

ADVISORY COMMITTEE ON NOVEL FOODS AND PROCESSES

DRAFT OPINION ON AN APPLICATION UNDER THE NOVEL FOODS REGULATION FOR CLOSTRIDIUM BUTYRICUM PROBIOTIC

Applicant: Miyarisan Pharmaceutical Co. Ltd
Responsible Person: Elinor McCartney
EC Classification: 2.2

Introduction

1. An application was submitted to the Food Standards Agency in January 2012 by Miyarisan Pharmaceutical Co. Ltd. for the authorisation of *Clostridium butyricum* (strain CBM 588) as a probiotic food supplement under the novel foods regulation (EC) No 258/97. A copy of the application was placed on the Agency's website for public consultation.
2. Clostridium is a large bacterial genus with more than 150 species. Although the genus contains pathogenic species, notably *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens* and *Clostridium tetani*, the applicant points out that less than 10% of this genus produces toxins. The applicant draws attention to the fact that most clostridial species, especially gut-associated clostridial species are non-pathogenic gut commensals which form an important part of the lower gut flora of humans and animals.
3. The *C. butyricum* strain (CBM 588) intended to be marketed by the applicant is a Gram positive, spore forming, obligate anaerobic, non pathogenic, non genetically modified bacterium.
4. The applicant's intention is to market CBM 588 as viable spores in tablet form intended for use as a probiotic food supplement to support, maintain or restore healthy gut flora physiology and/or function. The applicant intends to make a parallel application for assessment under the EU Nutrition and Health Claims Regulation.
5. The applicant has marketed preparations of CBM 588 for use as a probiotic in Japan and other Asian countries for several decades. This strain of *C. butyricum* has also received EU approval as a microbial feed additive for chickens for fattening, weaned piglets and minor avian and porcine species in 2009 and 2011, respectively.

6. This is the first time that a live microorganism has been assessed under the Novel Foods Regulation. Commission Recommendation 97/618/EC does not address the specific information that should be supplied by applicants for this type of ingredient. However, the European Food Safety Authority (EFSA) has established a framework known as the Qualified Presumption of Safety (QPS) concept which provides a generic assessment system that can be applied to all requests received for the safety assessments of microorganisms deliberately introduced into the food chain. Microorganisms granted QPS by EFSA have been placed on a list thus avoiding the extensive investigation of organisms known not to cause concern. Microorganisms not considered suitable for QPS remain subject to a full safety assessment.
7. *C. butyricum* was considered by EFSA's BIOHAZ Panel in its 2011 update to the QPS list. EFSA concluded that "the safety of *Clostridium butyricum* is a strain-related property, therefore *Clostridium butyricum* should not be recommended for the QPS list." This conclusion was based on the observation that a minority of strains contain a gene coding for botulinum neurotoxin type E and there is only limited knowledge of human and animal exposure to this species. As QPS does not apply, the microorganism should undergo a full novel food assessment.
8. CBM 588 has been classified as a complex novel food from a non-GM source, the source of the novel food has no history of food use in the Community (Class 2.2) according to the scheme in Commission Recommendation 97/618 (EC).

I. Specification of the novel food

Information on this aspect is provided on p. 14-22 of the application dossier

9. The applicant intends to market *Clostridium butyricum* tablets in two forms, standard and strong, containing a minimum of 3×10^5 and 4.5×10^5 viable cells per tablet, respectively. The tablets in different strengths are intended to suit the needs of the consumer as the need for this probiotic may vary amongst individuals. Data on five individual batches indicate that the actual content of the tablets is substantially higher than the quoted minimum (standard: 5 to 7.1×10^6 ; strong: 1.1 to 1.7×10^7). According to a certificate of analysis the content of "strong" tablets should not exceed 4.5×10^7 CFU. The specification for tablets containing CBM 588 has been established by the applicant and can be found in the table below.

Specification	Detail
Appearance	Round tablet, 9mm diameter, white or pale grey, with characteristic odour and sweet taste.
Total aerobic count	< 10 ³ CFU/g
<i>E. coli</i> in 1 g sample	not detected
<i>Staphylococcus aureus</i> in 1 g sample	not detected
<i>Pseudomonas aeruginosa</i> in 1 g sample	not detected
Yeasts & moulds	< 10 ² CFU/g

10. The applicant states that the product complies with the limits for food supplements that are set out in Commission Regulation (EC) 1881/2006 on maximum levels for certain contaminants in foodstuffs. The product specifications also comply with the Japanese Pharmacopoeia. A certificate of analysis is provided in Appendix 6 to the application dossier.

11. The applicant states that the original wild strain of *C. butyricum* MIYAIRI (CBM 588) was isolated in 1963 from a soil sample sourced in Nagano, Japan. This strain is deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan under the strain name *Clostridium butyricum* MIYAIRI 588 strain, deposit number FERM BP-2789. The applicant has preserved their collection of *Clostridium butyricum* MIYAIRI strains by freeze-drying and freezing methods since 1986. Subculture of CBM 588 master cell banks and working cell banks is performed at appropriate intervals. The applicant has provided details of quality control procedures employed for each lot of the novel ingredient including methods to confirm strain identity.

12. Genetic and biochemical stability of CBM 588 has been accepted by EFSA in the context of its use in animal feed. The applicant states that the strain of *C. butyricum* intended to be marketed does not carry any genes encoding any toxins and virulence factors associated with clostridium or other enteropathogens

13. Absence of neurotoxin production was demonstrated by PCR and Southern blot hybridisation for type E botulinum toxin gene. The absence of genes encoding botulinum neurotoxin A,B,F and genes encoding non-toxic haemagglutinin (NTNH) and genes encoding *Clostridium perfringens* toxins (alpha, beta, epsilon and iota) was demonstrated by PCR assay. The applicant acknowledges that the presence of a single cryptic plasmid of 6.5 kb has been noted in this strain of *C. butyricum* but the nucleotide sequence of this plasmid was analysed and none of the nine putative open reading frames encoded any

known virulence factor of *Clostridium* spp. (EFSA 2009, 2011). The applicant provided further details of these analyses in Appendices 12 and 13 to the dossier.

14. The susceptibility of this strain of *C. butyricum* to key antibiotics as recommended by EFSA was tested. The applicant reports that the minimum inhibitory concentrations of these key antibiotics were lower than the EFSA breakpoints confirming that CBM 588 is not resistant to antibiotics of human or veterinary importance (EFSA 2008, 2009, 2011).

Discussion: *The Committee was not sufficiently reassured that the data provided by the applicant conclusively demonstrated the absence of pathogenic clostridial toxins and other virulence factors in CBM 588. The Committee requested that the applicant provide a genome sequence for CBM 588 in comparison to other related species and strains, and a comprehensive bioinformatics analysis to ensure the absence of functional or partial virulence genes. The Committee emphasised that it was necessary to review the full dataset for the genome sequencing exercise, including information on the quantitative homology with other clostridial genome sequences for all open reading frames (ORFs) identified in CBM 588. The applicant had provided genome sequence data. The final assembly of the genomic sequence was initially hampered by the presence of redundant DNA sequences, for example ribosomal RNA (rRNA) genes and 200 bp direct repeat sequences, which create gaps in the deduced genomic sequence. The applicant did nonetheless state that 100% of the protein coding sequences of the genome (4208) had been sequenced. Subsequently, the sequences obtained were assembled into 157 contiguous sequences (contigs) which the applicant has listed. Details of the complete nucleotide sequences were also provided.*

The 157 sequences were uploaded to the software “GENOME GAMBLER” and the ORFs were predicted following the procedure described by Shimizu et al., 2002. The applicant has provided BLAST (Basic Local Alignment Search Tool) search results of CBM 588 ORFs against a non redundant protein database, which includes all bacterial protein sequences. The degree of homology is reported, based on the sequence alignment between CBM588 and all bacteria and the percentage of identical aligned amino acids between the ORF of CBM588 and the most identical sequence of all other bacteria.

The applicant performed a bioinformatics comparison of genomic sequences of CBM 588 with other available bacterial genomic sequences including clostridial species, to identify known virulence factors or clostridial toxins. The applicant provided nucleic acid sequences and amino acid sequences of the three putative virulence genes identified in CBM 588 (haemolysin A, haemolysin 3 and fibronectin-binding protein) and a detailed explanation as to why these sequences are not a cause for concern, including evidence to demonstrate lack of haemolytic activity in CBM 588. The Committee was content that the putative virulence genes are non-functional in CBM 588.

The applicant has also provided bioinformatics data to show that similar or related haemolysin sequences are present in the genomes of several Lactobacillus species; thus the presence of these genes, particularly when non-functional, does not necessarily indicate pathogenicity.

The Committee was satisfied that the applicant's additional data addressed its concerns and no further information was requested.

The Committee noted that several antibiotic resistance determinants were identified in the genome sequence of CBM 588 (tetracycline, chloramphenicol, beta-lactams, vancomycin) and the genome sequence suggests there may be others. The Committee requested further clarification from the applicant.

The applicant provided updated antimicrobial resistance data on CBM 588 strains (C. butyricum FERM BP-2789 as originally deposited, and the current CBM 588 working strain), the type strain (C. butyricum ATCC 19398^T) and Bacteroides fragilis ATCC 25285 as a positive control.

Resistances to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, tetracycline, vancomycin, metronidazole and acriflavine were tested. Both C. butyricum ATCC 19398^T and CBM 588 were susceptible to all of the antimicrobials used except aminoglycosides (i.e. gentamicin, kanamycin and streptomycin) and acriflavine.

The applicant highlighted in its response that anaerobes are intrinsically resistant to aminoglycosides and possibly acriflavine, in addition to clarifying that acriflavine is a topical antiseptic, thus any tolerance would be of little clinical significance.

The applicant's response highlights that the specific risk of transferring non-functional antibiotic resistance genes from CBM 588 to other bacteria where they may be functional, seems low, given the well documented use in humans in Japan since the 1960s which has been supported by pharmacovigilance

carried out by the applicant, the Japanese medical profession and Japanese regulatory authorities. Gene transfer issues are discussed further below in Section XIII.

The Committee was reassured by the applicant's new data relating to antibiotic resistance and no further information was requested on this point.

II. Effect of the production process applied to the novel food

Information on this aspect is provided on p 23-24 of the application dossier

15. The applicant's *Clostridium butyricum* supplement is produced by submerged anaerobic fermentation followed by centrifugation, drying, blending and packaging to produce either strong or standard tablets. The process complies with Japanese Good Pharmaceutical Manufacturing Practice and details can be found in the dossier.

***Discussion:** The Committee asked whether the quality control procedures employed during production are adequate to ensure the safety of individual batches of the novel ingredient. The applicant explained that fermentation of CM588 is carried out under strict conditions of monoculture, under certified pharmaceutical-quality GMP. The purity of every lot of CBM powder concentrate is tested by appropriate traditional and molecular microbiological methods to ensure no contamination by other Clostridial strains, which ensures a low risk of gene transfer events to CBM 588 during manufacture. The Committee was satisfied with this section of the dossier.*

III. History of the organism used as a source of the novel food

Information on this aspect is provided on p 24-25 of the application dossier

16. The applicant has marketed preparations of CBM 588 for use as a probiotic in Japan and other Asian countries for several decades.

***Discussion:** The Committee was reassured by the knowledge that CBM 588 preparations have been sold in Japan since the 1960s but requested further information on monitoring of side effects in Japan. The applicant has provided updated post-market monitoring data to replace the data in the original dossier to demonstrate that between 2005 and 2012, there have been no confirmed adverse effects or adverse drug reactions related to CBM 588, as defined by WHO pharmacovigilance procedures.*

IX. Anticipated intake/extent of use of the novel food

Information on this aspect is provided on p 25 of the application dossier

17. The applicant has considered historical and current consumption patterns of CBM 588 in non-EU countries in order to derive appropriate daily intakes of this food supplement in the EU. The applicant states that daily intake of CBM 588 as a food supplement in the EU as intended for market is expected to be within the range of 3×10^5 to 1.35×10^8 CFU/day (one standard tablet to three strong tablets per day). The supplement is intended for healthy adults.
18. The applicant states that the optimum daily dose may vary between adults but the appropriate daily dose is anticipated to provide gut health benefits such as improved gut transit time, improved faecal bulk and consistency and more comfortable bowel movements. The Committee's assessment focussed only on safety and labelling and does not address any nutrition or health benefits that may be claimed for the novel ingredient or for foods that contain it. Nutrition or health claims may only be made if they are specifically authorised under the EU Nutrition and Health Claims Regulation (EC) 1924/2006.
19. The applicant states that CBM 588 does not establish permanently in the gut.

Discussion: The Committee did not raise any concerns relating to this section of the dossier.

XI. Nutritional information on the novel food

Information on this aspect is provided on p 26 of the application dossier

20. The applicant states that CBM 588 is not intended to replace any other foods or nutrients in the diet, and does not supply significant dietary macro or micro nutrients.

Discussion: The Committee did not raise any concerns about this section of the dossier.

XII. Microbiological information on the novel food

Information on this aspect is provided on p 26 of the application dossier

21. Microbiological specifications are presented above in Section I. The applicant has also acknowledged the possibility that CBM 588 may have effects on the intrinsic gut flora of animals and humans but has highlighted a number of published studies illustrating that CBM 588 has no adverse effects on beneficial gut flora of humans or animals.

***Discussion:** The Committee questioned the effects of CBM 588 on the host gut epithelium, microbiome and immune system, as the dossier does not specifically address these issues and data from animal feeding studies presented in the toxicology section of the dossier indicate that CBM 588 may have immune effects. For example, the study by Yuzawa et al. 1987a refers to increased platelet and white blood cell counts in rats fed higher doses of CBM powder. The Committee also requested further information to rule out any possibility that CBM 588 has any detrimental effects on the host microbiome.*

*The applicant highlighted that CBM 588, in common with other probiotic bacteria, interacts with host microbiota and immune functions, but does not exert harmful, pro-inflammatory or inflammatory effects. The genomic data confirm that CBM 588 is a typical *Clostridium butyricum*, closely related to the type strain, *C. butyricum* ATCC 19398. Wild-type *Clostridium butyricum* strains are common commensal inhabitants of the gut of healthy individuals.*

*The applicant has therefore concluded that CBM 588 consumption is not expected to adversely affect the host microbiota, drawing on the presence of strains of *C. butyricum* in the healthy gut and three efficacy studies which demonstrated that CBM 588 did not have any adverse effects on human microbiota.*

The Committee accepted the applicant's information and no further information was requested.

XIII. Toxicological information on the novel food

Information on this aspect is provided on p. 27-29 of the application dossier

22. The applicant highlights that the safety of CBM 588 has been reviewed by EFSA in 2009 and 2011 when EFSA concluded that its use in animal nutrition is safe for animals, consumers, industrial workers/users and the environment. The applicant reiterates that CBM 588 does not pose a risk to humans as the strain does not carry genes encoding relevant toxins and virulence factors, nor does it harbour acquired or transferable antibiotic resistance.
23. The applicant has described a series of toxicological studies using CBM powder (the dried fermentation concentrate of CBM 588). An acute oral toxicity study in rats showed that the acute oral toxicity of CBM powder is in excess of 5000 mg/kg body weight. A subacute (5 week) oral toxicity study investigating the effects of CBM powder in beagle dogs showed a NOEL (No Observed Effect Level) of 2000 mg/kg body weight/day (the highest dose

tested). Chronic oral toxicity of CBM powder was investigated in SPF Fischer 344 rats over a twelve month period. Some effects (increased blood glucose and increased urine volume and kidney weights in males) were observed at the highest dose tested (50 g/kg diet) but macroscopic and microscopic pathological examinations revealed no differences between treated and untreated rats. The NOEL was therefore determined to be 5 g/kg diet (equivalent to 241 mg/kg body weight/day in male rats and 288 mg/kg body weight/day in female rats).

24. The applicant highlights that the optimum CBM 588 intake may vary between individuals but emphasises that the maximum intake envisaged in healthy adults in the EU is 100 fold less than the NOEL calculated from toxicological studies in laboratory animals. The lowest NOEL for CBM powder was determined as 241 mg/kg bodyweight per day in male rats. From these data, the NOEL can be extrapolated to a 60 kg human as 14.46 g/day ($0.241 \text{ g} \times 60 = 14.46 \text{ g}$). CBM powder contains $\geq 1 \times 10^9$ CFU CBM 588 per g, so the NOEL is equivalent to a dose of 1.45×10^{10} CFU/day for a 60 kg adult. The highest anticipated dose of CBM 588 (3 Strong Tablets per 60 kg adult per day, each containing up to 4.5×10^7 CFU) is 1.35×10^8 CFU/adult/day. This is 107 times less than the human equivalent of the NOEL.
25. The applicant also details a study looking into mutagenicity of CBM powder by way of reverse mutation assays and chromosome aberration assays; the study highlights that CBM 588 did not exhibit any mutagenicity or clastogenicity in this study.
26. Although CBM 588 does not have a history of consumption as a food ingredient in the EU, the applicant draws attention to other examples of previous human exposure to *Clostridium butyricum*. The applicant refers to studies which show that *C. butyricum* strains are commensals in the gut of humans and may colonise the gut of infants after birth.
27. The applicant has sold preparations of CBM 588 in Japan and other Asian countries for both human and animal use since the 1960s and the applicant states that there have been no confirmed adverse effects related to CBM 588 consumption nor any reports of allergenicity.

Discussion: *The Committee was satisfied that the toxicology data presented in the dossier did not give cause for concern. The Committee however, discussed the possibility of transfer of any possible virulence genes from CBM 588 to other bacteria in the gut or vice versa and requested further reassurance from the applicant on this aspect.*

The Committee highlighted that some toxin-encoding genes have been reported to reside on chromosomally-located conjugative transposons in Clostridia and such transposons can mediate efficient transfer to bacteria.

The applicant submitted additional data from bioinformatic analyses illustrating that CBM 588 does not harbour any chromosomally-encoded toxins of concern, so such genes cannot be transferred to other bacteria. Only three putative virulence genes were identified for CBM 588 which are non-functional in CBM 588 (the Committee was relatively content with the applicant's BLAST analyses data demonstrating that the putative haemolysin genes are non-functional and likely encode other proteins such as channel or membrane proteins or be involved in tRNA synthesis and FtsJ-like methyltransferase activity and are also found in gut commensals such as Lactobacillus spp).

The Committee considered that it is largely unknown whether these sequences and partial sequences could be transferred to other bacteria in the gut where they could become functional. However, to put the matter in perspective, the Committee highlighted that many gut bacteria harbour a multiplicity of pathogenicity determinants, which are being swapped back and forth (at least those borne on plasmids) and, in this environment, the non-functioning chromosomal sequences detected in CBM 588 would be an irrelevance. The Committee noted that CBM 588 has been marketed in Japan for several decades and requested further information on any adverse effects monitoring data that the applicant may hold. Following a response from the applicant to this, the Committee requested further details relating to the small number of suspected adverse effects mentioned in the applicant's response and the rationale for concluding that none were related to CBM 588 consumption.

The applicant categorised suspected adverse effects or adverse drug reactions into different groups in order to provide the Committee with more details. The applicant states that in most cases, no probable causal relationships between the reported adverse effect and CBM 588 was determined. In other cases, not enough information was provided to evaluate the possible relationship. The applicant therefore concluded that there are no confirmed adverse drug reactions related to its novel ingredient. The applicant emphasised that the number of adverse effect reports represent a tiny proportion of the total sales of CBM 588.

The Committee was satisfied with the applicant's response. The Committee suggested that, given that this is the first live micro-organism to be assessed as a novel ingredient, it could be useful for the applicant to establish a mechanism for monitoring adverse effects, should the novel ingredient be authorised in the EU for use in food supplements.

CONCLUSION

The ACNFP has completed its assessment of *Clostridium butyricum* CBM588 as a novel ingredient to be added to supplements and concluded that it did not have any unanswered safety concerns relating to this novel ingredient.

These conclusions are based on the information in the applicant's dossier, supplemented by additional information that the applicant provided, relating to:

- Verification of the absence of toxins and other virulence factors in CBM588 to be demonstrated by genome sequence and bioinformatics data
- Verification that the antibiotic resistance determinants identified CBM 588 are non-functional
- Impact of CBM 588 on host gut epithelium, microbiome and immune system
- Reassurance that any toxin or virulence-encoding genes will not be transferred to other bacteria in the gut
- Further details on the small number of suspected adverse effects reported

Draft: March 2013