96.00	PNT-M-605 (Calculated)
Fructose (%)	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	PNT-MF-144 (HPLC- Refractive index. AOAC 984.22)
Glucose (%)	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	PNT-MF-144 (HPLC- Refractive index. AOAC 984.22)
Saccharose (%)	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	PNT-MF-144 (HPLC- Refractive index. AOAC 984.22)
Maltose (%)	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	PNT-MF-144 (HPLC- Refractive index. AOAC 984.22)
Lactose monohydrate (%)	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	PNT-MF-144 (HPLC- Refractive index. AOAC 984.22)
Heavy metals						
Cadmium (mg/kg)	< 0.20	< 0.20	< 0.20	< 0.20	< 0.20	EN 15510:2007
Lead (mg/kg)	< 2.50	< 2.50	< 2.50	< 2.50	< 2.50	EN 15510:2007
Arsenic (mg/kg)	< 2	< 2	< 2	< 2	< 2	EN 15510:2007
Mercury (mg/kg)	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	UNE-EN 13806:2003
Microbial						
Aerobic mesophilic plate count (APC) (CFU/g)	< 40	< 40	< 40	< 40	< 40	ISO 4833-1: 2014
Yeasts (CFU/g)	$< 1\times10^2$	$< 1\times10^2$	$< 1\times10^2$	$< 1\times10^2$	$< 1\times10^2$	ISO 21527-2: 2008
Moulds (CFU/g)	$< 1\times10^2$	$< 1\times10^2$	$< 1\times10^2$	$< 1\times10^2$	$< 1\times10^2$	ISO 21527-2: 2008
Salmonella spp. (in 25 g)	n.d.	n.d.	n.d.	n.d.	n.d.	ISO 6579-1:2017/A1
Total coliforms (CFU/g)	< 10	< 10	< 10	< 10	< 10	ISO 4832: 2006
E. coli (CFU/g)	< 10	< 10	< 10	< 10	< 10	ISO 16649-2: 2001

Abbreviations: AOAC, Association of Official Analytical Collaboration; APC, aerobic mesophilic plate count; CFU, colony forming units; EC, European Commission; EN, European Standard; n.d., not detected; HPLC, high-performance liquid chromatography; ICP-OES, Inductively Coupled Plasma Optical Emission Spectroscopy; rRNA, ribosomal ribonucleic acid; UNE, Spanish standardisation body; WGS, whole genome sequence.

[a]The Panel notes that 'dietary fibre' refers to non-digestible polysaccharides.

In addition to the proximate analyses, the applicant provided detailed analyses of the fatty acids, which were all below the limit of quantification (LOQ = 0.01 g/100 g). Analyses of pesticides, mycotoxins and aflatoxins, dioxins and PCBs in the NF were also provided. No residues of these pesticides, mycotoxins and aflatoxins were detected above their respective LOQ or limit of detection as demonstrated by the provided analytical results.

Silicon (Si) analysis was performed in order to detect any silicon-based residues from the antifoaming agent (i.e. dimethyl polysiloxane (E 900)) used during the manufacturing process, and levels of 18–19 mg/kg of Si were detected. These amounts correspond to about 50 mg of the antifoaming agent (i.e. dimethyl polysiloxane (E 900)). Considering the maximum proposed intakes of the NF, which could reach up to 1 g NF/day (see Table 3), the daily intake of dimethyl polysiloxane would be 50 µg (equivalent to 0.7 µg/kg bw per day for adults). Taking into account the ADI for dimethyl polysiloxane of 17 mg/kg bw per day established in 2020 (EFSA FAF Panel, 2020), the Panel considers that this does not raise a safety concern.

Information was provided on the accreditation of the laboratories that conducted the analyses presented in the application.

The Panel considers that the information provided on the composition is sufficient for characterising the NF.

3.4.1 | Stability

The applicant performed stability tests with five independently produced batches of the NF. The tests were carried out at normal storage conditions at 25°C in a dry place (< 60% relative humidity (RH)) for a period of 25 months. The batches were analysed for microbial contaminants and physicochemical characteristics.

All batches after 25 months of storage appeared to be solid in the form of fine white homogeneous powder. The CFU content in the NF decreased from an average of 3.8×10^9 CFU/g at the beginning of the storage period to an average of 1.7×10^9 CFU/g after 25 months. Moisture and volatile matter (%) increased from 1% to about 2.4%. The analysed microbial parameters (yeasts, moulds, total aerobic microbial count (TAMC), *Salmonella* spp., total coliforms) remained within the limits as set in the specifications. Based on these results, the applicant proposed a shelf-life of 25 months for the NF.

The Panel considers that the data provide sufficient information with respect to the stability of the NF.

3.5 | Specifications

The specifications of the NF, as proposed by the applicant, are indicated in Table 2.

TABLE 2 Specifications of the NF.

Description: The NF is produced by anaerobic fermentation of *Clostridium butyricum* TO-A, which is harvested by centrifugation, dried and mixed with potato starch **Appearance:** Odourless white/greyish powder	
Parameter	**Specification**
C. butyricum TO-A	$1–7 \times 10^9$ CFU/g
Moisture	< 5%
Coliforms	< 10 CFU/g
TAMC	$\leq 1 \times 10^3$ CFU/g
TYMC	$\leq 1 \times 10^2$ CFU/g
Salmonella spp.	Not detected in 25 g

Abbreviations: CFU, colony forming units; TAMC, total aerobic microbial count; TYMC, total yeast and mould count.

The Panel considers that the information provided on the specifications of the NF is sufficient and does not raise safety concerns.

3.6 | History of use of the NF and/or of its source

3.6.1 | History of use of the source

C. butyricum was reported to be widely used in Asia, particularly in Japan, Korea and China (Vos et al., 2009). *C. butyricum* is found in many environments, including vegetables, soured milk and cheeses (Cassir et al., 2016).

3.6.2 | History of use of the NF

C. butyricum TO-A has been marketed for humans as part of a combination of three bacteria (i.e. BIO-THREE®) in China, Taiwan and Japan for many decades.

BIO-THREE® (including *C. butyricum* TO-A) was assessed as a feed additive by the EFSA FEEDAP Panel in 2022. The FEEDAP Panel concluded that BIO-THREE® is safe for the target species (chickens, turkeys, all avian species) under the proposed conditions of use. The Panel also concluded that the additive is safe for the consumers of products derived from animals receiving the additive (EFSA FEEDAP Panel, 2022). Following that assessment, BIO-THREE®, which includes *C. butyricum* TO-A, was authorised as a feed additive by Commission Implementing Regulation (EU) 2024/2185.[10]

3.7 | Proposed uses and use levels

3.7.1 | Target population

The target population proposed by the applicant is children from 3 months onwards, adolescents and adults.

3.7.2 | Proposed uses and use levels

The applicant intends to market the NF as a food supplement. The maximum proposed doses per day, which range from 1×10^8 CFU/day for infants to 1×10^9 CFU/day for adults, as well as the corresponding maximum amounts of the NF in mg per day, are indicated in Table 3.

TABLE 3 Proposed use of the NF as a food supplement in various population groups.

Population group	Age	Max. use level (CFU/day)	Max. amount of NF (mg/day)
Infants excluding those below 3 months	3 to < 12 months	1×10^8 CFU/day	100 mg/day
Young children	1 to < 3 years	1×10^8 CFU/day	100 mg/day
Other children	3 to < 10 years	3×10^8 CFU/day	300 mg/day
Adolescents	10 to < 18 years	6×10^8 CFU/day	600 mg/day
Adults	≥ 18 years	1×10^9 CFU/day	1000 mg/day

Abbreviation: CFU, colony forming units.

3.8 | Absorption, distribution, metabolism and excretion (ADME)

No ADME data were provided for the NF.

The applicant stated that the NF, consisting of live microorganisms, can be expected to pass through the gastrointestinal tract unabsorbed and that ADME studies with the NF were therefore not considered necessary.

The applicant submitted a study performed with another *C. butyricum* strain, i.e. CBM588 (Sato & Tanaka, 1997). Following a single administration to rats of 10^7 spores of *C. butyricum* CBM588, the number of *C. butyricum* CBM588 cells in the intestinal contents was counted. The results of this study suggest that the administered *C. butyricum* spores germinated in the upper small intestine, grew mainly from the distal small intestine to the colon and were excreted from the rat intestine within 3 days (Sato & Tanaka, 1997).

3.9 | Nutritional information

The applicant provided a nutritional analysis of the NF as intended to be marketed (i.e. including the potato starch). According to this analysis, the NF is mostly composed of carbohydrates (95%–96%) with minor amounts of protein (about 0.6%) and fat (below 0.2%).

The Panel considers that, taking into account the composition of the NF and the proposed conditions of use, the consumption of the NF is not nutritionally disadvantageous.

3.10 | Toxicological information

The applicant provided nine toxicological studies, which were performed with lysed *C. butyricum* TO-A (without potato starch), culture supernatant of the NF (i.e. derived from the manufacturing process of the NF) and BIO-THREE®, respectively.

[10]Commission Implementing Regulation (EU) 2024/2185 of 3 September 2024 concerning the authorisation of a preparation of *Bacillus subtilis* FERM BP-07462, *Enterococcus lactis* FERM BP-10867 and *Clostridium butyricum* FERM BP-10866 as a feed additive for all poultry species for fattening, all poultry species reared for laying or breeding and ornamental birds (holder of authorisation: Toa Biopharma Co., Ltd.). C/2024/5963. OJ L, 2024/2185.

With the exception of the subacute study (Sakamaki et al., 1989), the studies were conducted in compliance with principles of good laboratory practice (GLP) (OECD, 1998) and followed respective test guidelines (OECD, 1997, 2010, 2016a, 2016b) and Ministry of Health and Welfare (Japan), respectively). The studies, which were claimed proprietary by the applicant, are listed in Table 4.

TABLE 4 List of provided toxicological studies.

Reference	Type of study	Test item	Test system	Dose
Study No. FSR-IPL 180806 (Unpublished, 2018a)	Bacterial reverse mutation test (GLP, OECD TG 471)	BIO-THREE® (DMSO-extract)	S. typhimurium TA98, TA100, TA102, TA1535 and TA1537	Up to 100 µL/plate (absence and presence of S9 mix)
Study No. FSR-IPL 180807 (Unpublished, 2018b)	In vitro mammalian cell micronucleus test (GLP, OECD TG 487)	BIO-THREE® (DMSO-extract)	TK6 lymphoblastoid human cells	Up to 1% of the DMSO extract
Study No. FSR-IPL 221204 (Unpublished, 2024a)	Bacterial reverse mutation test (GLP, OECD TG 471)	NF – lysate	S. typhimurium TA98, TA100, TA102, TA1535 and TA1537	Up to 100 µL/plate
Study No. FSR-IPL 221206 (Unpublished, 2024b)	In vitro mammalian cell micronucleus test (GLP, OECD TG 487)	NF – lysate	TK6 lymphoblastoid human cells	Up to 10%
Study No. FSR-IPL 221203 (Unpublished, 2023a)	Bacterial reverse mutation test (GLP, OECD TG 471)	NF – culture supernatant	S. typhimurium TA98, TA100, TA102, TA1535 and TA1537	Up to 500 µL/plate
Study No. FSR-IPL 221205 (Unpublished, 2023b)	In vitro mammalian cell micronucleus test (GLP, OECD TG 487)	NF – culture supernatant	TK6 lymphoblastoid human cells	Up to 10%
Study No. FSR-IPL 230701 (Unpublished, 2024c)	In vivo mammalian alkaline comet assay combined with an in vivo mammalian erythrocytes micronucleus test (OECD TG 474 and TG 489)	NF – culture supernatant	Sprague Dawley rats	See description of the study
Sakamaki et al. (1989)	28-day oral toxicity study	NF	Sprague Dawley rats	Up to 3000 mg/kg bw per day (10^{10} CFU/g)
Study No. AZ13349 (Unpublished, 2014)	90-day repeated dose oral toxicity study (GLP; Guidelines for Repeated Dose Toxicity Studies (Notification No. 655, 1999, Japan))	NF (and also BIO-THREE® in two study groups)	Sprague Dawley rats	Up to 3000 mg/kg bw per day (NF: 3×10^{8} CFU/g)

Abbreviations: bw, body weight; DMSO, dimethylsulfoxide; GLP, Good Laboratory Practice; OECD, Organization for Economic Co-operation and Development; TG, Test Guideline.

3.10.1 | Genotoxicity

Originally the applicant undertook genotoxicity testing by using as test item the food supplement BIO-THREE®, which includes the NF, i.e. *C. butyricum* TO-A (at 1×10^{8} CFU/g), but also contains *B. subtilis* TO-A (at 1×10^{8} CFU/g) and *E. faecium* T-110 (at 1×10^{9} CFU/g).

The bacterial reverse mutation test (Unpublished, 2018a) was carried out with five Salmonella typhimurium strains (TA1535, TA1537, TA98, TA100 and TA102), both with and without metabolic activation, in two independent assays (the second one according to the pre-incubation protocol). The test item (BIO-THREE®) was used in the form of a dimethylsulfoxide (DMSO) extract at 2 g/mL (i.e. 2.4×10^{9} CFU/mL). The top dose volumes were 100 and 50 µL/plate of DMSO extract in the conditions without and with pre-incubation, respectively. No increases in the number of revertants were noted with any strain under any condition.

In the in vitro mammalian cell micronucleus test (Unpublished, 2018b), the genotoxic activity of BIO-THREE® was assessed in TK6 lymphoblastoid human cells using a DMSO extract at 2 g/mL (i.e. 2.4×10^{9} CFU/mL). The top concentration tested was 1% of DMSO extract. Two lower concentrations (0.5 and 0.25%) were also analysed. Following a preliminary cytotoxicity assay, the genotoxicity assay was carried out using a short-term 3 h treatment with and without metabolic activation, followed by a recovery period and a 27 h continuous treatment without metabolic activation and no recovery period. BIO-THREE® induced no statistically or biologically significant increases in the number of micronucleated cells under any condition.

Following an EFSA request for additional genotoxicity testing of the NF, for both the lysate of the NF and the culture supernatant (in separate tests), the applicant submitted the studies as described below. For the lysis of the NF (after removing

the potato starch by 4 washing steps), a French Press was used, and the efficacy of lysis was assessed by scanning electron microscope (SEM). The lysis was performed without any use of a lysis buffer that may interfere with genotoxicity test systems. After four press cycles at 2700 bars, the percentage of lysis reached 84.78%, which was considered satisfactory. Information on this process and documentation of the degree of lysis were provided by the applicant.

The bacterial reverse mutation test with the cell lysate of the NF was carried out with five Salmonella typhimurium strains (TA1535, TA1537, TA98, TA100 and TA102), tested both with and without metabolic activation in two independent assays (Unpublished, 2024a). A dose volume of 100 µL cell lysate/plate was used, instead of 5 µL/plate as recommended for a liquid by the OECD test guideline. The applicant explained that this was a compromise between the treatment volume used to increase the level of each component of the lysate and the volume of lysate that could technically be prepared with the French Press. There were no increases in the number of revertants with any strain under any condition.

The cell lysate of the NF was also tested in an in vitro mammalian cell micronucleus test (Unpublished, 2024b). For the test, TK6 lymphoblastoid human cells were treated with the cell lysate up to 10%, the maximum concentration that was compatible with the system. Lower concentrations of 2.5% and 5% were also assessed. Short-term treatments were conducted for 3 h with and without metabolic activation plus 24 h recovery period. The long-term treatment was performed without metabolic activation and lasted for 27 h (no recovery period applied). There were no statistically significant increases in the number of micronucleated cells under any condition.

As requested by EFSA, the applicant also tested the culture supernatant of the NF in the standard genotoxicity test battery, which includes a bacterial reverse mutation test and an in vitro mammalian cell micronucleus test (EFSA Scientific Committee, 2011).

In the bacterial reverse mutation test (Unpublished, 2023a), the culture supernatant of the NF was tested with five strains of Salmonella typhimurium (TA1535, TA1537, TA98, TA100 and TA102), in the presence and in the absence of metabolic activation. In order to increase the concentration of each component of the culture supernatant, 500 µL was used per plate. A first assay was performed following the standard plate incorporation technique, with and without metabolic activation. In this first assay, significant increases in the number of colonies were noted in all strains tested, with and without metabolic activation. In order to investigate whether the increases in the number of colonies observed were due to the presence of amino acids and/or peptides in the test item (i.e. the culture supernatant), a second mutagenicity assay was performed using the treat & wash method concurrently with the standard plate incorporation technique. In the second assay, significant increases in the number of colonies were also observed with and without metabolic activation when the standard plate incorporation method was applied. However, when using the treat & wash method, statistically and/or biologically significant increases in the number of colonies were observed in all strains except TA100 in the presence of metabolic activation only. In the absence of metabolic activation, no biologically or statistically significant increases in the number of revertants were noted in any of the strains.

The culture supernatant of the NF was also tested in an in vitro mammalian cell micronucleus test (Unpublished, 2023b). Human lymphoblastoid TK6 cells were exposed to the supernatant up to a concentration of 10%, which was considered by the study authors as the highest concentration compatible with the test system, taking into account results of the preliminary cytotoxicity assay and osmolality. The highest actual concentrations tested in the main assay for the short-term (3 h) treatment with and without metabolic activation were 10% and 7%, respectively. In the long-term treatment (27 h; no metabolic activation), the highest concentration tested was 1.8%. A minimum of 2000 mononucleated cells were analysed per concentration. In the short-term treatments with and without metabolic activation, no statistically or biologically significant increases in the number of micronucleated cells were observed. On the contrary, in the long-term treatment (no metabolic activation), the culture supernatant induced statistically and biologically significant increases in the number of micronucleated cells.

Owing to the positive results of the two in vitro genotoxicity tests for the culture supernatant of the NF, the applicant proceeded to test the culture supernatant further in vivo by conducting an in vivo mammalian erythrocyte micronucleus test performed in rat bone marrow combined with an in vivo mammalian alkaline comet assay in liver and duodenum (Unpublished, 2024c).

Sprague–Dawley rats (males; 5/group) were treated orally with the culture supernatant once a day for 3 consecutive days, 24 h apart, up to the maximum acceptable dose volume for an aqueous test item, i.e. 20 mL/kg bw per day. Two lower doses of 10 and 5 mL/kg bw per day were also tested. The sampling time for the vehicle control (sterile water) and the three treatment groups was 2–6 h after the third treatment. The positive control for the micronucleus assay was cyclophosphamide, with a sampling time of 24 h after a single treatment. A total of 4000 polychromatic erythrocytes per animal were assessed for the presence of micronuclei. No statistically significant increase in the number of micronuclei was noted at any dose of the culture supernatant.

The positive control for the comet assay was methylmethane sulfonate and the sampling time was 2–6 h after the third treatment (the same as for the vehicle and the three treatment groups). A total of 150 cells were analysed per animal, i.e. 750 cells per dose. In both liver and duodenum there were no statistically significant increases in the percentage of tail DNA (% tail intensity) at the three doses tested. However, in the duodenum, the tail DNA intensity was significantly lower in the mid-dose group (10 mL/kg bw per day) compared to all the other groups (no dose response relationship). The Panel considers this finding not of concern and notes that, in this study, the culture supernatant did not induce DNA strand breaks and/or alkali-labile sites in liver or duodenum.

Taking into account the results of the genotoxicity tests provided, the Panel considers that there are no concerns regarding the genotoxicity of the NF.

3.10.2 | Subacute toxicity

The applicant provided a repeated dose 28-day oral toxicity study with a 14-day recovery period (Sakamaki et al., 1989). No information was provided on GLP or on any guideline (OECD or other) followed. Sprague Dawley rats (12 per sex and group) received by gavage for 28 days *Clostridium butyricum* TO-A, *Streptococcus faecalis* T-110, *Bacillus mesentericus* TO-A, BIO-THREE®-1 ('for clinical use') or BIO-THREE®-2 ('for veterinary use') at 3000 mg/kg bw per day. The bacterial counts for *C. butyricum* were $\geq 10^{10}$ CFU/g (resulting in $\geq 3 \times 10^{10}$ CFU/kg bw per day). An additional control group received distilled water.

Upon completion of the 28-day dosing period, five animals (per sex and group) were subjected to autopsy and two animals (per sex and group) underwent bacteriological testing. The remaining five animals (per sex and group) were subjected to autopsy upon completion of the recovery period.

No death of any animal occurred during the study. No effects were seen regarding general condition/appearance of animals, body weight changes or water intake. No differences were observed in haematology or clinical chemistry in the *C. butyricum* TO-A group when compared to controls. In the analysis of organ weights, statistically significant differences (compared to controls) were seen in females in the *C. butyricum* TO-A group, showing lower right adrenal weight/body weight ratios upon completion of treatment. In these animals, no histopathological findings were observed.

Blood samples (collected from the abdominal aorta) from the control group and all treatment groups were tested for the presence of bacteria. No bacteria were detected in this test, which suggests that it is unlikely that the administered bacteria were transferred from small bowel mucosa epithelial cells to body tissues.

3.10.3 | Subchronic toxicity

The applicant submitted a repeated dose 90-day oral toxicity study (Unpublished, 2014). According to the full study report, the study followed the GLP Standards for Non-Clinical Safety Studies on Drugs (Ministry of Health and Welfare Ordinance No. 21, Mar. 26, 1997, partially revised on Jun. 13, 2008, Ordinance No. 114, Japan) and the Guidelines for Repeated Dose Toxicity Studies (Notification No. 655, Apr. 5, 1999, Ministry of Health and Welfare, Japan).

The study comprised one control and five treatment groups. Sprague Dawley rats (10 per sex and group) received by gavage for 13 weeks (i) vehicle (potato starch (control)), (ii) *E. faecium*[11] T-110 (at 3000 mg/kg bw per day), (iii) *C. butyricum* TO-A (i.e. the NF) (at 3000 mg/kg bw per day; 3×10^8 CFU/g), (iv) *B. subtilis*[11] TO-A (at 3000 mg/kg bw per day) and BIO-THREE® (v) at 1500 mg/kg bw per day and (vi) at 3000 mg/kg bw per day. The bacterial count of *C. butyricum* TO-A in BIO-THREE® was 2×10^7 CFU/g. The counts of *B. subtilis* TO-A and *E. faecium* in BIO-THREE® were 3×10^6 CFU/g and 5×10^7 CFU/g, respectively.

No death occurred in any group. No clinical signs attributable to treatment with the test article were noted in any animal in any group. Some statistically significant differences between the control group and treatment groups were seen at some points during the study regarding body weight and food consumption, but the differences were small and inconsistent.

With regard to haematological parameters, mean corpuscular haemoglobin concentration was higher in *C. butyricum* TO-A-treated males compared with the controls (33.3 ± 0.6 g/dL vs. 33.7 ± 0.3 g/dL (mean $\pm$ SD)), which was considered incidental since no appreciable changes were noted in any other erythrocytic parameters. In clinical chemistry, creatine kinase concentrations were lower in *C. butyricum* TO-A treated females when compared to controls (102 ± 9 U/L vs. 92 ± 5 U/L (mean $\pm$ SD)); however, these were not associated with histopathological changes. There were no findings in the *C. butyricum* group for ophthalmology, urinalysis, gross pathology, histopathology or organ weights.

The Panel considers that *C. butyricum* TO-A tested at 3000 mg/kg bw per day, corresponding to 9×10^8 CFU/kg bw per day, did not induce adverse effects in this study. Thus, the Panel considers this dose level as the no observed adverse effect level (NOAEL) of the study.

3.10.4 | Human data

The applicant submitted nine human studies (Chen et al., 2010; Dharani Sudha et al., 2017; Huang et al., 2014; Joh et al., 1993; Naranayappa, 2008; Rammohan et al., 2015; Sharma et al., 2008; Urao et al., 1999; Yoshimatsu et al., 2015) in diseased population groups with BIO-THREE®, which included *C. butyricum* TO-A (i.e. the NF) plus two additional strains. The applicant also retrieved via a literature search a number of human studies performed with various other *C. butyricum* strains. The Panel notes that no human studies with the NF alone were provided and considers that no conclusions can be drawn from the human studies submitted to establish the safety of the NF.

[11]For updated accepted names, please consult: https://the-icsp.org/code-of-nomenclatur.

3.11 | Allergenicity

No evidence of allergic reactions to *C. butyricum* was found in the literature search performed by the applicant. In addition, according to the applicant, there were no reports of allergic reactions to the NF in the countries where *C. butyricum* TO-A has already been consumed (as part of BIO-THREE®) for decades.

The Panel considers that the risk of allergic reactions to the NF for the general population is unknown but is expected to be low.

4 | DISCUSSION

The NF, which is the subject of the application, is *C. butyricum* TO-A.

The applicant intends to market the NF as a food supplement. The target population proposed by the applicant is the general population excluding infants below 3 months of age.

The Panel considers that the NF is sufficiently characterised. The information provided on the production process, composition, stability and specifications of the NF is sufficient and does not raise safety concerns.

Taking into account the test results of the genotoxicity tests provided, the Panel considers that, overall, there are no concerns regarding the genotoxicity of the NF.

One 90-day oral toxicity study in Sprague–Dawley rats was submitted in which *C. butyricum* TO-A was tested at 9×10^8 CFU/kg bw per day, which the Panel identified as the NOAEL of this study. Considering an uncertainty factor of 200 to account for inter- and intraspecies variability (10×10) and study duration (from subchronic to chronic; factor of 2), the resulting safe dose in humans would be 4.5×10^6 CFU/kg bw per day.

Considering that appropriate initial bacteria colonisation of the gastrointestinal tract in humans, in particular during the first 3 years of life, profoundly affects health during infancy and childhood (Romano-Keeler & Sun, 2022; Taddei & Neu, 2023), that little is known on how the maternal microbiota shapes the maternal–fetal immune system during pregnancy (Koren et al., 2024), that disruptions to the microbiota early in life can have lasting health effects into adulthood and taking into account that the 90-day oral toxicity study was conducted in adult rats, which hampers the extrapolation of the results to early life stages in humans, the Panel considers that the target population for the NF should be restricted to children above 3 years of age, adolescents and adults, excluding pregnant and lactating women. The corresponding safe doses in the respective population groups are indicated in Table 5.

TABLE 5 Safe doses of the NF considering default body weights and a safe dose (on a body weight basis) of 4.5×10^6 CFU/kg bw per day.

Population group	Age (years)	Body weight[a] (kg)	Max. daily dose (CFU/day)
Other children	3 to < 10	23.1	1.0×10^8
Younger adolescents	10 to < 14	43.4	2.0×10^8
Older adolescents	14 to < 18	61.3	2.8×10^8
Adults, excluding pregnant and lactating women	≥ 18	70	3.2×10^8

Abbreviation: CFU, colony forming units.

[a]Default body weights for each population group are available in EFSA Scientific Committee (2012).

5 | CONCLUSIONS

The Panel concludes that the NF, *C. butyricum* TO-A, is safe at 1.0×10^8 CFU/day for other children (3 to < 10 years), 2.0×10^8 CFU/day for adolescents from 10 to < 14 years, 2.8×10^8 CFU/day for adolescents from 14 to < 18 years, and 3.2×10^8 CFU/day for adults, excluding pregnant and lactating women.

5.1 | Protection of proprietary data in accordance with Article 26 of Regulation (EU) 2015/2283

The Panel could not have reached the conclusion on the safety of the NF under the proposed conditions of use without the data claimed as proprietary by the applicant (Section 2.1).

ABBREVIATIONS

ADI	Acceptable Daily Intake
ADME	absorption, distribution, metabolism and excretion
ANI	average nucleotide identity
AOAC	Association of Official Analytical Collaboration

APC	aerobic mesophilic plate count
BIOHAZ	Panel on Biological Hazards
bw	body weight
C.	*Clostridium*
CFU	colony-forming units
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
EN	European Standard
FAF	Panel on Food Additives and Flavourings
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis Critical Control Points
HPLC	high-performance liquid chromatography
ICP-OES	Inductively coupled plasma optical emission spectroscopy
ISO	International Organization for Standardization
LOQ	limit of quantification
n.d.	not detected
NCBI	National Center for Biotechnology Information
NDA	Panel on Nutrition, Novel Foods and Food Allergens
NF	novel food
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
PCBs	Polychlorinated biphenyls
QPS	qualified presumption of safety
RH	relative humidity
rRNA	ribosomal ribonucleic acid
S	Svedberg unit
SD	standard deviation
SEM	Scanning Electron Microscope
Si	Silicium
TAMC	total aerobic microbial count
TG	Test Guideline
TYMC	total yeast and mould count
UNE	Spanish standardization body
VFD	Virulence Factor Database
WGS	whole genome sequence

REQUESTOR

European Commission

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