100 S. Wacker Dr. | Suite 2000
Chicago, IL 60606
AminTalati.com

312.466.1033 PHONE
312.884.7352 FAX

AMIN | TALATI WASSERMAN

CHICAGO · WASHINGTON D.C. · LOS ANGELES

October 25, 2021

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Re: Generally Recognized as Safe (GRAS) Notice for D-β-Hydroxybutyrate (D-BHB)

Dear Sir/Madam:

Pursuant to 21 C.F.R. part 170, subpart E, Osaka Gas Chemicals Co. Ltd., hereby submits the enclosed notice, that use of its D-β-Hydroxybutyrate (D-BHB) at levels of up to 6 g per serving in conventional foods is excluded from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because the notifier has determined that such use is generally recognized as safe (GRAS).

Sincerely,

Ashish Talati
Amin Talati Wasserman
100 S. Wacker Drive,
Suite 2000
Chicago, IL 60606
Email:
Ashish@AminTalati.com

EVALUATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF D-β-HYDROXYBUTYRATE (D-BHB) AS A FOOD INGREDIENT

Prepared for:
Osaka Gas Chemicals Co. Ltd.
2-37, Chiyozaki 3-cyome-minami,
Nishi-ku, Osaka, 550-0023
JAPAN

Panel Members

Robert L. Martin., Ph.D.

John A. Thomas, Ph.D., FACT, FATS

Madhusudan G. Soni, PhD, FACN, FATS

October, 2021

EVALUATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF D-β-HYDROXYBUTYRATE (D-BHB) AS A FOOD INGREDIENT

TABLE OF CONTENTS

1. PART I- SIGNED STATEMENTS AND CERTIFICATION

In accordance with 21 CFR § 170 Subpart E consisting of § 170.203 through § 170.285, Osaka Gas Chemicals Co. Ltd. (Osaka Gas Chemicals) hereby informs the FDA that D-β-Hydroxybutyrate derived from *Halomonas* sp., is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on Osaka Gas Chemicals' view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described below.

1.1. Basis of Conclusion:

This GRAS conclusion for the use of D-β-Hydroxybutyrate (D-BHB) has been reached in accordance with the requirements in 21 CFR 170.220.

1.2. Name and address of organization:

Osaka Gas Chemicals Co. Ltd.
5-11-61,Torishima Konohana-ku,
Osaka, 554 -0051
JAPAN

1.3. Name of substance:

The name of the substance of this GRAS assessment is D-β-Hydroxybutyrate. The substance is also known as D-BHB; 3-Hydroxybutyric acid; 3-Hydroxybutanoic acid.

1.4. Intended conditions of use:

D-β-Hydroxybutyrate (D-BHB) is intended to be used as a food ingredient in selected food categories such as Beverages and Beverage Bases (Sports and nutrition beverages) and Grain Products (Sports and nutrition bars) at a maximum level up to 6 g per serving of food (Reference Amounts Customarily Consumed Per Eating Occasion; 21 CFR § 101.12). The use of D-BHB is targeted to high performance athletes as well as for use in conventional foods for the general interested population. It is recognized that there are Standard of Identity requirements for some of these specified foods and these foods will not be referred by their commonly recognized names. The D-BHB, subject of this GRAS assessment, is not proposed for uses in foods that are intended for infants, such as infant formulas.

1.5. Statutory Basis for GRAS conclusion:

This GRAS conclusion is based on scientific procedures in accordance with 21 CFR 170.30(a) and 170.30(b).

1.6. Exemption from Premarket approval requirements:

Osaka Gas Chemicals has concluded that D-BHB is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on our conclusion

that D-BHB, meeting the specifications cited herein, and when used as nutrient and as a food ingredient in selected conventional food products, is GRAS and is therefore exempt from the premarket approval requirements.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that D-BHB, when used as described in this dossier, is GRAS based on scientific procedures.

1.7. Availability of data and information:

The data and information that are the basis for this GRAS conclusion will be made available to FDA upon request by contacting

Mariko Kato

Deputy Research Director
Frontier Materials Laboratories

Osaka Gas Chemicals Co. Ltd.
5-11-61,Torishima Konohana-ku,
Osaka, 554 -0051
JAPAN

Phone: +81-6-6467-1571
Email: kato@ogc.co.jp

Or

Amin Talati Upadhye, LLP
100 S. Wacker Dr., Suite 2000
Chicago, IL 60606

Phone: 312.327.3381
Email: Ashish@amintalati.com

The data and information will be made available to FDA in a form in accordance with that requested under 21 CFR 170.225(c)(7)(ii)(A) or 21 CFR 170.225(c)(7)(ii)(B).

1.8. Data exempt from Disclosure:

Parts II through VII of this GRAS notification does not contain data or information that is exempt from disclosure under the Freedom of Information Act. There is no privileged or confidential information such as trade secrets and/or commercial or financial information in this document. Therefore the information contained in this dossier can be made publicly available.

1.9. Certification:

Osaka Gas Chemicals certifies that, to the best of its knowledge, this GRAS conclusion is based on a complete, representative, and balanced dossier that includes all relevant information, available and obtainable by Osaka Gas Chemicals, including any favorable or unfavorable information, and pertinent to the evaluation of the safety and GRAS status of the use of D-BHB. Osaka Gas Chemicals accepts responsibility for the GRAS determination that has been made for D-BHB as described in this dossier.

1.10. Name, position/title of responsible person who signs dossier and signature:

Kazuhiro Fujiwara

Executive Officer
Research Director
Frontier Materials Laboratories

Osaka Gas Chemicals Co. Ltd.
5-11-61,Torishima Konohana-ku,
Osaka, 554 -0051
JAPAN

Phone: +81-6-6467-1571
Email: k-fujiwara@ogc.co.jp

Signature: 

1.11. FSIS/USDA – Use in Meat and/or Poultry:

Osaka Gas Chemicals does not intend to add D-BHB to any meat and/or poultry products that come under USDA jurisdiction. Therefore, 21 CFR 170.270 does not apply.

2. PART II- IDENTITY AND TECHNICAL INFORMATION

2.1. Description

The subject of this GRAS assessment, D-β-Hydroxybutyrate (D-BHB), is a standardized preparation derived from a specific strain derived from *Halomonas* sp.by a fermentation and extraction process. D-BHB, produced by fermentation as acid form from sugar and other natural ingredients, has 99% optical purity. The preparation is a clear to pale yellow liquid with characteristic sour taste. General descriptive characteristics of D-BHB are summarized in Table 1.

Table 1. General Descriptive Characteristics of D-β-Hydroxybutyrate

Parameter	Description *
Source	*Halomonas* sp. strain KM-1
Synonyms	(R)-3-hydroxybutyrate; (3R)-3-hydroxybutanoate; D-3-hydroxybutyrate; D-beta-hydroxybutyrate; 3-D-hydroxybutyrate; 3-hydroxy-butanoic acid
Trade name	OKETOA
Systematic name	beta-Hydroxybutyric acid
IUPAC name	(3*R*)-3-hydroxybutanoate
CAS No.	625-72-9
Chemical formula	$C_4H_7O_3$; $CH_3CH(OH)CH_2CO_2H$ (acid); in solution exist as $C_4H_7O_3$- and H^+
Molecular weight	104.10
Appearance	Solution
Color	Clear or Pale yellow
Odor	No odor
Taste	Sour
Storage	Cool and Dark Place
Shelf life	5 years

*Based on information provided Osaka Gas Chemicals

D-BHB is a conjugate base of β-hydroxybutyric acid. A conjugate base is what is left over after an acid has donated a proton during a chemical reaction. Hence, a conjugate base is a species formed by the removal of a proton from an acid, as in the reverse reaction it is able to gain a hydrogen ion. Thus, BHB is a hydroxy fatty acid anion that is the conjugate base of 3-hydroxybutyric acid, obtained by deprotonation of the carboxy group which is the major species at pH 7.3. BHB is a chiral compound with two enantiomers: D-β-hydroxybutyric acid and L-β-hydroxybutyric acid. The chemical structure of D-BHB is presented in Figure 1.

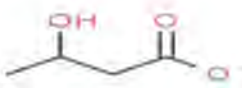


Figure 1. Chemical Structure of D-β-Hydroxybutyrate.

2.2. Specifications and Identity

In order to ensure a consistent and safe product, Osaka Gas Chemicals has established food grade specifications for D-BHB (Table 2). Analytical results from five lots of D-BHB (Appendix I) demonstrate that it is consistently manufactured and meets the standard

specifications. Please note that of the five lots, two lots only provide assay related information. The analytical methods used for the qualitative and quantitative analysis of the individual specification parameters are validated for their intended use. Generally, D-BHB, subject of present GRAS, is produced as a 40% solution; however, depending on the customer need it can be produced as a 60% or 80% solution.

Table 2. Specifications of D-β-Hydroxybutyrate

Parameters	Limits	Analysis method
Appearance	Clear of Pale yellow	Visual
Assay	NLT 93% HPLC substrate remains and fermentation by products (lactate, acetate) excluded	
β-Hydroxybutyrate (free)	>95%	HPLC[1]
Acetic acid	NMT 5%	HPLC[1]
BHB (free)	38 - 40%	HPLC[1]
Chirality (D-BHB %)	NLT 95%	HPLC[1]
Heavy metals		
Arsenic (As)	≤ 0.5 ppm	AAS
Cadmium (Cd)	≤ 0.5 ppm	AAS
Lead (Pb)	≤ 0.5 ppm	AAS
Mercury (Hg)	≤ 0.1 ppm	AAS
Microbial limits		
Aerobic mesophilic microorganisms	3,000 cfu/g	ISO 4833
Aerobic spores, thermophilic	100 cfu /g	Standard Agar Plating Method[2]
Enterobacteriaceae	0 /g	ISO 21528-1
Salmonella	0 /25 g	ISO 6579

NMT= not more than; NLT = not less than; ppm = parts per million; cfu = colony-forming unit
[1]HPLC: A copy of the HPLC method used for assessment of purity is provided in Appendix II.
[2]Heat-shocked conditions: in boiling water for 30 minutes, incubation conditions: 55 degree for 2 days

2.3. Manufacturing Process

D-β-Hydroxybutyrate (D-BHB) from Osaka Gas Chemicals is manufactured according to current good manufacturing practices (cGMP) for food ingredients at Osaka Gas Chemicals Co. Ltd. 2-37, Chiyozaki 3-cyome-minami, Nishi-ku, Osaka, 550-0023, Japan. As described below, D-BHB is produced by fermentation.

Production Organism:

A strain of the genus *Halomonas* is used in the production of BHB salt. The taxonomy of the production strain is shown in Table 3. Members of *Halomonas* are halophilic (salt-

tolerating), gram-negative, facultative aerobic, rod-shaped bacteria (Vreeland 2015). The production strain is wild type or naturally occurring mutants of *Halomonas sp.* strain KM-1. *Halomonas* sp. strain KM-1 has been deposited in the International Patent Organism Depositary (IPOD, AIST, Japan) as FERM BP-10995 (Kawata et al., 2012). *Halomonas* sp. strain KM-1 is moderately halophilic that exhibits higher level of poly(3-hydroxybutyrate) (PHB) production under aerobic conditions than other species. The 16S rRNA gene sequence of *Halomonas* sp. strain KM-1 exhibited a high level of sequence similarity (99.0%) to *Halomonas* species (Kawata and Aiba, 2010). *Halomonas* sp. KM-1 is not a genetically modified organism. *Halomonas* sp. KM-1 genome does not include any deleterious genes.

The *Halomonas* used for BHB production is maintained in-house and is subject to strict quality control for compliance with established internal specifications and is free of microbial contamination.

Table 3. Taxonomy of D-β-Hydroxybutyrate Production Organism

Taxonomy	Taxonomic Assignment
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Oceanospirillales
Family	Halomonadaceae
Genus	*Halomonas*
Species	*Halomonas* sp. strain KM-1

Raw Materials and Processing Aids:

The raw materials and processing aids used in the manufacture of BHB and its derivatives are listed in Table 4. All raw materials and processing aids are food-grade quality and are safe and suitable for use in the manufacture of food ingredients consistent with appropriate U.S. federal regulations, or have previously been determined to be GRAS.

Table 4. Processing Aids/Additives Used During Manufacturing

Material	Function	Regulatory Status
Fermentation Aids		
Ammonium sulfate (NH4)2SO4	Processing-aid (sulfate source for fermentation	§184- Direct food substances affirmed as generally recognized as safe Permitted for use in foods as a dough strengthener, firming agent, and processing aid in accordance to cGMP (21 CFR §184.1143)
Magnesium sulfate MgSO4•7H2O	Processing-aid (Fermentation nutrient)	21 CFR §184 - Direct food substances affirmed as generally recognized as safe Permitted for use in foods as a flavor enhancer, nutrient supplement, or processing aid in accordance to cGMP (21 CFR §184.1443)
Calcium chloride CaCl2· 2H20	Processing-aid (Fermentation nutrient	21 CFR §184 -Direct food substances affirmed as generally recognized as safe Permitted for use in foods as an anti-caking agent, antimicrobial agent, curing or pickling agent, firming agent, flavor enhancer, humectant, nutrient supplement, pH control agent, processing aid, stabilizer and thickener, surface-active agent, synergist, and texturizer not to exceed cGMP (21 CFR §184.11 93)

Potassium Phosphate K2HPO4	Processing-aid (Fermentation nutrient)	This substance is generally recognized as safe when used in accordance with good manufacturing practice (2 1CFR§182.6285)
Ammonia water	Processing-aid (nitrogen source for fermentation and pH control)	pH control- FCC 9th ed.
Sodium hydroxide	Processing-aid (pH control)	GRAS substance and permitted for use in accordance with cGMP (2 1 CFR §184.1763)
Iron sulfate FeSO4•7H2O	Processing-aid (Fermentation nutrient)	GRAS (21 CFR §184.1307)
Yeast extract	Processing-aid (Fermentation nutrient)	GRAS (21 CFR §184.1983)
Sorbitan Esters of Fatty Acids	Emulsifier	Emulsifier is permitted for use in the processing of foods (21 CFR §173.340)
Purification Aids		
Cation exchange resin	Purification	Ion-exchange resin permitted for use in the treatment of food under 21 CFR §173.25
Activated carbon	Adsorption	FCC 9th ed.

Manufacturing Details

The production of BHB is consistent with the principles of Hazard Analysis and Critical Control Points (HACCP) and Food Safety certification. A schematic overview of the manufacturing process of BHB is provided below in Figure 2.

Preparation of Working Cell Bank

The working cell bank (WCB) is prepared by dissolving food-grade raw materials in purified water. Following sub-loading in a clean flask, the tube well is packaged with Kraft paper and sterilized at 121°C for 20 minutes. After cooling, Iron Sulfate, which is separately sterilized at 121°C for 20 minutes, is added just before use. Under aseptic condition, the WCB is drawn from the master cell bank with a pipette and transferred to the prepared medium.

Culturing in Seed Tank and Fermentation

Food-grade raw materials are poured into the seed tank. Purified water is added to dissolve and dilute to scale. The mixture is sterilized at 121°C for 20 minutes. After cooling, Iron Sulfate, which is separately sterilized at 121°C for 20 minutes, is added just before use. The shaken flask culture is transferred to the seed tank under aseptic conditions.

Fermentation

To prepare the fermentation broth, raw materials are added to the fermentation tank and purified water is added as appropriate to dissolve and dilute. The broth is sterilized at 121°C for 20 minutes. After cooling, Iron Sulfate, which is separately sterilized at 121°C for 20 minutes, is added just before use. The seed tank broth is transferred to the fermentation tank by a transferring pipeline. During the fermentation, the proper volume of ammonia water and Sodium hydroxide is added to maintain the desired pH.

Extraction and Purification

BHB is isolated and purified through a series of filtration steps through a Micro, Ultrafiltration membrane followed by concentration and Ion exchange. After that, BHB is

further purified by Nanomembrane filtration and activated carbon followed by concentration.

The fermentation broth is filtered by Microfiltration membrane to remove the source organism. The filtrate is collected and filtered by Ultrafiltration membrane to remove the protein. The filtrate is concentrated under vacuum and applied to a cation exchange column to remove cations including NH4+ and Na+. Then, the permeated liquid is filtered by Nanofiltration for further purification and decolored by activated carbon. BHB is supplied as concentrated solution.

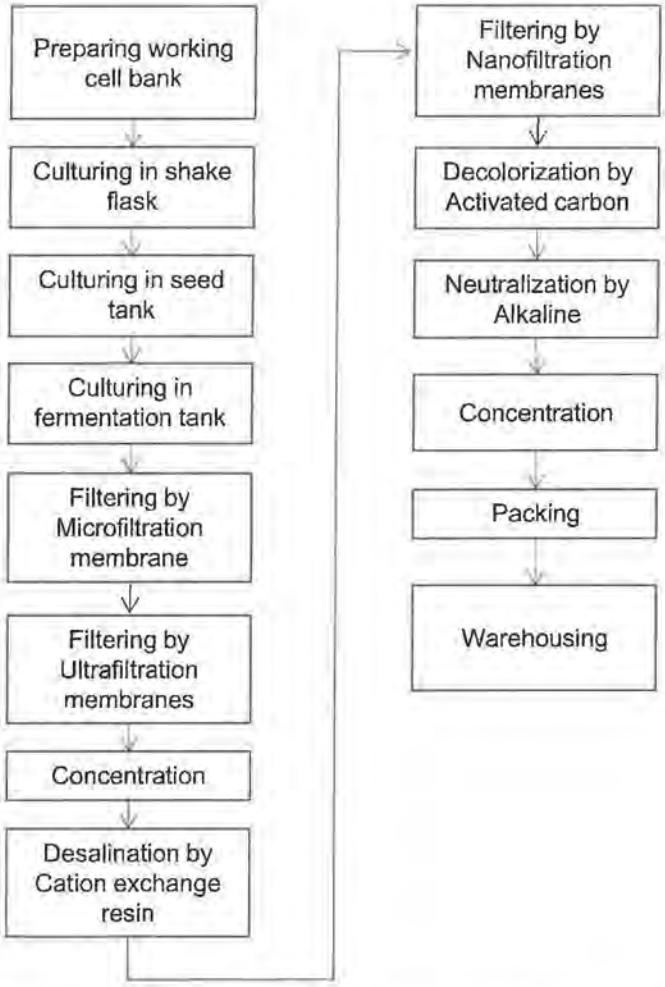


Figure 2. Manufacturing Flow Chart for D-β-Hydroxybutyrate from *Halomonas sp.* strain KM-1

In the manufacturing of D-BHB, high quality food grade materials are used. The manufacturing process assures a consistent and high quality D-BHB product. The production process ensures that the potential for contamination or the introduction of impurities is minimized. Processing aids and additives used as carrier in the manufacturing process are all food-grade quality and comply with specifications described in the current edition of the Food Chemicals Codex. Any potential impurities from fermentation are removed in three steps that include Micro, Ultra, and Nano membrane filtration.

3. PART III- DIETARY EXPOSURE

3.1. Intended Uses and Food Categories

Osaka Gas Chemicals intends to use D-β-Hydroxybutyrate (D-BHB) as a food ingredient in selected conventional food categories such as sports and nutrition beverages or powder, sports and nutrition bars, and sports and nutrient gels designed for consumption as an energy source. Intake estimates have been determined for both the target population (high performance athletes) and the general U.S. population using two assessment models: Assessment of the intake by the target population using a serving size-based approach ("target population assessment" hereafter); and Assessment of the intake by the general population based on a serving size (up to 6 g/serving) using consumption data from the U.S. National Center for Health Statistics' National Health and Nutrition Examination Surveys (NHANES) ("general population assessment" hereafter).

The details of food categories to which D-BHB is proposed for use are summarized in Table 5, along with descriptions of the types of foods within the category that was included in the assessment, the serving size associated with each food type, and the maximum use level of D-BHB. The intake analysis was conducted by Intertek Group PLC and the complete report is attached as Appendix III. The subject of this GRAS, D-BHB, will not be used in any foods for which food standards would preclude its use. Foods that are intended for infants, such as infant formulas and meat and poultry products that come under USDA jurisdiction are excluded from the list of intended food uses of D-BHB.

3.2. Methods Used for Estimated Daily Intake

For the target population intake estimates, no food consumption data was incorporated. However, worst-case estimates of intake were determined using the number of servings consumed per day. For the general population intake assessment, the NHANES dataset was used. For this population, consumption data from individual dietary records of the NHANES, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of BHB by the U.S. population. Estimates for the daily intake of BHB represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2017-2018. From these average amounts, a distribution was established from which the mean and percentile intake estimates for the cohort of interest were determined, which incorporated survey weights in order to provide representative intakes for the entire U.S. population. "*Per capita*" intake refers to the estimated intake of BHB averaged over all individuals surveyed, regardless of whether they consumed food products in which BHB is proposed for use, and therefore includes individuals with "zero" intakes (*i.e.*, including individuals who reported no intake of food products containing BHB during the 2 survey days). "Consumer-only" intake refers to the estimated intake of BHB by only those individuals who reported consuming food products of interest on either Day 1 or Day 2 of the survey.

For the general population assessment, food codes representative of each proposed food use were chosen from the NHANES 2017-2018. Food codes were grouped in the food use categories according to Title 21, Section §170.3 of the *Code of Federal Regulations*. Summary of the individual proposed food uses and use levels for BHB is provided in Table 5.

Table 5. Summary of the Individual Proposed Food Uses and Use Levels for D-β-Hydroxybutyrate.

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Food Uses[a]	BHB Use Level (g/serving)	RACC[c] (g or mL)	BHB Use Level, as Prepared (g/100 g)
Beverages and Beverage Bases	Sports and nutrition beverages[b]	6	360	1.7
Grain Products	Sports and nutrition bars	6	40	15

BHB = D-β-hydroxybutyrate; CFR = *Code of Federal Regulations*; NHANES = National Health and Nutrition Examination Surveys; RACC = Reference Amounts Customarily Consumed per Eating Occasion; U.S. = United States.

[a] BHB is intended for use in unstandardized products when standards of identity, as established under 21 CFR § 130 to 169, do not permit its addition.

[b] Food codes for the proposed use in "sports gels" were not available in the 2017-2018 NHANES and therefore this use was not included in Table 3-1. Furthermore, this intended use is considered to be substitutional for other products proposed to contain BHB and is excluded from the calculation of estimated daily intakes of BHB in the general U.S. population due to absence of consumption data.

[c] RACC based on values established in 21 CFR § 101.12. When a range of values is reported for a proposed food use, particular foods within that food use may differ with respect to their RACC.

3.3. Estimated Daily Intake of D-BHB from Proposed Uses

3.3.1. Estimated Daily Intake of D-BHB by the Target Population

As mentioned above, BHB will be used in selected categories of food and beverage products (beverages, bars, and gels) designed for consumption as an energy source by high-performance athletes (≥18 years) in the U.S. in accordance with the intended directions for use of 1 to 3 servings per day of any combination of products containing BHB, which will be indicated on product labels. In order to estimate daily intakes by this population cohort, it was assumed that between 1 and 3 servings per day could be consumed by the consuming individuals. This was combined with the proposed inclusion level of BHB in these at levels up to 6 g/serving, irrespective of the serving size. Table 6 presents the equivalent BHB intake on an absolute (g/day) and body weight basis (mg/kg body weight/day). The intakes of BHB range from 6 to 18 g/day, which is equivalent to 86 to 257 mg/kg body weight/day, as determined for a 70-kg adult.

Table 6. Total Daily Intake of D-β-Hydroxybutyrate Based on Number of Servings per Day

Number of Servings (beverage, gel, or bar)	BHB Level (g/serving)	Estimated BHB Intake	
		Absolute (g/day)	Body Weight Basis (mg/kg bw/day)[a]
1	6	6	86
2	6	12	171
3	6	18	257

BHB = D-β-hydroxybutyrate; bw = body weight.

[a] Estimated intakes on a per kilogram body weight basis were calculated using an average adult body weight of 70 kg.

3.3.2. Estimated Daily Intake of D-BHB by the General Population

The estimated total intake of BHB (g/person/day) from all proposed food uses is provided in Table 7, while this data on a per kilogram body weight basis (mg/kg bw/day) is summarized in Table 8. The percentage of users was low among all age groups evaluated in the current intake assessment, ranging from 9.4 to 22.7% of the population groups consisting of users of those food products in which BHB is currently proposed for use. The consumer-only estimates are more relevant to risk assessments as they represent exposures in only those individuals who reported the consumption of the food products of interest; consequently, only the consumer-only intake results are discussed below.

The mean and 90th percentile consumer-only intakes of BHB, among the total population (ages 2 years and older), were determined to be 6.3 and 13.7 g/person/day, respectively. Of the individual population groups, male adults were determined to have the greatest mean and 90th percentile consumer-only intakes of BHB on an absolute basis, at 7.8 and 16.9 g/person/day, respectively. Children aged 2 to 5 years had the lowest mean consumer-only intakes of 3.2 g/day, while female adults had the lowest statistically reliable 90th percentile estimates of 9.5 g/day.

Table 7. Summary of the Estimated Daily Intake of D-β-Hydroxybutyrate from Proposed Food Uses by Population Group

Population Group	Age (Years)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Children	2 to 5	0.3	na	9.4	45	3.2	6.6*
Children	6 to 11	0.7	2.7	14.6	84	4.9	11.4
Female Teenagers	12 to 19	0.5	1.3*	13.6	48	4.0	8.2*
Male Teenagers	12 to 19	1.4	5.8	22.7	91	6.3	12.5
Female Adults	20 and older	0.6	1.5	11.5	188	5.3	9.5
Male Adults	20 and older	1.3	4.5	16.6	258	7.8	16.9
Total Population	2 and older	0.9	3.2	14.2	714	6.3	13.7

n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.
* Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements (mean n<30; 90th percentile n<80).

On a body weight basis, the total population (ages 2 years and older) mean and 90th percentile consumer-only intakes of BHB were determined to be 92 and 198 mg/kg bw/day, respectively. Among the individual population groups, children aged 2 to 5 years were identified as having the highest mean consumer-only intakes of any population group, of 201 mg/kg bw/day (90th percentile values by this age group were not statistically reliable), while children aged 6 to 11 years were identified to have the highest statistically reliable 90th percentile intakes of 342 mg/kg bw/day. Female teenagers had the lowest mean consumer-only intakes of 63 mg/kg bw/day, while female adults had the lowest 90th percentile intake of 133 mg/kg bw/day (Table 8).

Table 8. Summary of the Estimated Daily Per Kilogram Body Weight Intake of D-β-Hydroxybutyrate from Proposed Food Uses by Population Group

Population Group	Age Group (Years)	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Children	2 to 5	18	na	9.1	43	201	446*
Children	6 to 11	22	65	14.6	84	147	342
Female Teenagers	12 to 19	9	24*	13.8	48	63	140*
Male Teenagers	12 to 19	20	80	22.7	90	88	179
Female Adults	20 and older	8	21	11.6	188	73	133
Male Adults	20 and older	15	54	16.6	256	92	191
Total Population	2 and older	13	42	14.3	709	92	198

bw = body weight; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.
* Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements (mean n<30; 90th percentile n<80).

3.4. Summary of Intake Analysis

The estimated daily intake of BHB in the target population, as assessed on a serving basis, ranged from 86 to 257 mg/kg bw/day when consuming 1 to 3 servings, respectively, by a 70-kg adult. In the general population assessment (*i.e.,* including non-target consumers), as calculated using the NHANES survey consumption data, the *per capita* and consumer-only intakes of BHB for specific demographic groups and for the total U.S. population were estimated. There were a number of assumptions included in the assessment which render exposure estimates that may be considered suitably conservative. For example, it is unlikely that BHB will have 100% market penetration in all identified food categories.

In summary, in the general population assessment, the mean and 90th percentile consumer-only intakes of BHB by the total population (ages 2 years and older) from all proposed food uses, were estimated to be 6.3 g/person/day (92 mg/kg bw/day) and 13.7 g/person/day (198 mg/kg bw/day), respectively. Among the individual population groups, the highest mean and 90th percentile consumer-only intakes of BHB were determined to be 7.8 g/person/day (92 mg/kg bw/day) and 16.9 g/person/day (198 mg/kg bw/day), respectively, as identified among male adults. When intakes were expressed on a body weight basis, children aged 2 to 5 years had the highest mean consumer-only intake of 201 mg/kg bw/day, while children aged 6 to 11 years were identified as having the highest statistically reliable 90th percentile consumer-only intake of 342 mg/kg bw/day. Although younger populations were identified as the groups having higher exposures to BHB on a body weight basis, it should be noted that products containing BHB will not be targeted towards the general population, and also will not be targeted to individuals under 18 years of age. As such, these estimates by young children are not considered representative of true potential intake. For safety assessment purposes, the highest intake of 18 g/person/day (resulting from intake of three servings of products containing D-BHB per day) is considered.

As part of the review of GRN 000515, the FDA's Office of Food Additive Safety (OFAS) calculated the estimated dietary exposure of D-BHB-ester for the general population

based on an assumption that the intended use applies to all sports drinks and bars at the maximum use levels described in GRN 000515 and that exposure would not be limited to high performance athletes. Based on food consumption data for sports drinks and bars from the 2009-2010 NHANES, the mean and 90th percentile average dietary exposure to D-BHB-ester for persons aged 2 years and older (users only) were determined to be 17 g/person/day (280 mg/kg bw/day) and 35 g/person/day (550 mg/kg bw/day), respectively. As compared to the above determined intake estimate of D-BHB from the proposed uses in food, the FDA estimate of D-BHB-ester of 35 g/person/day is almost double.

4. PART IV- SELF LIMITING LEVELS OF USE

D-β-Hydroxybutyrate (D-BHB) does not have any self-limiting intake levels under the conditions of use described in this GRAS notification.

5. PART V- EXPERIENCE BASED ON COMMON USE IN FOODS BEFORE 1958

The statutory basis for the conclusion of GRAS status of D-β-Hydroxybutyrate (D-BHB) in this document is not based on common use in food before 1958. However, BHB is found in dairy milk, making dairy products a natural source of BHB that have been routinely consumed orally prior to 1958. Notwithstanding this, it is reasonable to conclude that, since BHB has been part of the diet, it was present in food prior to 1958. This GRAS assessment for use of D-BHB as a food ingredient is based on scientific procedures.

6. PART VI- NARRATIVE

6.1. Natural Occurrence and Uses

D-β-Hydroxybutyrate (D-BHB) is reported to occur in nature. Technically, based on structure, BHB is not a ketone body. However, it has ketone like action in human body and is thus classified as one. Various ketone sources, including BHB, exist naturally in the food supply. BHB has been detected in cow's milk, with levels ranging from 10 to 631 μM (Larsen and Nielsen, 2005; Nielsen et al., 2005). This makes dairy products a natural source of BHB. BHB is also produced in the large intestine of animals from fiber via fermentation. Muscles and the central nervous system utilizes BHB for intracellular energy production. Dietary modulation can increase and maintain circulating ketone bodies, especially BHB, which is one of the most abundant ketone bodies in human circulation (Han et al., 2020). Normal postprandial BHB serum levels are less than 0.1 mmol/L (Fischer et al., 2018). In healthy subjects, these levels increase to approximately 0.1 - 0.2 mmol/L after overnight fasting.

Ketosis, meaning elevation of D-BHB and acetoacetate, has been central to starving man's survival by providing non-glucose substrate to his evolutionarily hypertrophied brain, sparing muscle from destruction for glucose synthesis. A mild ketosis is the natural adaption of the body to starvation, not to be confused with ketoacidosis, e.g., in untreated diabetes. Since ketone bodies are of mitochondrial origin, other foods of animal origin are likely to contain small amounts of D-BHB, though quantitative data were not identified in the literature. BHB is synthesized in the liver from acetyl CoA that is produced from fatty acids, and represents an essential carrier of energy from the liver to peripheral tissues when the supply of glucose is too low for the body's energetic needs, such as during periods of prolonged exercise, starvation, or absence of dietary carbohydrates (Newman and Verdin, 2017).

Based on an internet search of the worldwide supplement market, several products with BHB as the main ingredient are marketed in recent years, since 2015. The first available product was a simple mixture of calcium and sodium BHB salts with an added flavor (Fischer et al., 2018). In 2015, FDA completed review of a GRAS notice on D-BHB ester for its use in bars, gels, and beverages at a use level intended to provide 0.36 g/kg bw/serving, and provided a no question letter (FDA, 2015). In this GRAS notice, TdeltaS (2014) suggested that a maximum of 2 to 3 servings/day will be consumed, resulting in a maximum daily exposure of 75 g/person/day, which is equivalent to 1.1 g/kg bw/day for a 70 kg individual. This suggest that D-BHB ester can be used as a food ingredient in selected food categories.

As described in GRN 515 (TdeltaS, 2014; additional information or details not provided), D-BHB ester is considered GRAS in foods for special dietary purposes for the U.S. armed forces. Specifically, D-BHB ester was determined to be GRAS in specially formulated military ready-to-eat special dietary use foods, such as nutrition bars or beverages, by the military war fighters during brief periods of extreme physiological and cognitive duress (i.e., under conditions in combat). For these purposes, the daily dietary intake of the D-BHB ester was estimated be up to 200 g/day (equivalent to 2.9 g/kg bw/day for a 70 kg individual), consumed in small amounts over the course of the day.

In summary, the available information suggest that D-BHB is regularly consumed from dietary sources, such as milk products. In the U.S., BHB and its preparations are marketed as a dietary supplement and as discussed below it has received GRAS status as a food ingredient for uses in sports beverages (liquid or powder form), gels, and bars.

6.2. Data Pertaining to Safety

Given the natural occurrence of BHB in foods, such dairy products, and in the human body, the need for systematic toxicity studies of BHB has been diminished. BHB is the most abundant ketone body in mammals. Additionally, it also expresses a variety of molecular signaling functions. As an energy source, and the most abundant ketone in the body, it is absorbed as an alternative energy source by peripheral tissues, such as the muscles, heart, and brain. There has been significant efforts to elucidate the mechanism of action or physiological role of BHB. Published literature contains several studies on BHB. The available information suggest that BHB is synthesized in the liver from fatty acids and represents an essential carrier of energy from the liver to peripheral tissues when the supply of glucose is too low for the body's energetic needs, such as during periods of prolonged exercise, starvation, or absence of dietary carbohydrates.

For the present GRAS assessment, the safety determination of BHB is based on the totality of the available evidence, including human clinical observations/trials, animal experimental studies and *in vitro* studies. Efforts have been made to present both the data supporting BHB safety as well as any data on potential adverse effects. In addition to its role as a glucose-sparing energy carrier, BHB plays multiple roles in the human body that have been investigated in recent years. An attempt has been made to interpret these findings from relevant studies as it relates to the present GRAS assessment. The assessment of efficacy studies is limited to a review of the results related to safety and tolerability. In the following sections, relevant biological and toxicological studies on BHB and structurally related substances are described that provide support for the conclusions reached in this determination.

The safety of an ester of D-BHB, (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (D-β-hydroxybutyrate ester; D-BHB-ester) has been extensively investigated in animal and human studies. This ester is hydrolyzed to D-β-hydroxybutyrate (D-BHB) and (R)-1,3-butanediol, with the latter being further metabolized to D-β-hydroxybutyrate (D-BHB) and acetoacetate in the liver. The fact that this ester is primarily metabolized to D-BHB, the safety studies of the ester are applicable to the present GRAS assessment. The available studies with D-BHB-ester are described below and used to support the safety of D-BHB from the proposed uses described in this GRAS document.

6.2.1. Metabolic Fate

As mentioned earlier, ketone bodies, are small, lipid-derived molecules that provide energy to tissues when glucose is scarce, such as during fasting or prolonged exercise. The term ketone bodies usually includes three molecules that are generated during ketogenesis: BHB, acetoacetate, and acetone (Newman and Verdin, 2017). In humans, over 80% of the body's stored energy resides in the fatty acids contained in adipose tissue. Following fasting or starvation, once muscle and liver stores of glycogen are depleted, fatty acids are mobilized from adipocytes and transported to the liver for conversion to ketone bodies that are then distributed via blood circulation to metabolically active tissues, such as muscle or brain, where they are metabolized into acetyl-CoA and eventually ATP. In humans, serum levels of BHB are usually in the low micromolar range but begin to rise to a few hundred micromolar after 12 - 16 hours of fasting, reaching 1 - 2 mM after 2 days of fasting and 6 - 8 mM with prolonged starvation. Similarly, following intense exercise, serum levels of BHB can reach 1 - 2 mM after 90 min. Intake of a ketogenic diet that is almost devoid of carbohydrates can also lead to

consistent levels of BHB above 2 mM. Most of the dynamic range in ketone body levels is in the form of BHB. Once, ketogenesis is activated, such as during fasting, blood levels of BHB rise much faster compared to either acetoacetate or acetone (Newman and Verdin, 2017).

As regards the metabolism of D-BHB-ester, similar to other aliphatic esters, this ester undergoes complete hydrolysis via carboxylesterases or esterases distributed throughout the intestinal tract, blood, liver, and other tissues (Heymann, 1980; Anders, 1989). It is hydrolyzed to D-BHB and (R)-1,3-butanediol, with the latter being further metabolized to D-BHB and acetoacetate in the liver (Tate et al., 1971; Desrochers et al., 1992). Clarke et al. (2012a) investigated the metabolic fate of D-BHB-ester in a dose escalating study in healthy subjects (6/group). Following administration of a single drink of the D-BHB-ester (140, 357, and 714 mg/kg bw), plasma levels of D-BHB and acetoacetate were readily elevated, while the ketone ester was not detected. At the highest dose of the D-BHB-ester tested, maximum plasma levels of ketones were achieved within 1.5 to 2.5 hours, reaching 3.30 mM and 1.19 mM for D-BHB and acetoacetate, respectively. The elimination half-life was found to range from 0.77 to 3.06 hours for D-BHB, and from 8 to 14 hours for acetoacetate. No sex related differences in the pharmacokinetic parameters of D-BHB or acetoacetate were reported.

In addition to the dose escalation study, Clarke et al. (2012a) also studied the pharmacokinetics of D-BHB-ester following administration at doses of 140, 357, and 714 mg/kg bw, three times daily, over 5 days (equivalent to 0.42, 1.07, and 2.14 g/kg bw/day). In this study, the circulating D-BHB level did not exceed 5.5 mM, which is equivalent to physiological levels following a period of fasting. As such, the human pharmacokinetic data showed that a dosage of the D-BHB-ester at a value of approximately twice the estimated maximum level produced plasma levels that were considered to cause no safety concerns as they remained within the physiological normal range. The ketone ester was generally well-tolerated, although some gastrointestinal effects were reported, when large volumes of milk-based drink were consumed, at the highest ketone monoester dose. Together, these results suggest that ingestion of D-BHB-ester is a safe and simple method to elevate blood ketone levels, compared with the inconvenience of preparing and consuming a ketogenic diet.

In a human study, Shivva et al. (2015) attempted to investigate the pharmacokinetics of D-BHB and to quantify the sources of variability following a dose of D-BHB-ester. In this study, healthy volunteers (n = 37) were given a single drink of D-BHB-ester, following which, 833 blood BHB concentrations were measured. For these investigations, two formulations and five dose levels of D-BHB-ester were used. A nonlinear mixed effect modelling approach was used to develop a population pharmacokinetic model. A one compartment disposition model with negative feedback effect on endogenous BHB production provided the best description of the data. Absorption was best described by two consecutive first-order inputs and elimination by dual processes involving first-order (CL = 10.9 L/h) and capacity limited elimination (V_{max} = 4520 mg/h). Covariates identified were formulation (on relative oral bioavailable fraction and absorption rate constant) and dose (on relative oral bioavailable fraction). Lean body weight (on first-order clearance) and sex (on apparent volume of distribution) were also significant covariates. The pharmacokinetics of BHB is complicated by a complex absorption process, endogenous production and nonlinear elimination. Formulation and dose appear to strongly influence the kinetic profile following D-BHB-ester administration. These investigators reported the need for additional work to quantify mechanisms of absorption and elimination of ketones for therapeutic use in the form of the D-BHB-ester.

In a review article, Veech et al. (2001) reported that ingestion of approximately 100 to 150 g of ketone bodies (synthetic esters or polymers of BHB taken orally) is expected to result in blood ketone body levels of 2 to 7 mM. In a study in children with acyl-CoA dehydrogenase deficiency, Van Hove et al. (2003) reported that oral administration of sodium D,L-BHB at dose levels of 80 to 900 mg/kg bw/day resulted in peak blood levels of 0.19 to 0.36 mM of combined D,L-BHB and acetoacetate. Similarly, in another study, Plecko et al. (2002) reported treatment of two, 6-month-old infants with persistent hyperinsulinaemic hypoglycaemia with oral sodium D,L-BHB was reportedly tolerated with no side effects. Administration of 0.9 to 1.0 g sodium D,L-BHB/kg bw/day resulted in blood BHB concentrations comparable to those observed following a 16- to 24-hour fast. BHB levels also were observed to increase in the cerebrospinal fluid. The high ratio of BHB to acetoacetate supported that the increase in BHB levels was due to administration of exogenous BHB.

Cuenoud et al. (2020) compared the metabolism of a pure D-BHB oral supplement, i.e., the increase in blood D-BHB and acetoacetate after D-BHB administration, to that produced by the ingestion of the same amount of racemic D+L-BHB or medium chain triglycerides (MCT). A pilot study was also performed to assess the feasibility of using ^{11}C-acetoacetate PET (positron emission tomography) to observe organ ketone uptake after oral ingestion of D-BHB. These investigators reported that an oral D-BHB (14.1 g) supplement is rapidly absorbed and metabolized in humans and increases blood ketones to millimolar levels. At the same dose, D-BHB is significantly more ketogenic and provides fewer calories than a racemic mixture of BHB or medium chain triglyceride. In a whole body ketone positron emission tomography pilot study, these investigators observed that after D-BHB consumption, the ketone tracer ^{11}C-acetoacetate is rapidly metabolized, mostly by the heart and the kidneys.

In a randomized study, Stubbs et al. (2017) investigated the effects of BHB-salts (sodium plus potassium) and BHB-ester [(R)-3-hydroxybutyl (R)-3-hydroxybutyrate] on blood BHB and metabolite concentrations. For these investigations, healthy human volunteers took part in three randomized metabolic studies of drinks containing a BHB-salt or BHB-ester. In the first experiment, subjects (n=15) consumed BHB-ester or BHB-salt drinks that delivered ~12 or ~24g of BHB. Both drinks elevated blood D-BHB concentrations (D-BHB Cmax: BHB-ester 2.8 mM, BHB-salt 1.0 mM, $P < 0.001$), which returned to baseline within 3-4 hours. BHB-salt drinks were found to contain 50% of the L-BHB isoform, which remained elevated in blood for over 8hours, but was not detectable after 24 hours. Urinary excretion of both D-BHB and L-BHB was <1.5% of the total BHB ingested and was in proportion to the blood AUC. D-BHB, but not L-BHB, was slowly converted to breath acetone. The BHB-ester drink decreased blood pH by 0.10 and the BHB-salt drink increased urinary pH from 5.7 to 8.5. Both drinks mildly altered acid-base balance. These investigators also reported that the difference in peak blood D-BHB concentrations between matched amounts of BHB as ester or salts arose because the salt contained L-BHB, as the blood concentrations of D- plus L-BHB isoforms were similar for both compounds. It is unclear if kinetic parameters of BHB-ester and BHB-salt drinks would be similar if matched D-BHB were taken in the drinks.

In the second experiment, Stubbs et al. (2017) investigated the effect of a meal before a BHB-ester drink on blood D-BHB concentrations in 16 participants. In this study, food intake lowered blood D-BHB Cmax by 33% (Fed 2.2 mM, Fasted 3.3 mM, $P < 0.001$), but did not alter acetoacetate or breath acetone concentrations. All ketone drinks lowered blood glucose, free fatty acid and triglyceride concentrations, and had similar effects on blood electrolytes,

which remained normal. In the third and final experiment, subjects were given BHB-ester over a period of 9 hours as three drinks (n=12) or a continuous nasogastric infusion (n=4) to maintain blood D-BHB concentrations greater than 1 mM. Both drinks and infusions gave identical D-BHB AUC of 1.3 - 1.4 moles.min. The investigators concluded that exogenous drinks containing ketones are a practical, efficacious way to achieve ketosis.

Fischer et al. (2018) investigated the effects of a sodium and calcium DL-BHB salt in healthy adults. In this one-dose kinetic study, six healthy subjects (3 males and 3 females; 18 to 57 years; 4-normal, 1-overweight, and 1-obese; BMI 25.44 ± 5.99 kg/m²) received an intervention (0.5 g/kg bw) using a commercially available ketone body supplement. The dose translated to 30 - 57.5 g of the supplement per subject depending on their body weight. The supplement contained a mixture of sodium and calcium D-/L-BHB as well as food additives. The blood samples drawn in the study were tested for concentrations of D-BHB, glucose, and electrolytes, and blood gas analyses were done. Data on sensory evaluation and observed side effects of the supplement were collected. The product also went through chemical food analysis. The supplement led to a significant increase of D-BHB concentration in blood 2.5 and 3 hours after oral intake. The first significant effect was measured after 2 hours with a mean value of 0.598±0.300 mmol/L at the peak, which was recorded at 2.5 hours. Changes in serum electrolytes and blood gas analysis (pH, electrolytes, and metabolites) were largely unremarkable. Taking the supplement was not without side effects. One subject dropped out due to gastrointestinal symptoms and two others reported similar but milder problems. The dose used in this study was quite high as compared to the proposed dose for the present GRAS.

In summary, the available pharmacokinetic information demonstrate that D-BHB is readily absorbed from the gastrointestinal tract, is metabolized in humans, and increases blood ketones to millimolar levels. Following oral D-BHB consumption, the ketone tracer ^{11}C-acetoacetate is rapidly metabolized, mostly by the heart and the kidneys. D-BHB does not appear to lower the blood acetoacetate/D-BHB ratio, making it an efficient fuel compared to other ketone precursors.

6.2.2. Human Studies of D-BHB

In a randomized, double-blind, placebo-controlled trial, Stefan et al. (2021) investigated the safety of exogenous BHB salt supplementation as determined by hematological safety markers and other parameters in healthy adolescents. In this study, 22 healthy male and female adolescents (aged 10 to 17 years; non-obese; BMI ≤ 30 kg/m²) consumed 3.75 g of BHB salts (n=12; 6/sex) or maltodextrin as placebo (n=10; 6 male/4 female) twice daily for 90 days. As mentioned in the publication, "In addition, BHB, the experimental condition, contained: 750 mg of leucine, 112.5 mg of theanine, 375 mg of creatine, and vitamins B6 and B12." It is not clear, but apparently, the study product contains other ingredients. Comprehensive blood safety analysis, bone densitometry, happiness and emotional intelligence surveys, and blood pressure were assessed at Pre, Day 45, and Day 90.

There was no significant, between- or within-group, differences in the Complete Blood Count values ($p \geq 0.05$) or Automated Differential Cell Count. A significant group by time interaction was detected for Albumin:Globulin Ratio, Creatinine, and Carbon Dioxide. A post hoc analysis was carried out for the Albumin:Globulin Ratio, Creatinine, and Carbon Dioxide and it indicated that there were no significant differences between groups. However, in the placebo group, the Albumin: Globulin Ratio was significantly higher at Day 45 compared to

Pre and Day 90. No differences were observed in BHB. Additionally, no values exceeded their respective reference ranges indicating no clinical significance. There was no significant, between- or within-group, differences for the remaining values of the Comprehensive Metabolic Panel. There were no significant between- or within-group differences in any Body Composition or Bone Densitometry measure, in resting blood pressure or heart rate. The results indicate that exogenous BHB salt supplementation can induce mild ketosis with no negative effects on any investigated metrics of safety and well-being. There were no significant differences detected in subjects supplementing with BHB salts, indicating that exogenous BHB salts had no detrimental impact on fasting blood safety metrics, bone density, happiness, emotional intelligence, or blood pressure. The investigators concluded that supplementing with exogenous BHB salts is safe and well-tolerated by healthy adolescents (Stefan et al., 2021).

In an earlier study, Stefan et al. (2020) explored the safety of exogenous BHB in a healthy adult population and was determined to be safe. In this study, 22 healthy male and female adults consumed 12.75 g BHB salts (n=11) or maltodextrin placebo (n=11) twice daily for 90 days. Comprehensive blood safety analysis, body composition, bone densitometry, psychological and immune surveys, and blood pressure were administered at baseline, 30, 60, and 90 days. There were no significant differences in any measures collected, indicating that exogenous BHB had no detrimental impact on fasting blood values such as electrolyte levels, glucose, hemoglobin A1c, complete blood count, body composition, bone density, psychological well-being, immune status, or blood pressure. The investigators concluded that supplementing with exogenous BHB at dose levels of 25.50 g/day for 90 days is safe and well-tolerated by healthy adults.

In a randomized, double-blinded, placebo-controlled trial, Holland et al. (2019) examined the safety of BHB salt supplementation for 6 weeks in healthy, young adults. In this study, 23 men and women (aged 18-35 years old) supplemented with BHB salt preparation or a placebo twice per day for 6 weeks. The BHB salt preparation included 7 g of BHB combined with erythritol, L-taurine, fermented L-leucine, citric acid, natural flavor, vegetable juice color, stevia, xanthan gum, beta carotene and approximately 920 mg of sodium. Baseline and post-intervention measures included BMI, resting blood pressure and heart rate, questionnaires assessing mood and energy, urinalysis, and venous blood measures, including comprehensive metabolic panel (CMP), lipid panel, and complete blood count (CBC). Systolic blood pressure was significantly lower after supplementing with the BHB salt preparation. All other health parameters remained unchanged by the supplementation, including BMI, resting heart rate, urinalysis, CMP, lipid panel, and CBC. The investigators concluded that supplementation with BHB salt preparation seems safe.

6.2.3. Specific Toxicity Study of D-BHB

In a 4 week dose-response toxicity study, Kawata (2015; unpublished) investigated the effects of dried cells of *Halmonas* sp. KM-1 (provided by National Institute of Advanced Industrial Science and Technology, Japan) and 3-hydroxybutyric acid (BHB) (provided by Osaka Gas) in rats. For these investigations, the dried cells of *Halomonas* sp. KM-1 was suspended and BHB dissolved in water for injection individually, and administered orally by gavage to Crl:CD(SD) SPF rats (6/sex/group) at 6 weeks of age at dose levels of 0 (vehicle), 1000 and 2000 mg/kg bw/day for 4 weeks to examine their toxicity. For the dried cells of *Halomonas* sp. KM-1 and BHB, each 2 dose groups, dose levels 1000 and 2000 mg/kg bw/day, were provided and a total of 5 groups including a control group composed. All common

standard parameters generally investigated for toxicity were studied during the course of study (Clinical observation, body weight, feed consumption, water intake, urinalysis) and at termination (hematology, clinical chemistry, necropsy, organ weight, histopathology).

Except for one male in the 2000 mg/kg bw/day group receiving BHB that was necropsied moribund on study day 20, there were no deaths or clinical observations during the course of the study in any group. In both males and females, body weight in treatment groups was largely comparable to that of the control group during the administration period. In both males and females, feed consumption in these groups was largely comparable to that of the control group during the administration period. As regards urine analysis, a significantly low value in urine volume was recorded in females in the 2000 mg/kg bw/day group treated with *Halomonas* sp. KM-1. In BHB acid treatment groups, males in the 2000 mg/kg/day group showed a tendency toward a low value in pH, and increases in the number of protein positive (2+) and ketone body positive (+1) animals, and females in the 1000 mg/kg bw/day group and above groups showed a tendency toward low values in pH, increases in the number of protein positive (2+) animals, and significantly low values in urine volume.

Hematology analysis did not show any effects in groups treated with *Halomonas* sp. KM-1 groups, except for a significantly high values in the number of basophils were recorded in males in the 1000 mg/kg bw/day group. However, this was judged to be incidental change, as it was not dose related and the variations occurred only in the actual numbers. In the BHB treatment groups, there were no effects from administration of the test article in any group, except for significantly low values in hemoglobin concentration and hematocrit in males in the 2000 mg/kg bw/day group. However, these changes were judged to be incidental, as they were slightly low values and there were no changes in any other erythrocyte parameters.

Clinical chemistry parameters in the 2000 mg/kg bw/day male and female groups receiving *Halomonas* sp. KM-1 showed significantly low values in inorganic phosphorus and total cholesterol, respectively. However, these changes were judged to be incidental as they were slightly low values, and there were no abnormalities in the kidney or liver in organ weight and at necropsy. In the BHB groups, males treated with 2000 mg/kg bw/day group showed significantly low values in glucose, sodium and inorganic phosphorus. Females in the 2000 mg/kg bw/day group showed significantly low values in total protein and albumin. However, these changes were considered to be incidental as the values were slightly low, and there were no abnormalities in organ weight and at necropsy in the kidney or liver.

Organ weight changes that might be related to administration of *Halomonas* sp. KM-1 were recorded for the thymus in males, as significantly high values in the absolute and relative thymus weights compared to those of the control group were recorded in males in the 2000 mg/kg bw/day group. In the BHB groups, there were no effects from administration of the test article in any group. The other changes recorded were judged to be incidental, as these changes were not dose-related or ones either in the absolute or relative weight.

Necropsy from the animals treated with *Halomonas* sp. KM-1 did not reveal any effects from administration of the test article. In the 2000 mg/kg bw/day group, 1 male and 1 female showed white foci raised in the forestomach and dark red foci in the glandular stomach, respectively. However, these changes were judged to be incidental based on the incidence of their occurrence or pathological nature. In the BHB treatment groups, there were no effects from administration of the test article. One male in the 1000 mg/kg bw/day group showed

diverticulum in the jejunum. However, it was judged to be an incidental change, as it was not dose-related and based on its pathological nature. One male in the 2000 mg/kg bw/day group that became moribund showed undernourishment, pale skin, luminal stenosis from thickened mucosa in nasal cavity, dilatation of the stomach and intestines due to retention of gas, dark red foci in glandular stomach and small thymus and spleen. Histopathological examinations were not performed since there were no organs/tissues that showed changes thought to be caused by administration of the test article at scheduled necropsy.

In summary, there were no animals that showed toxic changes caused by administration of the dried cells of *Halomonas* sp. KM-1 or BHB in general condition, body weight, feed consumption, hematology, blood chemistry and necropsy. Based on these results, the investigators estimated that the no-observed adverse effect level (NOAEL) was higher than 2000 mg/kg bw/day in males and females for both the dried cells of *Halomonas* sp. KM-1 and BHB.

6.2.4. Other Toxicity Studies of D-BHB and Related Compounds

In a 28-day animal study, Kesl et al. (2016) investigated the effects of administration of five ketone supplements on blood glucose, ketones, and lipids in male Sprague-Dawley rats. The supplements used in this study included: 1,3-butanediol (BD), a sodium/potassium BHB salt (BMS), medium chain triglyceride oil (MCT), BMS + MCT 1:1 mixture, and 1,3 butanediol acetoacetate diester (KE). The rats (n=10-11/group) were divided in six study groups; five treatment groups and one control group that received water. The BHB salt used in the study is as a 50% solution containing approximately 375 mg/g of pure BHB and 125 mg/g of sodium/potassium. On days 1 - 14, rats received a 5 g/kg bw/day dose of their respective treatments via intragastric gavage. Dosage was increased to 10 g/kg bw/day for the second half of the study (days 15 - 28) for all groups except BD and KE to prevent excessive hyperketonemia (ketoacidosis). Each daily dose of BMS would equal approximately 1000 - 1500 mg of BHB, depending on the weight of the animal. Weekly whole blood samples were taken for analysis of glucose and BHB at baseline and, 0.5, 1, 4, 8, and 12 hours post-gavage, or until BHB returned to baseline. At 28 days, triglycerides, total cholesterol and high-density lipoprotein (HDL) were measured.

Ketone supplementation caused a slight change in triglycerides and lipoproteins over a 4-week investigation (Kesl et al., 2016). Blood BHB levels in the BMS group did not show significant elevation at any time point, even after dose escalation. Rats supplemented with BMS had lower blood glucose compared to control at 12 hours in week 4. As compared to controls, BMS + MCT, BMS, and BD supplemented rats gained significantly less weight than controls during weeks 2 - 4. The BMS ketone supplement did not significantly induce blood hyperketonemia or reduced glucose in the rats. The findings from this study suggest that exogenous ketone supplementation, including BMS, caused a rapid and sustained elevation of BHB, reduction of glucose, and little change to lipid biomarkers compared to control animals. This study demonstrates the tolerability of oral exogenous ketone supplementation in inducing nutritional ketosis independent of dietary restriction.

6.2.5. Toxicity Studies of D-BHB-Ester

6.2.5.1. Repeat Dose Studies of D-BHB-Ester

In addition to the above described pharmacokinetic study, Clarke et al. (2012b) also investigated the toxicity of D-BHB-ester in a 28-day repeat dose study. The study was conducted in accordance with FDA and OECD GLP and as per FDA Redbook guidance. In this study, Crl:WI (Wistar) rats (10/sex/group) received diets containing, as 30% of the calories, D-BHB-ester (12 and 15 g/kg bw/day for male and female rats, respectively). Control groups received either carbohydrate- or fat-based diets. During the course of study, rats were monitored for clinical signs, body weight, and feed consumption. At the end of the study period, hematology, coagulation, clinical chemistry, and urinalysis parameters were assessed, organs were weighed, and gross and microscopic examination was performed. Rats in the test group consumed less feed and gained less weight than control animals. Decreased feed consumption may have resulted from the palatability of the diet containing ketone monoester. The investigators stated that similar findings have been documented in studies of ketogenic diets. All animals survived to the scheduled necropsy date.

Hematological and clinical chemistry analyses revealed significant between-group differences in several hematology parameters (i.e., increased RBC, Hb, and Hct, and decreased MCV values in D-BHB-ester-fed rats compared to controls) with increased cholesterol in ketone ester-fed rats; though values were within normal physiological ranges. LDH levels were significantly higher in ketone ester-fed rats (both sexes compared to control animals). However, the increases were small in magnitude and were not associated with changes in hemolytic or histological findings. LDH levels vary greatly in rats, as demonstrated by the historical control ranges for Sprague Dawley rats in the testing facility. LDH levels in the ketone ester-fed rats were within these ranges and, thus, were not considered to be of toxicological relevance (Clarke et al., 2012b).

Histological examination, revealed liver vacuolation in all female rats in all three groups; likewise, necroinflammatory foci were observed in some animals (males and females) in all groups. Given that these findings were present in all groups and that liver function enzyme levels were within normal ranges, it is unlikely that they were related to consumption of the D-BHB-ester. The diets were formulated by the separate addition of macronutrients (fat, CHO, ketone ester) to the rat chow; the vitamin and mineral compositions of each diet were diluted, which may explain the observed findings (Clarke et al., 2012b).

In summary, the findings from a 28-day oral toxicity study in rats indicated that consumption of D-BHB-ester at a level of 11.4% (12.0 g/kg bw/day and 15.1 g/kg bw/day in male and female rats, respectively) in the diet did not cause adverse effects (Clarke et al., 2012b). The findings from this study are applicable to the subject of present GRAS and indicate that D-BHB is likely to be safe.

In the above described publication, Clarke et al. (2012a) also described findings from unpublished preliminary studies. In these studies, pair-feeding a diet containing D-BHB-ester (13.7 g/kg bw/day) to Wistar rats for 66 days did not significantly impact body weights in comparison to a Western diet or a CHO-based diet (unpublished). Plasma free fatty acid levels and lactate dehydrogenase (LDH) activity were also unaffected. Additionally, the ester was not detected in the blood of rats administered BHB-ester in the diet for 66 days. The additional description of the 66 day study is provided in GRN 515 (pages 14 and 15) and are incorporated here by reference.

6.2.5.2. Reproductive Developmental Toxicity of D-BHB-Ester

In addition to the above described 28-day study, Clarke et al. (2012b) also investigated developmental toxicity of D-BHB-ester. The study was conducted in accordance with FDA and OECD GLP and as per FDA Redbook guidance. In this study, 25 pregnant Crl:WI(Han) rats per group were administered 2 g/kg bw of D-BHB-ester or deionized water (as a control substance) once daily by oral gavage on Days 6 through 20 of gestation (DGs 6 to 20). On DG 21, blood samples were collected for hematology and clinical chemistry analysis and the rats were euthanized, Caesarean-sectioned, and examined for gross lesions. All fetuses were examined for external abnormalities. Approximately one-half of the fetuses in each litter were examined for visceral abnormalities, while the remaining fetuses were examined for skeletal abnormalities.

In dams administered D-BHB-ester decreased body weight gains and body weight corrected for gravid uterine weight were noted as compared to control. Feed consumption was similarly reduced. These findings are expected given that the test article provided caloric value whereas the control article (water) did not. Maternal alanine aminotransferase (ALT) and alkaline phosphatase (ALP) values were lower in the test group relative to controls. However, in the above described 28-day study, no significant effects on ALT were noted in female rats fed the D-BHB-ester diet and ALP levels were only lower in comparison to the fat control group but not the other control group. Moreover, no gross or histopathological effects in the liver toxicity were noted.

In the control group, pregnancy occurred in 22 rats, while in the treatment group it occurred in 24 rats. The litter averages for corpora lutea, implantations, the percentage of pre-implantation loss, litter sizes, live and dead fetuses, early and late resorptions, the percentage of post-implantation loss, the percentage of resorbed conceptuses, and the percentage of live male fetuses were comparable among the groups. In the D-BHB-ester treated group, male fetal body weights were significantly lower as compared to controls. However, combined fetal weights did not significantly differ, the percent difference from the control group was less than 5%, and the average value was within historical ranges at the test facility.

There were no significant differences between the groups as it relates to the litter or fetal incidences of any gross external, soft tissue, or skeletal abnormalities (malformations or variations), nor were there differences in fetal ossification site averages. The number of fetuses with any alteration observed and percentage of fetuses within a litter with any alteration observed were significantly higher in the D-BHB-ester group compared to controls. These findings were driven by skeletal variations; however, it should be noted that the incidences of skeletal variations did not significantly differ between groups. Consequently, the observed skeletal variations were not considered to be of toxicological concern.

In summary, in the developmental toxicity study, pregnant Crl:WI (Han) rats were administered 2 g/kg bw/day D-BHB-ester or water (control) via gavage on days 6 through 20 of gestation. D-BHB-ester did not affect Caesarean-sectioning or litter parameters. The overall incidence of fetal alterations was higher in the treatment group; however, there were no specific alterations attributable to D-BHB-ester treatment. The investigators reported that D-BHB-ester did not adversely affect the development of rats exposed in utero at a level of 2 g/kg bw/day.

6.2.5.3. Human Studies of D-BHB-Ester

As mentioned earlier, safety and tolerability of D-BHB-ester is also investigated in a human study (Clarke et al., 2012a). Ingestion of the D-BHB-ester in a meal replacement milkshake beverage was without adverse effects in participants administered a single dose at up to 714 mg/kg bw in the pharmacokinetic study. Also, as described in GRN 515, a human study was undertaken to assess the tolerability of D-BHB-ester. In this randomized, blinded, placebo-controlled, cross-over study, citrus-flavored sports water drinks were used as the matrix for the ingredient as they mask the flavor of D-BHB-ester more effectively compared to milkshake. In this study, 42 adult men consumed a vitamin water drink to which 1.23 g D-BHB-ester/kg bw or 1.44 g dextrose/kg bw was added (both drinks also contained 0.1 g fructose/mL). The subjects drank a total volume of 7.6 mL/kg bw (approximately 578 mL total volume), divided into 3 drinks of equal volume, at 10 min before starting the exercise session, and at 65 min and 130 min of the cycling session (so 1.23 g d-BHB-ester/kg bw consumed within 140 minutes). There was a 72-hour washout period between each test protocol.

As described in GRN 515, subjects completed a Gastrointestinal Symptoms Questionnaire 7 times during the exercise session, where the subjects rated the severity of symptoms on a scale of 0 (none) to 8 (unbearable). Blood samples were collected concurrently with the completion of the questionnaire for the measurement of D-BHB, glucose, lactate, glycerol, insulin, and free fatty acid levels. Blood D-BHB levels increased to about 1 mM, 10 min after the first drink of D-BHB-ester, rising with each successive drink to reach about 4 mM at the end of exercise. Levels of glucose, lactate, glycerol, insulin, and free fatty acids in the blood were not adversely impacted by the consumption of D-BHB-ester. Gastrointestinal symptoms occurred in a few subjects, and there were no significant differences in symptom severity between the D-BHB-ester and placebo arms of the study. No severe adverse events occurred. The findings of this study demonstrate that D-BHB-ester is well-tolerated under the intended conditions of use.

Clarke et al. (2012a) also described the study in which healthy volunteers drank only the D-BHB-ester in a milkshake drink for 5 days. In this study, the subjects (6/sex/group) consumed a meal replacement milkshake beverage containing 140, 357, or 714 mg/kg bw three times daily (at 0, 4, and 9 h) for five consecutive days. Each 100 g serving of the meal replacement milkshake contained approximately 84 kcal and 5.2 g of D-BHB-ester. The highest daily dose administered in this study was 2,142 mg/kg bw/day. In this study, the same safety evaluations were conducted at screening; on each day of dosing (prior to administration of the test product); at 1-2 hours following administration of the third dose; at discharge (24 hours after the last dose); and, at the 7-day follow-up. Adverse events that were deemed to be treatment-related were observed in 4 out of 12 participants in the low dose group (420 mg/kg bw/day), 1 out of 12 participants in the mid-dose group (1071 mg/kg bw/day), and 12 out of 12 participants in the high dose group (2142 mg/kg bw/day). The low and mid- doses of D-BHB-ester were generally well tolerated, and the few adverse events were all considered to be mild and only "possibly" related to treatment.

At the highest dose of D-BHB-ester (2142 mg/kg bw/day), two participants dropped out of the study as a result of adverse events. These included severe vomiting in one individual, and nausea, diarrhea, chest pain, abdominal distension, and upper abdominal pain in the other. A number of gastrointestinal symptoms were also reported by participants in the high dose group who completed the study. These include flatulence, nausea, diarrhea, constipation, vomiting, abdominal distension, and abdominal pain ranging from mild to moderate in severity.

Some of these events were deemed to be "highly probable" in their relation to D-BHB-ester drink. Headaches, dizziness, lethargy, and somnolence were also reported in some participants, although these were considered to be mild in severity and were deemed to be "probable" in relation to the D-BHB-ester treatment. All other adverse events reported were considered mild in severity with "probable" or "possible" relation to the treatment. The investigator noted that, at this dose, the subjects were consuming about 1.1 liters of the milk-based drink within 30 min (*i.e.*, 3.3 liters of drink per day). The adverse events reported at all doses of D-BHB-ester resolved by the end of the study, with the exception of the positive fecal occult test observed in one individual in the lowest dose group (420 mg/kg bw/day).

In GRN 515, the notifier has further described the above two human studies (pages 16-20 of the GRAS dossier; incorporated here by reference TdeltaS, 2014). The notifier summarized the results of the physical endurance study (Clarke et al., 2013- unpublished as cited in GRN 515) and concluded that the study supports the safety of D-BHB-ester under the proposed conditions of use (provided at a total daily amount of 1.23 g/kg bw, consumed before, during and/or following exercise, and consumed in reasonable volumes of less than 200 mL in a sports drink matrix). While gastrointestinal effects were noted in the high-dose group (2,142 mg/kg bw/day) from the study reported by Clarke et al. (2012a), these effects may be attributed to the study design (i.e., matrix, volume, and directions for use). Additionally, only 2 mild adverse effects were reported in the mid-dose group (1,071 mg/kg bw/day), in subjects who consumed D-BHB-ester at levels consistent with the intended uses of the ingredient described in GRN 515. The notifier also stated that the findings of the Clarke et al. (2012a) study provide corroborative evidence of safety.

6.2.6. Safety of Metabolites of D-BHB-Ester

As mentioned earlier, D-BHB-ester is metabolized to D-BHB and (R)-1,3-butanediol. The available information also shows that 1,3-butanediol is further metabolized to D-BHB. In the published literature, several studies with 1,3-butanediol have appeared.

The findings from some of the *in vitro* studies (with isolated embryos) indicate that physiologically relevant levels of BHB, particularly the L-isomer, may disrupt normal embryogenesis (Sheehan et al., 1985; Hunter et al., 1987; Moley et al., 1994). However, as discussed below, the results of an *in vivo* study with R,S-1,3-butanediol indicate that it is not teratogenic (Hess et al., 1981). As 100% of the R enantiomers and 30% S enantiomer of 1,3-butanediol are metabolized to ketones (Desrochers et al., 1992), studies on 1,3-butanediol indirectly provide information on the reproductive and developmental safety of BHB. Additionally, as described above, findings from the rat developmental toxicity study of D-BHB-ester did not indicate adverse effects on development.

In a reproductive toxicity study, Hess et al. (1981) investigated the effects of 1,3-butanediol. There was no mention of any chirality (R or S form) or of any value for optical rotation for the 1,3-butandiol was identified. The results of this study with R,S-1,3-butanediol indicate that it is not teratogenic (Hess et al., 1981). These investigators demonstrated that in 5 successive breedings of Wistar rats, that consumption of 5 to 24 g/kg bw/day of 1,3-butanediol was not associated with teratological effects. The control and test groups were comparable with respect to gestation, viability, and lactation indices. In majority of the experimental studies, both the (R)- and (S)- forms of butanediol were used, except for the chronic toxicity studies reported by Scala and Paynter (1967). In this study, it was demonstrated that the 2-year oral

administration of (R)-1,3- butanediol to Sprague-Dawley rats or to pure bred-beagles, at dose levels of 5 and 0.8 g/kg bw/day, respectively, was not associated with toxicity.

Short-term human experimental studies indicate that 1,3-butanediol consumption may result in statistically, but not clinically significant, reductions in blood glucose levels (Kies et al., 1973; JECFA, 1980). However, as described earlier by Clarke et al. (2012a), no clinically significant effects on blood glucose levels were reported in the 5-day human study in which subjects consumed up to 2 g D-BHB-ester/kg bw/day.

6.2.7. Human Studies and Observations with D-BHB

As described earlier in Section 6.2.1., sodium D,L-BHB has been investigated for its metabolic fate. In these studies, oral administration of sodium D,L-BHB to children with acyl-CoA dehydrogenase deficiency or with persistent hyperinsulinemic hypoglycemia at dose levels up to 1000 mg/kg bw/day did not cause adverse effects (Plecko et al., 2002; Van Hove et al., 2003). In the study by Plecko et al. (2002), two infants were treated and monitored for five and seven months. Four and eight gram doses, respectively, were administered and well tolerated. It should be noted that extreme therapeutic dosing such as these requires medical monitoring.

6.2.8. Production of Ketone bodies in Humans

The available information suggest that, during food deprivation, the human body obtains energy from ketone bodies to lower glucose utilization and muscle protein breakdown. During this process, circulating ketone bodies levels rise to fuel the body's need for energy to levels (2 to 3 mM). This suggest that the increase of circulating ketone bodies is a natural mechanism that is used for human survival and shows clearly that elevated circulating ketones protect the body during adverse conditions or dietary manipulation. The rate of ketone formation is low when an adequate supply of carbohydrates is available. However, when blood glucose levels decrease, the rate of ketogenesis is increased, and ketones replace glucose as the primary source of energy. As mentioned earlier, the normal 6- to 8-hour fasting blood ketone concentration of a healthy individual is reported to be approximately 0.5 mM, while after a 5- to 7-day fast, total blood ketone levels as high as 5 to 7 mM have been documented (Owen et al., 1967; Mensink et al., 1992; VanItallie and Nufert, 2003).

Generally, the blood levels of D-BHB are reported to be approximately 0.2 mM, with levels increasing by up to 50 times during periods of limited calorie intake (Hall et al., 1984). In pregnant women, the normal blood ketone level is slightly higher as compared to other individuals, and pregnant women, because of their greater caloric requirements, achieve greater ketone levels during times of limited glucose availability (Paterson et al., 1967; Gin et al., 2006). In a healthy adult, the maximal ketone body production by liver and kidney is approximately 185 g/day, with ketones accounting for between 2 and 6% of an individual's energy needs after an overnight fast and up to 40% of energy needs after a 3-day fast (Reichard et al., 1974; Laffel, 1999).

The fatty acid oxidation product, acetyl-CoA, formed in the liver of most mammals can enter the citric acid cycle or, alternatively, can be converted to ketone bodies, a metabolic process called ketogenesis (Nelson and Cox, 2000). Primarily, ketogenesis occurs in the mitochondria of liver cells during times of limited glucose availability, when there is an increase in lipolysis and a concomitant reduction or saturation in acetyl-CoA oxidation in the

citric acid cycle. The latter occurs as a result of the need for citric acid cycle intermediates to enter the gluconeogenic pathway to maintain blood glucose levels. The primary substrate in the citric acid cycle, acetyl-CoA is then diverted into the ketogenic pathway, resulting in the formation of the ketone bodies, acetoacetate and D-BHB. Acetoacetate and D-BHB are transported to extra-hepatic tissues, where they can be oxidized in the citric acid cycle and thus serve as an alternate source of energy. D-BHB is oxidized to acetoacetate by D-β-hydroxybutyrate dehydrogenase, which is subsequently converted to acetoacetyl-CoA and finally to two acetyl-CoA molecules. While D-β-hydroxybutyrate dehydrogenase is found in the liver, 3-oxoacid-CoA transferase, which converts acetoacetate to acetoacetyl-CoA, does not occur in hepatic tissue. Thus, the liver is unable to utilize ketone bodies as an alternate source of energy. In the fed state, the brain uses glucose as its primary source of energy. However, the brain also can use acetoacetate and D-BHB when these metabolites are available. Production of ketone bodies, and their transport to other tissues for conversion to acetyl-CoA, allows for continued oxidation of fatty acids, particularly when acetyl-CoA oxidation slows down. A reduction in acetyl-CoA oxidation may occur when intermediates of the citric acid cycle are being used in gluconeogenesis or during coenzyme A saturation.

6.2.9. Ketogenic Diets

The ketogenic diet is a high-fat, adequate-protein, low-carbohydrate diet that is used mainly to treat hard-to-control epilepsy in children. This type of diet induces and maintains ketosis in the body. The "classic" ketogenic diet consists of fats and carbohydrates in a 4:1 ratio. It was developed in 1921 for use in the treatment of pediatric refractory epilepsy (Wilder, 1921), and continues to be utilized as an anticonvulsant dietary regimen. The ketogenic diet enjoyed a place in the medical world as a therapeutic diet for pediatric epilepsy and was widely used until its popularity ceased with the introduction of antiepileptic agents.

Given the carbohydrate restriction, the ketogenic diet may have some undesirable side effects which, in turn, can make it difficult to adhere to. Some of the undesirable side effects are gastrointestinal discomfort, constipation, vomiting, lack of energy, hunger, and increases in apoB-lipoproteins. For these reason, it may be beneficial to have rapid and temporary rises in ketone concentrations with no dietary modifications. As an alternative, or to supplement a well-formulated ketogenic diet, exogenous ketones have been shown to be a reliable method of rapidly increasing blood ketone levels and promoting a state of ketosis.

In addition to their established role in the treatment of pediatric epilepsy, ketogenic diets have potential therapeutic applications in neurodegenerative diseases, such as Alzheimer's (Reger et al., 2004) and Parkinson's disease (Vanitallie et al., 2005). The ketogenic diet with variations has also been investigated for their therapeutic effects in weight management (Dashti et al., 2003, 2006; Yancy et al., 2004), diabetes (Westman et al., 2008; Al-Khalifa et al., 2009), cancer (Freedland et al., 2008; Otto et al., 2008; Seyfried et al., 2008), and a number of other conditions. In a review article, Cavaleri and Bashar (2018) reported the therapeutic applications for exogenous ketones and ketone bodies, like BHB, have merit in multiple disease models.

Similar to ketogenic diets, D-BHB can be used to elevate blood ketone levels, given that it is absorbed following ingestion. The long history of use of ketogenic diets provides corroborative evidence of D-BHB safety for its intended use as an ingredient in food supplement products.

6.2.10. FDA Review of D-BHB-Ester GRAS Notice

As indicated earlier, in a 2014 GRAS notice (GRN 000515) the safety of D-BHB-ester has been extensively summarized (TdeltaS, 2014). In this GRAS notice, the notifier concluded that the use of D-BHB-ester as an ingredient in selected foods products sports beverages (liquid or powder form), gels, and bars, at levels up to 40% (in these foods) is GRAS through scientific procedures. D-BHB-ester was produced via a lipase-catalyzed transesterification reaction of ethyl-D-hydroxybutyrate and (R)-1,3-butanediol as an odorless, colorless oil with a slight bitter, sharp taste containing ≥ 97.5% D-BHB-ester. The proposed use is estimated to result in intakes that will not exceed 0.36 g/kg bw/serving and a maximum of 2 to 3 servings/day (d) will be consumed, resulting in a maximum daily exposure of 75 g/person (1.1 g/kg bw/day for a 70 kg individual). The use of D-BHB-ester is as a specialty product targeted to high performance athletes rather than for use in conventional foods for the general population.

During the review of GRN 000515, the OFAS, FDA calculated the estimated dietary exposure of D-BHB-ester for the general population based on an assumption that the intended use applies to all sports drinks and bars at the maximum use levels described in GRN 000515 and that exposure would not be limited to high performance athletes. The mean and 90th percentile average dietary exposure to D-BHB-ester for persons aged 2 years and older (users only) were determined to be 17 g/person/day (280 mg/kg bw/day) and 35 g/person/day (550 mg/kg bw/day), respectively.

In the GRAS Notice, the available data and information to support the safety of the intended use of D-BHB-ester has been extensively summarized and discussed. The available information shows that D-BHB-ester is completely hydrolyzed to its metabolites, D-β-hydroxybutyrate (D-BHB) and (R)-1,3-butanediol, the latter being further metabolized to D-BHB and acetoacetate. The metabolic profile of D-BHB-ester shows that the resulting metabolic product of the ester is identical to the subject of this present GRAS assessment and the safety of D-BHB-ester is applicable to the present GRAS determination.

The published human pharmacokinetic data (Clarke et al., 2012a) suggest that the consumption of D-BHB-ester in healthy adult volunteers resulted in circulating D-BHB levels that did not exceed 5.5 millimolar at the highest dose tested (up to 2.1 g/kg bw/day) that is equivalent to physiological levels observed during fasting states. In the same study, D-BHB-ester consumption for 5 days was well-tolerated, with no adverse effects. In a published 28-day oral toxicity rat study, no significant treatment-related toxicity was observed in rats consuming diets containing D-BHB-ester at intake levels of 12 and 15 g/kg bw/day in male and female rats, respectively. The notifier also discussed the findings from a published developmental toxicity study in rats indicating that D-BHB-ester did not adversely affect the development of rats in utero at a level of 2 g/kg bw/day. The safety of D-BHB-ester was corroborated by the results of an unpublished 66-day oral toxicity study described in the GRAS notice. In this study, rats were fed diets containing D-BHB-ester at 13.7 g/kg bw/day. Based on the totality of the scientific evidence, the notifier concluded that D-BHB-ester is GRAS under the conditions of its intended use.

Following its review, the FDA responded to the notifier of GRN 000515 that, based on the information provided in the Notification, as well as other information available to the FDA,

the agency had no questions at the time regarding TdeltaS's conclusion that D-BHB is GRAS under the intended conditions of use.

6.3. GRAS Panel Review, Summary and Discussion

At the request of Amin Talati Upadhye, LLP (AminTalati), USA and its client Osaka Gas Chemicals Co. Ltd. (Osaka Gas Chemicals), an independent panel of recognized experts (hereinafter referred to as the Expert Panel)[1], qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened to evaluate the Generally Recognized As Safe (GRAS) status of D-β-Hydroxybutyrate (D-BHB) derived from *Halomonas* sp. for use as a food ingredient in selected conventional foods such as Beverages and Beverage Bases (Sports and nutrition beverages) and Grain Products (Sports and nutrition bars) at a maximum level of up to 6 g per serving of food (Reference Amounts Customarily Consumed Per Eating Occasion; 21 CFR § 101.12). A comprehensive search of the scientific literature for safety and toxicity information on D-BHB and related compounds such as its esters, salts or metabolites was conducted through August 2021 and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by AminTalati and Osaka Gas Chemicals and other information deemed appropriate or necessary. Following an independent, critical evaluation, the Expert Panel conferred on October 15, 2021 and unanimously agreed to the decision described herein.

AminTalati and Osaka Gas Chemicals ensured that all reasonable efforts were made to identify and select a balanced Expert Panel with expertise in food safety, toxicology, and nutrition. The Expert Panel was selected and convened in accordance with the Food and Drug Administration (FDA)'s guidance for industry on "Best Practices for Convening a GRAS Panel"[2]. Efforts were placed on identifying conflicts of interest or relevant "appearance issues" that could potentially bias the outcome of the deliberations of the Expert Panel and no such conflicts of interest or "appearance issues" were identified. The Expert Panel members received a reasonable honorarium as compensation for their time; the honoraria provided to the Expert Panel members were not contingent upon the outcome of their deliberations.

Osaka Gas Chemicals intends to market the standardized D-BHB preparation, produced by a fermentation and extraction process, using microbial strain *Halomonas sp.* KM-1. The production process and the specifications of D-BHB have been fully developed. The chirality of the molecule is not less than 95%. Batch analyses for five lots of D-BHB demonstrate compliance with product specifications and production of consistent products. D-BHB is produced according to the current good manufacturing practices. D-BHB will be used in selected food categories such as Beverages and Beverage Bases (Sports and nutrition beverages) and Grain Products (Sports and nutrition bars), at a maximum use level of 6 g/serving (RACC). The estimated daily intake of BHB in the target population, ranged from 86 to 257 mg/kg bw/day when consuming 1 to 3 servings, respectively, by a 70-kg adult. For the general population, the mean and 90th percentile consumer-only intakes of BHB from the proposed food uses, were estimated to be 6.3 g/person/day (92 mg/kg bw/day) and 13.7 g/person/day (198 mg/kg body weight/day), respectively. For safety assessment purposes, highest intake of 18 g/person/day is considered.

---

[1]Modeled after that described in section 201(s) of the Federal Food, Drug, and Cosmetic Act, As Amended. See also attachments (curriculum vitae) documenting the expertise of the Panel members.

[2] Available at: https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ucm583856.htm

D-BHB is found in nature and in the food supply. It is a fatty acid derivative, which is present in milk and milk products. It is also produced in the large intestine of animals from fiber via fermentation. Muscles and the central nervous system utilizes BHB for intracellular energy production. Dietary modulation can increase and maintain circulating ketone bodies, especially BHB, which is one of the most abundant ketone bodies in human circulation. BHB is quickly distributed and incorporated into extrahepatic tissue by monocarboxylate transporters, which are increasingly expressed in times of fasting/starvation. BHB is subsequently converted to Acetyl-CoA and fed into the citrate cycle. BHB and its preparations in salt and ester form are marketed as a dietary supplement and for uses in selected foods as GRAS ingredient.

The available evidence suggests that following oral exposure, D-BHB is readily absorbed from the gastrointestinal tract and increases blood ketones to millimolar levels. Following oral D-BHB consumption, the ketone tracer ^{11}C-acetoacetate is rapidly metabolized, mostly by the heart and the kidneys. D-BHB does not appear to lower the blood acetoacetate/D-BHB ratio, making it an efficient fuel compared to other ketone precursors. Similarly, one of the well-known esters of D-BHB, (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, is fully hydrolyzed following oral exposure to D-BHB and (R)-1,3-butanediol in the gut. The latter is further metabolized to D-BHB and acetoacetate in the liver. The hydrolysis of D-BHB-ester occurs via carboxylesterases or esterases distributed throughout the intestinal tract, blood, liver, and other tissues. The available data suggest ready bioavailability and rapid elimination of the metabolites. As orally administered D-BHB-ester is primarily metabolized to D-BHB, the available safety studies of the ester are applicable to D-BHB.

In two separate human studies, the safety of exogenous BHB salt supplementation as determined by hematological safety markers and other parameters in adult and adolescents has been investigated. In these studies, D-BHB salt was administered at dose levels of 25.50 and 7.50 g/person/day to adults and adolescents, respectively, for 90 days. In both of these studies, the BHB salt was found to be safe and well tolerated. In an unpublished 4 week animal toxicity study, administration of the dried cells of *Halomonas* sp. KM-1 (source material of D-BHB) or D-BHB to rats did not reveal adverse effects. The NOAEL was reported as higher than 2000 mg/kg bw/day in males and females for both the dried cells of *Halomonas* sp. KM-1 and D-BHB. In a published 28 day toxicity study in rats (Kesl et al., 2016) the effects of administration of five ketone supplements, including BHB salts, on blood glucose, ketones, and lipids were investigated in rats. The findings from this study supports tolerability of oral exogenous D-BHB salts.

In a safety tolerability study in humans, administration of D-BHB-ester at dose levels up to 2.1 g/kg bw/day for 5 days was well-tolerated, with no adverse effects. The findings from a 28-day oral toxicity study in rats indicated that consumption of D-BHB-ester at a level of 11.4% (12.0 g/kg bw/day and 15.1 g/kg bw/day in male and female rats, respectively) in the diet did not cause adverse effects. In a developmental toxicity study in pregnant rats, administration of D-BHB-ester did not adversely affect the development of rats exposed in utero at a level of 2 g/kg bw/day. The findings from these human and animal studies of D-BHB-ester are applicable to the subject of present GRAS and indicate that D-BHB is unlikely to cause adverse effects.

The available evidence shows that, during times of limited glucose availability, the maximal ketone body production can be as high as 185 g/day, indicating that the human body

has the ability to handle large amounts of ketones. Additionally, there is a long history of use of ketogenic diets, which have been used for over a century in the treatment of pediatric refractory epilepsy, and more recently for other therapeutic applications. Ketogenic diets, similar to D-BHB, result in elevated ketone levels.

FDA reviewed the safety of D-BHB-ester as part of GRN 000515 and did not question the proposed uses in foods resulting in a maximum daily exposure of 75 g/person (1.1 g/kg bw/day for a 70 kg individual). The use of D-BHB-ester is as a specialty product targeted to high performance athletes rather than for use in conventional foods for the general population. The FDA calculated the estimated 90th percentile intake of D-BHB-ester for the general population to be 35 g/person/day (550 mg/kg bw/day). As compared to the above determined intake estimate of D-BHB from the proposed uses in food, the FDA estimate of D-BHB-ester of 35 g/person/day is almost double. These observations also suggest that the proposed use of D-BHB is unlikely to be of any safety concern.

In summary, the totality of the available evidence from *in vitro,* animal and human studies, including studies for D-BHB-ester that is primarily metabolized to D-BHB, the historical dietary exposure to BHB from foods like dairy products, and the presence of BHB in the human body support the safety-in-use of D-BHB, at the intended use levels (maximum 6 g/serving). On the basis of scientific procedures[3], and exposure from natural dietary sources, the consumption of D-BHB as an added food ingredient is considered safe at use levels up to 6 g/serving resulting in the highest 90th percentile intake of 18 g/person/day. The intended uses are compatible with current regulations, *i.e.,* D-BHB is used in selected (targeted) food categories and is produced according to current good manufacturing practice (cGMP).

[3] 21 CFR §170.3 Definitions. (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

6.4. GRAS Panel Conclusion

Based on a critical evaluation of the publicly available data summarized herein, the Expert Panel members whose signatures appear below, have individually and collectively concluded that D-β-Hydroxybutyrate (D-BHB), meeting the specifications cited herein, and when used as a food ingredient at maximum use levels of up to 6 g/serving (when not otherwise precluded by a Standard of Identity) in foods such as Beverages and Beverage Bases (Sports and nutrition beverages) and Grain Products (Sports and nutrition bars), described in this assessment and resulting in the 90th percentile all-user estimated intake of 18 g D-BHB/person/day is safe.

It is also the opinion of the Expert Panelists that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that D-BHB, when used as described, is GRAS based on scientific procedures.

Signatures

[redacted]

Robert L. Martin, Ph.D. — Date: Oct. 15, 2021

[redacted]

John A. Thomas, Ph.D., F.A.C.T., F.A.T.S. — Date: OCT 18 2021

[redacted]

Madhusudan G. Soni, Ph.D., F.A.C.N., F.A.T.S. — Date: Oct 18, 2021

7. PART VII- LIST OF SUPPORTING DATA AND INFORMATION

Al-Khalifa, A., Mathew, T.C., Al-Zaid, N.S., Mathew, E., Dashti, H.M., 2009. Therapeutic role of low carbohydrate ketogenic diet in diabetes. Nutrition 25(11/12):1177-1185.

Anders, M.W., 1989. Biotransformation and bioactivation of xenobiotics by the kidney. In: Intermediary Xenobiotic Metabolism in Animals: Methodology, Mechanisms and Significance. Editors: Hutson, D., Caldwell, J., Paulson, G.D. New York (NY): Taylor and Francis, pp. 81- 97.

Cavaleri, F., Bashar, E., 2018. Potential Synergies of β-Hydroxybutyrate and Butyrate on the Modulation of Metabolism, Inflammation, Cognition, and General Health. Journal Nutrition Metabolism 2018:7195760. https://doi.org/10.1155/2018/7195760

Cuenoud, B., Hartweg, M., Godin, J. P., Croteau, E., Maltais, M., Castellano, C. A., Carpentier, A. C., Cunnane, S.C., 2020. Metabolism of Exogenous D-Beta-Hydroxybutyrate, an Energy Substrate Avidly Consumed by the Heart and Kidney. Frontiers Nutrition, 7:13. https://doi.org/10.3389/fnut.2020.00013

Clarke, K., Tchabanenko, K., Pawlosky, R., Carter, E., Todd King. M., Musa-Veloso, K., Ho, M., Roberts A, Robertson J, Vanitallie T.B., Veech R.L., 2012a. Kinetics, safety and tolerability of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate in healthy adult subjects. Regul Toxicol Pharmacol 63(3):401-408.

Clarke, K., Tchabanenko, K., Pawlosky, R., Carter, E., Knight, N.S., Murray, A.J. et al., 2012b. Oral 28- day and developmental toxicity studies of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate. Regul Toxicol Pharmacol 63(2):196-208.

Clarke, K. et al., [unpublished]. Gastrointestinal effects of drinking the ketone ester, deltaG™. [See Appendix H in DRAFT GRAS Ketone Ester Report Dated July 10, 2013]. Cited in GRN 000515.

Dashti, H.M., Bo-Abbas, Y.Y., Asfar, S.K., Mathew, T.C., Hussein, T., Behbahani, A., et al., 2003. Ketogenic diet modifies the risk factor of heart disease in obese patients. Nutrition 19(10):901-902.

Dashti, H.M., Al-Zaid, N.S., Mathew, T.C., Al-Mousawi, M., Talib, H., Asfar, S.K., et al., 2006. Long term effects of ketogenic diet in obese subjects with high cholesterol level. Mol Cell Biochem 286(1/2):1-9.

Desrochers, S., David, F., Garneau, M., Jetté, M., Brunengraber, H., 1992. Metabolism of (R)- and S1,3-butanediol in perfused livers from meal-fed and starved rats. Biochem J 285(2):647- 653.

FDA, 2015. Agency Response Letter GRAS Notice No. 000515. D-beta-hydroxybutyrate ester. Available at: https://wayback.archive-

it.org/7993/20171031002904/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm443267.htm

Fischer, T., Och, U., Klawon, I., Och, T., Grüneberg, M., Fobker, M., Bordewick-Dell, U., Marquardt, T., 2018. Effect of a Sodium and Calcium DL-β-Hydroxybutyrate Salt in Healthy Adults. J Nutr Metab 2018:9812806. doi: 10.1155/2018/9812806.

Freedland, S.J., Mavropoulos, J., Wang, A., Darshan, M., Demark-Wahnefried, W., Aronson, W.J., et al., 2008. Carbohydrate restriction, prostate cancer growth, and insulin-like growth factor axis. Prostate 68(1):11-19.

Gin, H., Vambergue, A., Vasseur, C., Rigalleau, V., Dufour, P., Roques, A., et al., 2006. Blood ketone monitoring: a comparison between gestational diabetes and non-diabetic pregnant women. Diabetes Metab 32(6):592-597.

Hall, S.E., Wastney, M.E., Bolton, T.M., Braaten, J.T., Berman, M., 1984. Ketone body kinetics in humans: the effects of insulin-dependent diabetes, obesity, and starvation. J Lipid Res 25(11):1184-1194.

Han, Y.M., Ramprasath, T., Zou, M.H., 2020. β-Hydroxybutyrate and its metabolic effects on age-associated pathology. Exp. Mol. Med. 52:548-555. https://doi.org/10.1038/s12276-020-0415-z

Hess, F.G. Jr., Cox, G.E., Bailey, D.E., Parent, R.A., Becci, P.J., 1981. Reproduction and teratology study of 1,3-butanediol in rats. J. Appl. Toxicol. 1: 202-209.

Heymann, E., 1980. Carboxylesterases and amidases. In: Enzymatic Basis of Detoxification. (Biochemical Pharmacology and Toxicology, vol 2). Editor: Jakoby, W. New York (NY)/Toronto (ON): Academic Press, pp. 291-323.

Holland, A., Qazi, A., Beasley, K., Bennett, H., 2019. Blood and cardiovascular health parameters after supplementing with ketone salts for six weeks. Journal of Insulin Resistance 4:1. DOI: https://doi.org/10.4102/jir.v4i1.47

Hunter, E.S. III, Sadler, T.W., Wynn, R.E., 1987. A potential mechanism of DL-β-hydroxybutyrate induced malformations in mouse embryos. Amer. J. Physiol. 253: E72-E80.

JECFA, 1980. Butane-1,3-diol. In: Toxicological Evaluation of Certain Food Additives. 23rd Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Apr. 2-11, 1979, Geneva, Switz. (WHO Food Additives Series, no 14). Geneva, Switz.: World Health Organization (WHO), pp. 76-83.

Kawata, Y., 2015. A 4 week oral gavage repeated dose toxicity study of the dried cells of Halomonas sp KM-1 and 3-hydroxybutyric acid in rats. Study No: N-TT140003. Tsukuba Research Institute, Japan. Study report provided by Osaka Gas Chemicals. pp. 1-265.

Kawata Y, Aiba, S., 2010. Poly(3-hydroxybutyrate) production by isolated Halomonas sp. KM-1 using waste glycerol. Biosci. Biotechnol. Biochem. 74:175-177.

Kawata, Y., Kawasaki, K., Shigeri, Y., 2012. Draft genome sequence of *Halomonas* sp. strain KM-1, a moderately halophilic bacterium that produces the bioplastic poly(3-hydroxybutyrate). J. Bacteriol. 194(10):2738-2739. https://doi.org/10.1128/JB.00237-12

Kesl, S.L., Poff, A.M., Ward, N.P., Fiorelli, T.N., Ari, C., Van Putten, A.J., Sherwood, J.W., Arnold, P., D'Agostino, D.P., 2016. Effects of exogenous ketone supplementation on blood ketone, glucose, triglyceride, and lipoprotein levels in Sprague-Dawley rats. Nutrition Metabolism 13:9. https://doi.org/10.1186/s12986-016-0069-y

Kies, C., Tobin, R.B., Fox, H.M., Mehlman, M.A., 1973. Utilization of 1,3-butanediol and nonspecific nitrogen in human adults. J Nutr 103(8):1155-1163.

Laffel, L., 1999. Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. Diabetes Metab Res Rev 15(6):412-426.

Larsen T., Nielsen N.I., 2005. Fluorometric determination of β-hydroxybutyrate in milk and blood plasma. Journal Dairy Science 88(6):2004-2009. doi: 10.3168/jds.s0022-0302(05)72876-9.

Mensink, R.P., Meijer, G.W., Zock, P.L., Katan, M.B., Beynen, A.C., 1992. Effects of dietary saturated, cis-and trans-monounsaturated and polyunsaturated fatty acids on fasting blood ketone levels in man. In: Sinclair A, Gibson R, editors. Essential Fatty Acids and Eicosanoids. Invited Papers from the Third International Congress, March, 1-5, 1992, Adelaide, Australia. Champaign (IL): American Oil Chemists' Society (ACS), pp. 274-278.

Moley, K.H., Vaughn, W.K., Diamond, M.P., 1994. Manifestations of diabetes mellitus on mouse preimplantation development: Effect of elevated concentration of metabolic intermediates. Human Reproduction 9:113-121.

Nelson, D.L., Cox, M.M., 2000. Oxidation of fatty acids (chapter 17). In: Lehninger Principles of Biochemistry, 3rd edition. New York (NY): Worth Publishers, pp. 598-622.

Newman, J.C., Verdin, E., 2017. β-Hydroxybutyrate: A Signaling Metabolite. Annual Review Nutrition 37:51-76. https://doi.org/10.1146/annurev-nutr-071816-064916

Nielsen N.I., Larsen T., Bjerring M., Ingvartsen, K.L., 2005. Quarter health, milking interval, and sampling time during milking affect the concentration of milk constituents. Journal Dairy Science. 88(9):3186-3200. doi: 10.3168/jds.s0022-0302(05)73002-2.

Otto, C., Kaemmerer, U., Illert, B., Muehling, B., Pfetzer, N., Witting, R., et al., 2008. Growth of human gastric cancer cells in nude mice is delayed by a ketogenic diet supplemented with omega-3 fatty acids and medium-chain tryglicerides. BMC Cancer 8:122. 122. doi: 10.1186/1471-2407-8-122.

Owen, O.E., Morgan, A.P., Kemp, H.G., Sullivan, J.M., Herrera, M.G., Cahill, G.F. Jr. 1967. Brain metabolism during fasting. J Clin Invest 46(10):1589-1595.

Paterson, P., Sheath, J., Taft, P., Wood, C., 1967. Maternal and foetal ketone concentrations in plasma and urine. Lancet 289(7495):862-865.

Plecko, B., Stoeckler-Ipsiroglu, S., Schober, E., Harrer, G., Mlynarik, V., Gruber, S., et al., 2002. Oral β-hydroxybutyrate supplementation in two patients with hyperinsulinemic hypoglycemia: Monitoring of β-hydroxybutyrate levels in blood and cerebrospinal fluid, and in the brain by *in vivo* magnetic resonance spectroscopy. Pediatr Res 52(2):301-306.

Reger, M.A., Henderson, S.T., Hale, C., Cholerton, B., Baker, L.D., Watson, G.S., et al., 2004. Effects of beta-hydroxybutyrate on cognition in memory-impaired adults. Neurobiol Aging 25(3):311-314.

Reichard, G.A. Jr., Owen, O.E., Haff, A.C., Paul, P., Bortz, W.M., 1974. Ketone-body production and oxidation in fasting obese humans. J Clin Invest 53(2):508-515.

Scala, R.A., Paynter, O.E., 1967. Chronic oral toxicity of 1,3-butanediol. Toxicol Appl Pharmacol 10(1):160-164.

Seyfried, T.N., Kiebish, M., Mukherjee, P., Marsh, J., 2008. Targeting energy metabolism in brain cancer with calorically restricted ketogenic diets. Epilepsia 49(Suppl. 8):114-116.

Sheehan, E.A., Beck, F., Clarke, C.A., Stanisstreet, M., 1985. Effects of β-hydroxybutyrate on rat embryos grown in culture. Experientia 41: 273-275.

Shivva, V., Cox, P.J., Clarke, K., Veech, R.L., Tucker, I.G., Duffull, S.B., 2016. The Population Pharmacokinetics of D-β-hydroxybutyrate Following Administration of (R)-3-Hydroxybutyl (R)-3-Hydroxybutyrate. AAPS J. 18(3):678-688. https://doi.org/10.1208/s12248-016-9879-0

Stefan, M., Sharp, M., Gheith, R., Lowery, R., Wilson, J., 2021. The Effect of Exogenous Beta-Hydroxybutyrate Salt Supplementation on Metrics of Safety and Health in Adolescents. Nutrients 13(3):854. https://doi.org/10.3390/nu13030854

Stefan, M., Sharp, M., Gheith, R., Lowery, R., Wilson, J., 2020. The Effects of Exogenous Beta-Hydroxybutyrate Supplementation on Metrics of Safety and Health. Int. J. Nutr. Food Sci. 9:154. doi: 10.11648/j.ijnfs.20200906.13.

Stubbs, B.J., Cox, P.J., Evans, R.D., Santer, P., Miller, J.J., Faull, O.K., Magor-Elliott, S., Hiyama, S., Stirling, M., Clarke, K., 2017. On the Metabolism of Exogenous Ketones in Humans. Front. Physiol. 8:848. doi: 10.3389/fphys.2017.00848

Tate, R.L., Mehlman, M.A., Tobin, R.B., 1971. Metabolic fate of 1,3-butanediol in the rat: conversion to -hydroxybutyrate. J Nutr 101(12):1719-1726.

TdeltaS, 2014. GRAS Exemption Claim for (R)-3-hydroxybutyl (R)-3-hydroxybutyrate. GRAS Notice 000 515 submitted by TdeltaS Limited. Available at: http://wayback.archive-it.org/7993/20171031042051/https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/UCM403846.pdf

Van Hove, J.L., Grunewald, S., Jaeken, J., Demaerel, P., Declercq, P.E., Bourdoux, P., et al., 2003. D,L,3-Hydroxybutyrate treatment of multiple acyl-CoA dehydrogenase deficiency (MADD). Lancet 361(9367):1433-1435.

VanItallie, T.B., Nonas, C., Di Rocco, A., Boyar, K., Hyams, K., Heymsfield, S.B., 2005. Treatment of Parkinson disease with diet-induced hyperketonemia: a feasibility study. Neurology 64(4):728-730.

VanItallie, T.B., Nufert, T.H., 2003. Ketones: metabolism's ugly duckling. Nutr Rev 61(10):327-341.

Veech, R.L., Chance, B., Kashiwaya, Y., Lardy, H.A., Cahill, G.F. Jr., 2001. Ketone bodies, potential therapeutic uses. IUBMB Life 51(4):241-247.

Vreeland, R.H., 2015. "*Halomonas*". In: Bergey's Manual of Systematics of Archaea and Bacteria. Editor: Whitman WB.., pp. 1-19. doi:10.1002/9781118960608.gbm01190. ISBN 9781118960608.

Westman, E.C., Yancy, W.S. Jr., Mavropoulos, J.C., Marquart, M., McDuffie, R., 2008. The effect of a low-carbohydrate, ketogenic diet versus a low-glycemic index diet on glycemic control in type 2 diabetes mellitus. Nutr Metab (Lond) 5:36.

Wilder, R.M., 1921. The effects of ketonemia on the course of epilepsy. Mayo Clin Proc 2:307-308.

Yancy, W.S. Jr., Olsen, M.K., Guyton, J.R., Bakst, R.P., Westman, E.C., 2004. A low-carbohydrate, ketogenic diet versus a low-fat diet to treat obesity and hyperlipidemia: a randomized, controlled trial. Ann Intern Med 140(10):769-777.

8. Appendix I

Certificate of Analysis from Five Lots of D-β-Hydroxybutyrate

(Please note that of the five lots, two lots only provide assay related information and not the heavy metals or microbiological parameters)

OSAKA GAS CHEMICALS CO.,LTD.

5-11-61,Torishima, Konohana-Ku, Osaka, 554-0051,Japan

Tel:+81-6-6467-1224 /Fax:+81-6-6466-4568

To whom it may concern

Certificate of Analysis

We hereby certify the specific of our material as follows.

Name of product		40% BHB solution	
Lot No.		B2003004	
test	Specification	Observation	Test Method
Appearance	Clear or Pale yellow	Pale yellow	NA
Assay	NLT93% HPLC substrate remains (sucrose) and fermentation by-product (lactate, acetate) excluded	BHB 96.1% Acetic acid 3.9%	HPLC
BHB(free)	38-42%	41%	HPLC
Chirality(D-BHB %)	>95%.	99.8%	HPLC
Mercury	≦0.1ppm	≦0.01ppm	AAS
Lead	≦0.5ppm	≦0.05ppm	AAS
Cadmium	≦0.5ppm	≦0.01ppm	AAS
Arsenic	≦0.5ppm	≦0.1ppm	AAS
Aerobic mesophilic microorganisms	≦3,000 cfu/g	≦300 cfu/g	ISO 4833
Aerobic Spores thermophilic	≦100 cfu/g	≦1cfu/g	Standard Agar Plating Method; Heat-shocked conditions: in boiling water for 30 minutes, Incubation conditions: 55 degree for 2 days
Enterobacteriaceae	0 cfu/g	0 cfu /g	ISO 21528-1
Salmonella	0 cfu/25g	Negative/25g	ISO 6579

Sincerely yours

Yuichiro Yasuda

Team Manager

New Business Development Dept

Fine Materials Business Div.

OSAKA GAS CHEMICALS CO.,LTD.

5-11-61,Torishima, Konohana-Ku, Osaka, 554-0051,Japan

Tel:+81-6-6467-1224 /Fax:+81-6-6466-4568

To whom it may concern

Certificate of Analysis

We hereby certify the specific of our material as follows.

Name of product		40% BHB solution	
Lot No.		B2006007	
test	Specification	Observation	Test Method
Appearance	Clear or Pale yellow	Clear	NA
Assay	NLT93% HPLC substrate remains (sucrose) and fermentation by-product (lactate, acetate) excluded	BHB 95.9% Acetic acid 4.1%	HPLC
BHB(free)	38-42%	40%	HPLC
Chirality(D-BHB %)	>95%	100.0%	HPLC
Mercury	≦0.1ppm	≦0.01ppm	AAS
Lead	≦0.5ppm	≦0.05ppm	AAS
Cadmium	≦0.5ppm	≦0.01ppm	AAS
Arsenic	≦0.5ppm	≦0.1ppm	AAS
Aerobic mesophilic microorganisms	≦3,000 cfu/g	≦10 cfu/g	ISO 4833
Aerobic Spores thermophilic	≦100 cfu/g	≦10cfu/g	Standard Agar Plating Method; Heat-shocked conditions: in boiling water for 30 minutes, Incubation conditions: 55 degree for 2 days
Enterobacteriaceae	0 cfu/g	0 cfu /g	ISO 21528-1
Salmonella	0 cfu/25g	0 cfu/25g	ISO 6579

Sincerely yours

Yuichiro Yasuda

Team Manager

New Business Development Dept

Fine Materials Business Div.

OSAKA GAS CHEMICALS CO.,LTD.

5-11-61,Torishima, Konohana-Ku, Osaka, 554-0051,Japan
Tel:+81-6-6467-1224 /Fax:+81-6-6466-4568

To whom it may concern

Certificate of Analysis

We hereby certify the specific of our material as follows.

Name of product		40% BHB solution	
Lot No.		B2006008	
test	Specification	Observation	Test Method
Appearance	Clear or Pale yellow	Clear	NA
Assay	NLT93% HPLC substrate remains (sucrose) and fermentation by-product (lactate, acetate) excluded	BHB 95.5% Acetic acid 4.5%	HPLC
BHB(free)	38-42%	38%	HPLC
Chirality(D-BHB %)	>95%.	100.0%	HPLC
Mercury	≦0.1ppm	≦0.01ppm	AAS
Lead	≦0.5ppm	≦0.05ppm	AAS
Cadmium	≦0.5ppm	≦0.01ppm	AAS
Arsenic	≦0.5ppm	≦0.1ppm	AAS
Aerobic mesophilic microorganisms	≦3,000 cfu/g	≦10 cfu/g	ISO 4833
Aerobic Spores thermophilic	≦100 cfu/g	≦10cfu/g	Standard Agar Plating Method; Heat-shocked conditions: in boiling water for 30 minutes, Incubation conditions: 55 degree for 2 days
Enterobacteriaceae	0 cfu/g	0 cfu /g	ISO 21528-1
Salmonella	0 cfu/25g	0 cfu/25g	ISO 6579

Sincerely yours

Yuichiro Yasuda

Team Manager

New Business Development Dept

Fine Materials Business Div.

OSAKA GAS CHEMICALS CO.,LTD.

2-37, Chiyozaki 3-chome-minami, Nishi-Ku, Osaka, 550-0023, Japan

Tel:+81-6-4393-0184 /Fax:+81-6-4393-0192

To whom it may concern

Certificate of Analysis

We hereby certify the specific of our material as follows.

Product name	40% (D)-β-Hydroxybutyrate
Product code	40% D-BHB
Lot No.	B1907033
Appearance	pale yellow
Assay (BHB)	95.6%
Assay (acetic acid)	0.7%
BHB (free %)	42
Chirality (D-BHB %)	99.7

Sincerely yours

Takahiro Nishino

Team Manager

New Business Development Dept.

Fine Materials Business Div.

OSAKA GAS CHEMICALS CO.,LTD.

2-37, Chiyozaki 3-chome-minami, Nishi-Ku, Osaka, 550-0023, Japan

Tel:+81-6-4393-0184 /Fax:+81-6-4393-0192

To whom it may concern

Certificate of Analysis

We hereby certify the specific of our material as follows.

Product name	40% (D)-β-Hydroxybutyrate
Product code	40% D-BHB
Lot No.	B1903019
Appearance	pale yellow
Assay (BHB)	100.0%
Assay (acetic acid)	0.0%
BHB (free %)	46
Chirality (D-BHB %)	99.8

Sincerely yours

[redacted]

Takahiro Nishino

Team Manager

New Business Development Dept.

Fine Materials Business Div.

9. Appendix II

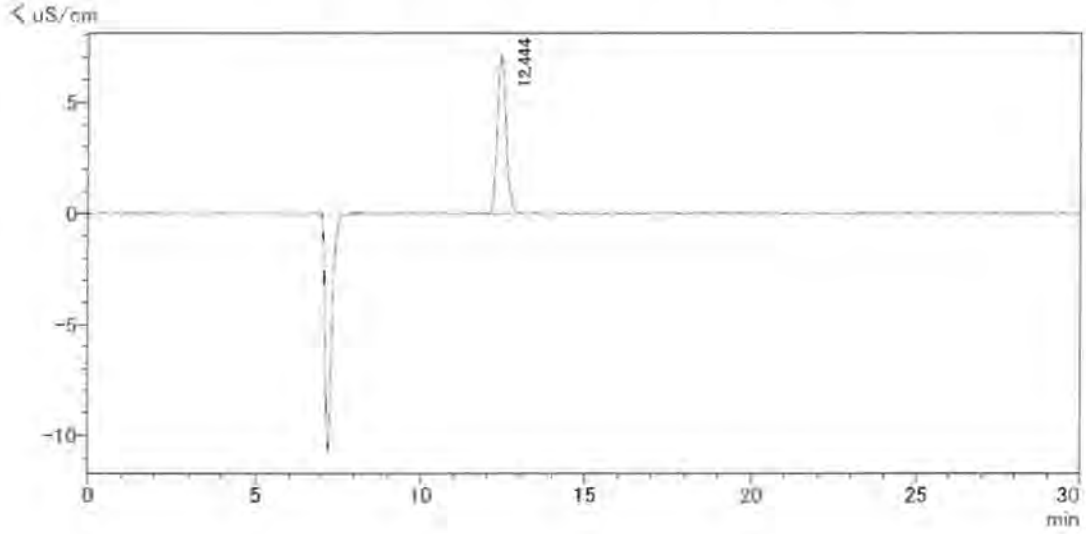
Assay for D-β-Hydroxybutyrate by HPLC

1. Analytical method of purity of β-Hydroxy butyrate

We use the analytical system of organic acids offered by SHIMADZU Co. Ltd.
(Reference: https://www.shimadzu.com/an/hplc/aplsys/organic.html)

Analysis equipment of HPLC: Prominence (SHIMADZU Co. Ltd.)
Column: Shim-pack SCR-102H (Particle size: 7μm 8.0mm i.d. × 300mm+Guard column)
(SHIMADZU GLC, Co. Ltd)
Mobile phase A: 5 mM p-toluenesulfonic acid
Mobile phase B: 5 mM p-toluenesulfonic acid, 20 mM Bis-Tris, 100 μM EDTA
Flow rate A: 0.8 mL/min
Flow rate B: 0.8 mL/min
Column temperature: 40°C
Detector: Electrical conductivity (CDD-10A VP)
Injection volume: 10 μL (Analysis range of concentration: 50ppm～1000ppm)
Analytical time: 30 min
Purity of β-Hydroxy butyrate was estimated from peak area (%).

Chromatogram of R-(-)-ß-Hydroxy butyrate (Sigma-Aldrich 298360-1G)

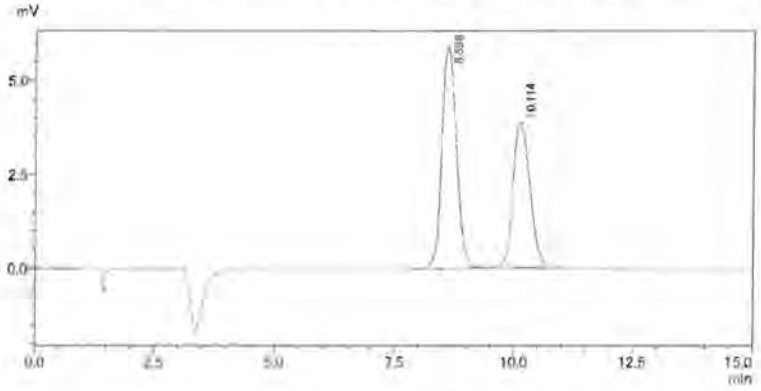


Peak	R.T.	Area	Area%	Concenration	Unit	Chemical substance
1	12.444	131112	100.000	497.028	mg/L	3HB
Total		131112	100.000			

2. Analytical method of optical purity of β-Hydroxy butyrate

Analysis equipment of HPLC: Nexera X2 (SHIMADZU Co. Ltd.)
Column: Sumichiral OA6100 (Particle size: 5μm 4.6mm i.d. × 150mm+Guard column)
Sumika Chemical Analysis Service, Co. Ltd.
Mobile phase: 1 mM Cooper (II) sulphate in water
Flow rate: 1 mL/min
Column temperature: 25°C
Detector: 254nm
Injection volume: 10 μL (Analysis range of concentration: 50ppm～1000ppm)
Analytical time: 15 min
Chiral purity of β-Hydroxy butyrate was estimated from peak area (%).

Chromatogram of Sodium-β-Hydroxy butyrate (Sigma-Aldrich 54965-10G-F))



Peak	R.T.	Area	Area%	Chemical substance
1	8.596	126016	56.559	S-3HB
2	10.114	96788	43.441	R-3HB
Total		222804	100.000	

10. Appendix III

ESTIMATED DAILY INTAKE OF D-β-HYDROXYBUTYRATE BY THE U.S. POPULATION FROM CURRENT DIETARY SOURCES AND PROPOSED FOOD USES (2017-2018 NHANES)

Complete report from Intertek.

Attached separately

Santos, Marissa

From: Ashish Talati <ashish@amintalati.com>
Sent: Tuesday, December 14, 2021 3:42 PM
To: Santos, Marissa
Subject: [EXTERNAL] RE: Question Regarding GRAS Notice for D-BHB

We confirm, that intended conditions of use includes gel and the maximum use level is also 6 g/serving. Sorry for the oversight.

Thanks

Ashish

From: Santos, Marissa [mailto:Marissa.Santos@fda.hhs.gov]
Sent: Tuesday, December 14, 2021 1:57 PM
To: Ashish Talati <ashish@amintalati.com>
Subject: Question Regarding GRAS Notice for D-BHB

Dear Mr. Talati,

We are working towards filing the GRAS notice you submitted on behalf of Osaka Gas Chemicals Co. Ltd. for D-β-hydroxybutyrate (D-BHB); however, we have a question regarding the intended uses of D-BHB. We note that in section 1.4 of your notice, the intended uses include sports and nutritional beverages and bars at up to 6 g/serving. However, in section 3 of your notice, the intended uses also include use in gels. Please confirm that the intended conditions of use includes gels and that the maximum level is also 6 g/serving.

Please let me know if you have any questions.

Regards,
Marissa

Marissa Santos, M.S.
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
Tel: 240.402.8160
marissa.santos@fda.hhs.gov

Dear Dr. Santos,

RE: Questions/Comments regarding GRN 001032 (D-β-Hydroxybutyrate)

This responds to your email of February 18, 2022, regarding your queries that need to be addressed for D-β-Hydroxybutyrate (D-BHB) GRAS Notice (GRN 1032) submitted on behalf of Osaka Gas Chemicals Co. Ltd. We are providing a point-by-point response to all your queries along with some additional relevant clarifications/discussion.

CHEMISTRY

FDA Query: (1) The identity of the notified substance is described as a solution that is approximately 40% D-β-hydroxybutyrate (D-BHB). Please confirm that the solvent is water.

Response: Yes, we confirm the solvent is water.

FDA Query: (2) The intended use level of D-BHB is described in the notice as up to 6 g/serving in sports and nutritional beverages, bars, and gels. The subject of the notice is described as a solution that is approximately 40% D-BHB, and the notifier states that the ingredient may be produced at other concentrations (e.g., 60 or 80%). Please confirm that the maximum intended use level is on the basis of D-BHB and not the solution.

Response: We confirm that the maximum use level is on the basis of D-BHB content and not the solution. We are sorry for not making this clear in the GRAS notice.

FDA Query: (3) Please address the following questions regarding the specifications and batch analyses for D-BHB provided in the notice:

A. A limit of not less than (NLT) 93% is provided for the "assay" specification. This limit includes the statement that "substrate remains, and fermentation byproducts (lactate, acetate) excluded." Please confirm the identities of the analyte(s) that are included in this parameter and confirm that the HPLC method used is the same as provided for other specifications.

Response: We confirm that the analyte included in this parameter is D-BHB. The other analytes that are measured by HPLC, but excluded in reporting as part of specification includes lactate as L-lactate with CAS No.: 79-33-4, and acetate with CAS No.: 64-19-7. We confirm that the HPLC method used is the same as provided for other specifications.

Excluded analytes: lactate, acetate, sucrose
Included analyte: D-BHB
Other matter: protein, pigment (not detected by HPLC and less than 0.1%).

B. A limit of > 95% for "β-hydroxybutyrate (free)" is provided. Please confirm whether this limit is on a dry matter only basis, applies to the assay analysis or to the whole ingredient, and whether this limit includes both D- and L-β-hydroxybutyrate.

Response: We confirm that this limit of > 95% is on a dry matter only basis and the limit includes both D- and L-β-hydroxybutyrate.

C. In Table 2 of the notice, a limit of 38-40% for "BHB (free)" is provided. Please confirm whether this limit includes both D- and L-β-hydroxybutyrate. We note that in the certificates of analysis for lot B1903019, the measured level of "BHB (free %)" is reported as 46%, lot B1907033 has a level of 42%, and lot B2003004 has a level of 41%. We also note that the specification for this parameter is listed in the certificates of analysis for lots B2003004, B2006007, and B2006008 as 38-42%. Please address the results for these lots that exceed the specification provided in Table 2 and clarify this specification.

Response: We confirm that this limit of 38-40% for "BHB (free)" includes both D- and L-β-hydroxybutyrate. Thank you for bringing to our attention the discrepancy between the established specifications and the certificate of analysis. We are sorry for the oversight. As provided in the specifications (Table 2), we will limit the levels of BHB (free) 38-40%. In the future for each batch that comes under this category, we will make sure the BHB (free) level is within the specific limit (38-40%). We are providing two additional certificates of analysis to support the established specification limits (Annexure I). In the manufacturing it is likely that some batches may have higher levels (>40%) of BHB (free). However, in these cases the product will be diluted such that the final product limit of BHB (free) remains between 38-40%.

D. A limit of NLT 95% for "Chirality (D-BHB %)" is provided. Please confirm whether this limit is on a dry matter only basis.

Response: We confirm that this limit is on a dry matter only basis.

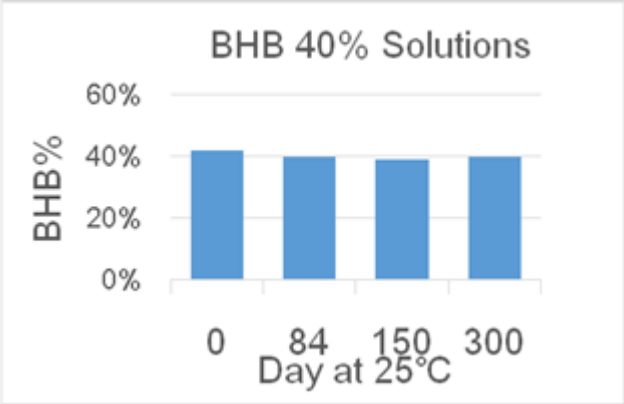
E. Please confirm whether the five lots of D-BHB described in Appendix I of the notice are consecutive or non-consecutive batches.

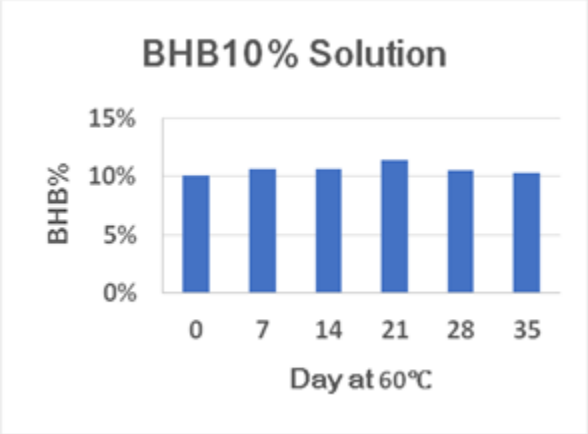
Response: We confirm that five lots of D-BHB described in Appendix I of the notice are non-consecutive. B2006007 and B2006008 is continuous number but different production lot from fermentation. Based on our understanding, consecutive lots mean that the production is done continuously. Our production records show that for batch number B2006007, the fermentation started on May 26, 2020 and ended May 28, 2020. For batch number B2006008, the fermentation started on June 08, 2020 and ended on June 10, 2020. The interval between that batches was a 12 days interval. This is considered as non-consecutive.

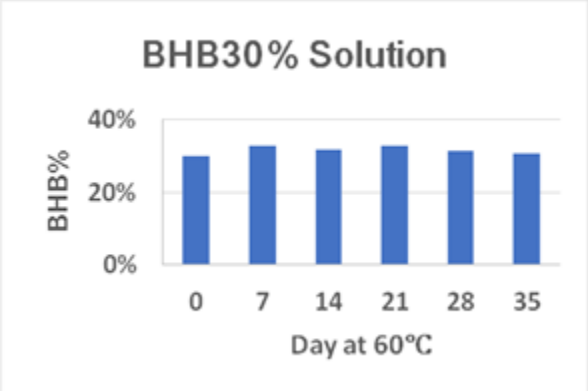
FDA Query: (4) Please provide a discussion of the stability of D-BHB at the various concentrations, including any studies of stability conducted with the D-BHB produced by the method described in the notice.

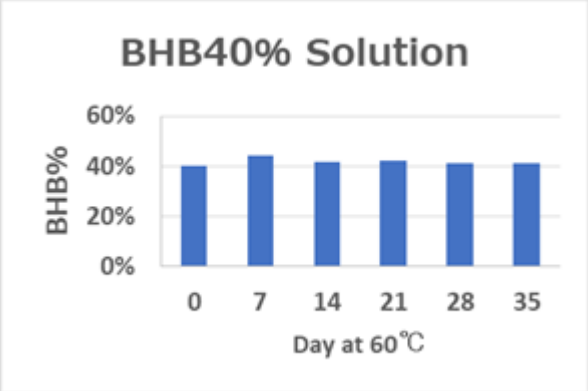
Response: The stability of D-BHB solution at a temperature of 25°C for 300 days has been investigated (please see below Figure). The results of this study suggest that D-BHB is stable for 300 days at 25°C. Additionally, we are also providing following data (please see below Figures) of accelerated stability test at 60°C for D-BHB solution at concentrations of 10, 20, 30 and 40% BHB. Considering acceleration ratio of 50 times at 60°C according to ICH Q1A - Q1F Stability Guidelines, D-BHB is stable no less than 1,750 days (4.8 years) at levels ranging from 10% to 40% BHB solution.

In a published report, Bruss (2008) noted that the available information suggest that 3-hydroxybutyric acid and its salts are relatively stable compounds.









TOXICOLOGY

FDA Query: (1) Please note that marketing to high-performance athletes aged 18 and older is a business decision and not relevant to a GRAS conclusion for intended uses in food. Generally recognized safety of the intended use of D-BHB cannot be predicated on prescribed conditions of use in select populations of consumers. A GRAS conclusion must establish safety of the intended use in the general population and should consider vulnerable subpopulations.

Response: Osaka Gas agrees that marketing to high-performance athletes is a business decision and not relevant to the GRAS conclusion. In the GRAS notice, the totality of the available evidence is considered to establish the safety of D-BHB at the proposed use levels in the general population, including any vulnerable subpopulation. Please note that we are not aware of any subpopulations that may be "vulnerable" to D-BHB.

FDA Query: (2) The notice includes estimates of dietary exposure to D-BHB based on the intended uses and cites information on dietary exposure provided in a previous GRAS notice for the use of DBHB ester. Please clarify if the intended uses of D-BHB will be substitutional or if these uses will be additive to the previously notified uses of D-BHB ester. If the intended use will be additive, please provide a cumulative dietary exposure for D-BHB ingredients in the diet and discuss the relevant safety information that supports the safe use of D-BHB ingredients at the cumulative estimated dietary exposure.

Response: Sorry, we forgot to mention that the intended uses will be substitutional to those of D-BHB ester described in the previous GRAS notice (GRN 515).

FDA Query: (3) Due to insufficient insulin signaling, diabetics are considered susceptible to onset of diabetic ketoacidosis. This is considered an adverse and possibly life-threatening condition characterized by supraphysiological ketone levels and decreases in blood pH. Diabetics may be at an increased risk of diabetic ketoacidosis related to elevated ketone levels.

A. Please address that the intended use of D-BHB will not present a safety concern for diabetic consumers.

Response:

Ketoacidosis is a metabolic state associated with pathologically high serum and urine concentrations of ketone bodies, namely acetone, acetoacetate, and D-BHB. Diabetic ketoacidosis is a potentially life-threatening complication of uncontrolled diabetes. The available evidence from published studies indicates that in patients with type 1 and type 2 diabetes, a ketogenic diet improves glycemic control and diabetic complications. In patients with type 2 diabetes, a ketogenic diet has been reported to be able to stop or reduce their diabetes medications. The ketogenic diet could reduce weight, triglycerides and blood pressure (Han et al., 2020).

A common concern of exogenous ketone supplementation particularly in subjects with diabetes is the risk of ketoacidosis (White et al., 2021; Batch et al., 2020; Kang et al., 2004). In the published literature, there are no reports of diabetic ketoacidosis resulting from intravenous ketone supplementation which appears to improve glucose control in the short term, although some trials excluded diabetic patients. Furthermore, data from trials using enteral ketone supplementation to induce ketosis suggest the incidence of diabetic ketoacidosis is exceedingly low and limited to case reports. In another recent review article, Bolla et al. (2019) reviewed the literature pertaining to ketogenic diets in diabetics, both type 1 and 2. These data suggests that ketogenic diets were safe although close monitoring for diabetic ketoacidosis was necessary. The available information of case reports from children with diabetes and epilepsy managed with a ketogenic diet suggest the treatment was safe provided patients are closely monitored. The few case reports of patients developing diabetic ketoacidosis secondary to ketogenic diets appears to be limited to individuals with undiagnosed type 1 insulin-dependent diabetes mellitus who initiated the ketogenic diets and subsequently developed diabetic ketoacidosis (Charoensri et al., 2021; Shaikh et al., 2020; Dressler et al., 2010).

The available information indicate that during ketoacidosis, the serum and urine levels of ketone bodies can be as high as 15-25 mM. In a human study, D-BHB supplementation at dose level of 12 g has been reported to increase plasma ketone level to 1.2 mM which is less than one tenth of the ketoacidosis plasma ketone level (Bernard et al., 2020). It is assumed that diabetics are under the control and advice of a physician. As such, it is expected that their diets are closely monitored as part of their medical regimen.

In a rat study, Kinzig et al. (2010) reported that maintenance of a ketogenic diet for 8 weeks decreased sensitivity to peripheral insulin and impaired glucose tolerance. However, a return to the normal chow diet after a ketogenic diet resulted in a dramatic reversal of these effects. Thus, long-term maintenance of a ketogenic diet negatively affects glucose homeostasis, but this effect can be rapidly reversed upon cessation of a ketogenic diet. Based on these observations, it can be postulated that direct consumption of ketone bodies, specifically BHB, is a more efficient way to control metabolic syndrome.

Interestingly, Kesl et al. (2016) hypothesized that exogenous ketone supplements could produce sustained hyperketonemia (>0.5 mM) without dietary restriction and without negatively influencing metabolic biomarkers, such as blood glucose, total cholesterol, HDL, LDL, and triglycerides. As described in the GRAS notice, these investigators studied the effects of administration of five ketone supplements on blood glucose, ketones, and lipids in male Sprague-Dawley rats. The supplements used in this study included: 1,3-butanediol (BD), a sodium/potassium BHB salt (BMS), medium chain triglyceride oil (MCT), BMS + MCT 1:1 mixture, and 1,3 butanediol acetoacetate diester (KE). The rats (n=10-11/group) were divided in six study groups; five treatment groups and one control group that received water. The BHB salt used in the study is as a 50% solution containing approximately 375 mg/g of pure BHB and 125 mg/g of sodium/potassium. On days 1 - 14, rats received a 5 g/kg bw/day dose of their respective treatments via intragastric gavage. Dosage was increased to 10 g/kg bw/day for the second half of the study (days 15 - 28) for all groups except BD and KE to prevent excessive hyperketonemia (ketoacidosis). Each daily dose of BMS would equal approximately 1000 - 1500 mg of BHB, depending on the weight of the animal. Weekly

whole blood samples were taken for analysis of glucose and BHB at baseline and, 0.5, 1, 4, 8, and 12 hours post-gavage, or until BHB returned to baseline. At 28 days, triglycerides, total cholesterol and high-density lipoprotein (HDL) were measured.

The findings from the above study revealed that ketone supplementation caused a slight change in triglycerides and lipoproteins over a 4-week investigation (Kesl et al., 2016). Blood BHB levels in the BMS group did not show significant elevation at any time point, even after dose escalation. BMS (5 g/kg bw/day) did not elicit a significant elevation in blood βHB at any time point. Rats supplemented with BMS had lower blood glucose compared to control at 12 hours in week 4. As compared to controls, BMS + MCT, BMS, and BD supplemented rats gained significantly less weight than controls during weeks 2 - 4. The BMS ketone supplement did not significantly induce blood hyperketonemia or reduced glucose in the rats. The findings from this study suggest that exogenous ketone supplementation, including BMS, caused a rapid and sustained elevation of BHB, reduction of glucose, and little change to lipid biomarkers compared to control animals. This study demonstrates the tolerability of oral exogenous ketone supplementation in inducing nutritional ketosis independent of dietary restriction.

In summary, findings from the above described human and animal studies indicate that D-BHB or its salts are unlikely to be of safety concern for individuals with diabetes.

MICROBIOLOGY

FDA Query: (1) Please provide a detailed description (with relevant references) of *Halomonas* sp. KM-1 "FERM BP-10995" including genotypic (e.g., pathogenicity and toxigenicity) and phenotypic characteristics (e.g., production of antimicrobials, production of secondary metabolites and toxins, antimicrobial resistance), and whether this poses a safety concern.

Response: In recent years, the salt-tolerating bacteria, *Halomonas* spp., are becoming the attractive candidate hosts for microbial cell factory engineering due to their strong metabolism of diverse substrates and fast growth under high salt and high pH conditions, making possible the contamination free, non-food raw materials- and seawater-consuming fermentation processes (Ye and Chen, 2021). Among the several predominant *Halomonas* strains of industrial potential, *Halomonas* sp. KM-1 (Jin et al., 2013; Yoshikazu et al., 2010; Yoshikazu et al., 2016) is being developed for producing different metabolic targets, demonstrating *Halomonas* as powerful chassis in biomanufacturing based on next-generation industrial biotechnology.

As described in the GRAS notice, members of *Halomonas* are halophilic (salt-tolerating), gram-negative, facultative aerobic, rod-shaped bacteria (Vreeland 2015). The production strain is wild type or naturally occurring mutants of *Halomonas sp.* strain KM-1. *Halomonas* sp. strain KM-1 has been deposited in the International Patent Organism Depositary (IPOD, AIST, Japan) as FERM BP-10995 (Kawata et al., 2012). This strain is moderately halophilic

that exhibits higher level of poly(3-hydroxybutyrate) (PHB) production under aerobic conditions than other species. The 16S rRNA gene sequence of *Halomonas* sp. strain KM-1 exhibited a high level of sequence similarity (99.0%) to *Halomonas* species (Kawata and Aiba, 2010).

Kawata et al. (2012) reported the draft genome sequence of *Halomonas* sp. strain KM-1 that produces the bioplastic poly(3-hydroxybutyrate). The total length of the assembled genome was reported as 4,992,811 bp, and 4,220 coding sequences were predicted within the genome. Genes encoding proteins, involved in the production and depolymerization of poly(3-hydroxybutyrate) were identified. The identification of these genes has been claimed to be useful in the production of the bioplastic poly(3-hydroxybutyrate) and its monomer 3-hydroxybutyrate.

All the *Halomonas* species are listed as Risk Groups 1 by the 'Classification of Prokaryotes (Bacteria and Archaea) into Risk Groups' by BAuA[1].

The genomic analysis shows that *Halomonas* sp. strain KM-1 does not include genes that code for toxins or virulence factors, but does include genes that code for resistance to some antibiotics[2]. This genomic testing was conducted using MiFuP, a database (in Japanese) that searches for genes related to harmfulness from the genomic information of microorganisms and estimates the harmfulness of microorganisms. It is maintained by National Institute of Technology and Evaluation (NITE), Japan. The safety related genes for specific toxins that are evaluated by MiFuP are summarized in Annexure II along with findings of the homologous gene analysis. The *Halomonas* sp. strain KM-1 genome includes homologous genes that code for resistance to bacitracin, beta-lactam, fosfomycin**,** macrolide, penicillin and streptogramin but this observation does not pose a safety concern because the nanofiltration step will remove any residual genomic DNA from the final product.

As regards antibiotic resistance of *Halomonas* sp. strain KM-1, it should be noted that processing and purification step, such as nanofiltration, used in the production of BHB, is likely to remove any bacterial cells, cell debris, proteins, DNA fragments and nucleic acids or other impurities whose molecular weight is larger than 300Da. The final product is >98% pure.

As stated above, the *Halomonas* sp. strain KM-1 genome does not contain sequences that code for any known virulence factor or toxin that is active via the oral route. Additionally, as described in the GRAS notice, both D-BHB, the subject of this GRAS Notice, as well as the source material (dried cells of *Halomonas* sp. KM-1) were tested in a 4 week rat toxicity study. In this study, administration of BHB in water or dried cells of *Halomonas* sp. KM-1 suspended in water, via oral gavage at dose levels of 0 (vehicle), 1000 and 2000 mg/kg bw/day for 28 days did not reveal adverse effects as evaluated by changes in general condition of animal, body weight, feed consumption, hematology, blood chemistry and necropsy. Histopathological examinations were not performed in this study, as there were no organs/tissues that showed changes thought to be caused by administration of BHB or dried

[1] Available at: https://www.baua.de/EN/Service/Legislative-texts-and-technical-rules/Rules/TRBA/pdf/TRBA-466.pdf?__blob=publicationFile&v=2.
[2] Available at: https://www.nite.go.jp/nbrc/mrinda/mifup_safety/potentials# (in Japanese)

cells of *Halomonas* sp. KM-1. Based on the findings from this study, the NOAEL was determined to be the highest dose tested, 2000 mg/kg bw/day in rats for both the dried cells of *Halomonas* sp. KM-1 and D-BHB. This supports the safety of source organism (*Halomonas* sp. KM-1) and BHB.

In summary, the available information, as discussed above suggest that *Halomonas* sp. KM-1, the source organism for the production of BHB is unlikely to pose any safety concern. Also, it is unlikely that any unknown impurity in the final product will be of any safety concern.

FDA Query: (2) Please provide a discussion on how the purity of the initial inoculum is determined.

Response: We tested purity by direct culture, Gram stain, and DNA sequencing. *Halomonas* sp. KM-1 does not carry any plasmid or bacteriophage.

FDA Query: (3) Please specify whether the fermentation process is continuously monitored for purity.

Response: Yes, at every step of the seed fermentations and main fermentation processes, the bacterial purity of the broth is checked by both microscopy and plating on standard agar.

FDA Query: (4) Please state whether any of the raw materials used in the fermentation process are major allergens or derived from major allergens. If any of the raw materials used are major allergens or derived from major allergens, please discuss why these materials do not pose a safety concern.

Response: We confirm that the raw materials used in the fermentation process do not contain any major allergens nor the raw material derived from major allergens.

FDA Query: (5) Please state whether the fermentation process is conducted in a contained, sterile environment.

Response: The fermentation process is conducted in a contained, sterile environment. The fermenter and its connecting line is sterilized before fermentation and kept pressured by sterile air during fermentation.

FDA Query: (6) On page 8 of the notice, the microbial specifications for *Enterobacteriaceae* and *Salmonella* are listed as 0/g and 0/25 g, respectively. Please clarify the units of measure for these specifications.

Response: The units of measure for *Enterobacteriaceae* and *Salmonella* are colony forming units (CFU):

Enterobacteriaceae : 0 CFU in 1g of 40% BHB solution

Salmonella : 0 CFU in 25g of 40% BHB solution

FDA Query: (7) In the Certificate of Analysis on page 44 of the notice, the observation for Salmonella is listed as "Negative/25 g". The specification on the same page is listed as "0 CFU/25 g". Please clarify what "negative" means for this observation.

Response: Negative means 0 CFU.

We hope the above information and clarification addresses your queries. If you have any questions or need additional explanation, please let me know.

Thank you for the opportunity to provide this explanation to your questions.

Best regards,

Ashish Talati

References:

Bernard, C., Mickaël, H., Jean-Philippe, G., Etienne, C., Mathieu M., Christian-Alexandre C., André C., Stephen C., 2020. Metabolism of Exogenous D-Beta-Hydroxybutyrate, an Energy Substrate Avidly Consumed by the Heart and Kidney. Front. Nutr. 19 February 2020 : https://doi.org/10.3389/fnut.2020.00013.

Bruss, M.L., 2008. Lipids and Ketones. In Clinical Biochemistry of Domestic Animals (Sixth Edition).

Charoensri, S., Sothornwit, J., Trirattanapikul, A., Pongchaiyakul, C., 2021. Ketogenic diet-induced diabetic ketoacidosis in a young adult with unrecognized type 1 diabetes. Case Rep Endocrinol. 2021:6620832. 10.1155/2021/6620832

Dressler, A., Reithofer, E., Trimmel-Schwahofer, P., Klebermasz, K., Prayer, D., Kasprian, G., et al., 2010. Type 1 diabetes and epilepsy: efficacy and safety of the ketogenic diet. Epilepsia. 51:1086–1089.

Han, Y.M., Ramprasath, T., Zou, M.H., 2020. β-Hydroxybutyrate and its metabolic effects on age-associated pathology. Exp Mol Med 52, 548–555.

Jin Y.X., Shi L.H., Yoshikazu K., 2013. Metabolomics-based component profiling of *Halomonas* sp. KM-1 during different growth phases in poly(3-hydroxybutyrate) production. Bioresour. Technol. 140:73–79.

Kesl, S.L., Poff, A.M., Ward, N.P., Fiorelli, T.N., Ari, C., Van Putten, A.J., Sherwood, J.W., Arnold, P., D'Agostino, D.P., 2016. Effects of exogenous ketone supplementation on blood

ketone, glucose, triglyceride, and lipoprotein levels in Sprague-Dawley rats. Nutrition Metabolism 13:9. https://doi.org/10.1186/s12986-016-0069-y

Kinzig, K.P., Honors, M.A., Hargrave, S.L., 2010. Insulin sensitivity and glucose tolerance are altered by maintenance on a ketogenic diet. Endocrinology, 151(7):3105–3114.

Shaikh, S., Mohamed, M.M., Mujeeb, A., Shaikh, F., Harris, B., 2020. Euglycemic Diabetic Ketoacidosis Precipitated by a Keto Diet: Importance of Dietary History in Diagnosis. Cureus 12:e10199. 10.7759/cureus.10199

Ye, J.W., Chen, G.Q., 2021. *Halomonas* as a chassis. Essays Biochem. 65(2):393-403.

Yoshikazu K., Aiba S., 2010. Poly(3-hydroxybutyrate) production by isolated *Halomonas* sp. KM-1 using waste glycerol. Biosci. Biotechnol. Biochem. 74:175–177.

Yoshikazu K., Taku N., Isao M., Jun T., 2016. Efficient production and secretion of pyruvate from *Halomonas* sp. KM-1 under aerobic conditions. AMB Express 6:22 10.1186/s13568-016-0195-y.

Yoshikazu K., Kazunori K., Yasushi S., 2012. Draft genome sequence of *Halomonas* sp. strain KM-1, a moderately halophilic bacterium that produces the bioplastic poly(3-Hydroxybutyrate). J. Bacteriol. 194:2738–2739.

Annexure I

Two additional Certificate of Analysis

OSAKA GAS CHEMICALS CO.,LTD.

5-11-61,Torishima, Konohana-Ku, Osaka, 554-0051,Japan
Tel:+81-6-6467-1224 /Fax:+81-6-6466-4568

To whom it may concern

Certificate of Analysis

We hereby certify the specific of our material as follows.

Name of product		40% BHB solution	
Lot No.		B2110012	
test	Specification	Observation	Test Method
Appearance	Clear or Pale yellow	Clear	NA
Assay	NLT93% HPLC substrate remains (sucrose) and fermentation by-product (lactate, acetate) excluded	BHB 99.5% Acetic acid 0.5%	HPLC
BHB(free)	38-40%	39%	HPLC
Chirality(D-BHB %)	>95%.	99.5%	HPLC

Sincerely yours

Yuichiro Yasuda
Team Manager
New Business Development Dept
Fine Materials Business Div.

OSAKA GAS CHEMICALS CO.,LTD.

5-11-61,Torishima, Konohana-Ku, Osaka, 554-0051,Japan
Tel:+81-6-6467-1224 /Fax:+81-6-6466-4568

To whom it may concern

Certificate of Analysis

We hereby certify the specific of our material as follows.

Name of product		40% BHB solution	
Lot No.		B2202005	
test	Specification	Observation	Test Method
Appearance	Clear or Pale yellow	Pale yellow	NA
Assay	NLT93% HPLC substrate remains (sucrose) and fermentation by-product (lactate, acetate) excluded	BHB 98.8% Acetic acid 0.6% Lactic acid 0.6%	HPLC
BHB(free)	38-40%	40%	HPLC
Chirality(D-BHB %)	>95%.	100.0%	HPLC

Sincerely yours

Yuichiro Yasuda

Team Manager

New Business Development Dept

Fine Materials Business Div.

Annexure II

Details available at: https://www.nite.go.jp/nbrc/mrinda/mifup_safety/#agree

MiFuP Safety

Toxins

Alpha-toxin of *Staphylococcus aureus* / Anthrax toxin / Beta-pore-forming toxin / Beta-toxin of *Clostridium perfringens* / Bicomponent pore-forming leukocidin / Binary Enterotoxin of *Clostridium perfringens* / Binary actin-ADP-ribosylating toxin / Botulinum toxin / CFTR inhibitory factor / Cereulide synthetase / Cholera enterotoxin / Cholesterol-dependent cytolysin family / Cholix toxin / Clostridium perfringens enterotoxin / Cytolethal distending toxin / Dermonecrotic toxin / Diphtheria toxin / Epsilon-toxin of *Clostridium perfringens* / Exfoliative toxin / Exotoxin A of *Pseudomonas aeruginosa* / Gamma-hemolysin AB / Gamma-hemolysin CB / Heat-labile enterotoxin / Heat-stable enterotoxin / Insecticidal crystal protein / Leukocidin AB / Leukocidin ED / Leukocidin MF' / Listeriolysin O / Panton-Valentine leukocidin / Perfringolysin O / Pertussis toxin / Phospholipase C / RTX toxin / Shiga toxin / Sphingomyelinase C / Staphylococcal and group A streptococcal superantigens / Streptolysin O / Tetanolysin / Tetanus toxin / Thermostable direct hemolysin / Vibrio cholerae cytolysin / Vibrio vulnificus hemolysin

Drug Resistance

Aminoglycoside resistance / Bacitracin resistance / Beta-lactam antibiotic resistance / Bleomycin resistance / Chloramphenicol resistance / Fosfomycin resistance / Macrolide antibiotic resistance / Polymyxin resistance / Streptogramin resistance / Sulfonamide resistance / Trimethoprim resistance / Vancomycin resistance

Others

Aromatic azo compounds degradation / CAMP factor / Histamine biosynthesis / Hop resistance / Hydrogen cyanide biosynthesis / Lysozyme resistance / Phosphorylcholine decoration / Type III secretion system / Tyramine biosynthesis / Urease

Findings from the homologues gene analysis are provided in below table:

ID	Function name	Function ID	Type	Pattern	Rule name	Rule ID	Protein name	CDS
1	Aromatic azo compounds degradation	NFUNC_0073	Require	A	AZOR	NRULE_0236	FMN-dependent NADH-azoreductase	scf7180000000004_quiver_4194; scf7180000000004_quiver_1237; scf7180000000004_quiver_1208
2	Bacitracin resistance	NFUNC_0022	Require	A	UPPP	NRULE_0097	Undecaprenyl-diphosphatase	scf7180000000004_quiver_270
3	Beta-lactam antibiotic resistance	NFUNC_0033	Require	D	BLAD	NRULE_0125	Beta-lactamase class D	scf7180000000004_quiver_4026
4	Fosfomycin resistance	NFUNC_0023	Require	A	FOSA	NRULE_0098	Glutathione transferase FosA	scf7180000000004_quiver_1549
5	Lysozyme resistance	NFUNC_0107	Require	N	MLIC	NRULE_0424	C-type lysozyme inhibitor MliC	scf7180000000004_quiver_4196
6	Macrolide antibiotic resistance	NFUNC_0045	Require	D	ERE	NRULE_0168	Erythromycin esterase	scf7180000000004_quiver_3491
7	Penicillin resistance	NFUNC_0047	Require	B	BLAD	NRULE_0125	Beta-lactamase class D	scf7180000000004_quiver_4026
8	Streptogramin resistance	NFUNC_0027	Require	A	VAT	NRULE_0113	Streptogramin A acetyltransferase	scf7180000000004_quiver_1092
9	Urease	NFUNC_0130	Require	A	UREA	NRULE_0472	Urease subunit gamma	scf7180000000004_quiver_2754
10	Urease	NFUNC_0130	Require	A	UREB	NRULE_0474	Urease subunit beta	scf7180000000004_quiver_2753
11	Urease	NFUNC_0130	Require	A	UREC	NRULE_0475	Urease subunit alpha	scf7180000000004_quiver_2752
12	Urease	NFUNC_0130	Require	A	URED	NRULE_0476	Urease accessory protein UreD	scf7180000000004_quiver_2755
13	Urease	NFUNC_0130	Require	A	UREE	NRULE_0477	Urease accessory protein UreE	scf7180000000004_quiver_2751
14	Urease	NFUNC_0130	Require	A	UREF	NRULE_0478	Urease accessory protein UreF	scf7180000000004_quiver_2750
15	Urease	NFUNC_0130	Require	A	UREG	NRULE_0479	Urease accessory protein UreG	scf7180000000004_quiver_2749