CHR. HANSEN
Improving food & health

Chr. Hansen, Inc.

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Division of Biotechnology and GRAS Notice Review
Center for Food Safety & Applied Nutrition (HFS-255)
U.S. Food & Drug Administration

Reference: *Leuconostoc carnosum* DSM 32756

April 20, 2020

Dear Sir or Madam,

In accordance with the Federal Register [81 Fed. Reg. 159 (17 August 2016)] issuance on Generally Recognized as Safe (GRAS) notifications (21 CFR Part 170), Chr. Hansen is pleased to submit a notice that we have concluded, through scientific procedures that *Leuconostoc carnosum* DSM 32756 is generally recognized as safe and is not subject to the pre-market approval requirements for use to enhance the quality of packed bacon throughout shelf-life by inhibiting spoilage. The culture preparation is recommended to be used at levels that will result in a final concentration up to and including 9.0 log Colony forming unit per g (CFU/g) on the finished food product.

We also request that a copy of the notification be shared with the United States Department of Agriculture's Food Safety (USDA) and Inspection Service (FSIS), regarding the use of *Leuconostoc carnosum* DSM 32756 as a safe and suitable ingredient in bacon.

If there are any questions or concerns, please contact us.

Yours sincerely,

Arie Carpenter
Senior Regulatory Affairs Specialist
usarbr@chr-hansen.com
414-777-7526

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Table of Contents

LIST OF TABLES AND FIGURES

LIST OF APPENDICES

ADDITIONAL SUPPORTING MATERIAL

SafePro® B-SF-77 Bacon challenge study
SafePro® B-SF-77 Bacon shelf-life study

SafePro® B-SF-77 705725 Batch records:

1. Batch 3376991
2. Batch 3393628
3. Batch 3434345
4. Batch 3413130

ABBREVIATIONS

BA: Biogenic Amine
BIOHAZ: Panel on Biological Hazards
CDS: Coding sequences
CFU: Colony forming units
CFR: Code of federal regulations
CLSI: Clinical and Laboratory Standards Institute
CNS: Coagulase negative Staphylococci
EFFCA: European Food and Feed Cultures Association
EFSA: European Food Safety Authority
FDA: Food and Drug Administration
FSIS: Food Safety and Inspection Service
FSSC: Food safety system certification
GMO: Genetically Modified Organism
GMO: Genetically Modified Organism
GMP: Good Manufacturing Practices
GRAS: Generally recognized as safe
IDF: International Dairy Federation
ISO: International Organization for Standardization
LAB: Lactic Acid Bacteria
Leu. (carnosum): Leuconostoc
MIC: Minimum Inhibitory Concentration
NCBI: National Center for Biotechnology Information
NR: non-redundant
RAST : Rapid Annotations using Subsystems Technology
RAST: Rapid Annotations using Subsystems Technology
S. (carnosus): Staphylococcus
SOP: standard operation procedure
USDA: United States Department of Agriculture

Part 1: Signed Statements and Certification

1.1 Statement of Intent

In accordance with the 21 Code of Federal Regulation (Code of Federal Regulation (CFR)) 170 Subpart E, regulations for GRAS notifications, Chr. Hansen, Inc. is pleased to submit a notice that we have concluded, through scientific procedures, that *Leuconostoc carnosum* DSM 32756 (currently commercially sold under the tradename SafePro®) is GRAS and is not subject to the premarket approval requirements under the intended use conditions described within this notification.

1.2 Name and Address of Notifier

Chr. Hansen, Inc.
9015 W Maple St.
Milwaukee, WI 53214
Tel: (414) 607-5700

Fax: (414) 607-59591.3 Common or Usual Name

Food culture, bacterial culture, *Leuconostoc carnosum, Leu. carnosum, L. carnosum*

(Leu. carnosum DSM 32756 is currently sold under the tradename SafePro®).

1.4 Conditions of Use

Leu. carnosum DSM 32756 is intended for use to enhance the quality of packed bacon throughout shelf-life inhibiting slime formation by spoilage organisms It is applied by diluting in water and injecting or sptraying onto the surface of food at use levels up to and including 9.0 log cfu/g of the finished food product. The applications covered in this GRAS notice are cured meat products, including but not limited to cured ham and bacon.It is applied by diluting in water and injecting or spraying onto the surface of the food at use levels up to and including 9.0 log cfu/g of the finished food product. The applications covered in this GRAS notice are cured meat products, including but not limited to cured ham and bacon.

1.5 Basis for GRAS Determination

Pursuant to the GRAS rule [81 Fed. Reg. 159 (17 August 2016)], Chr. Hansen, Inc. has concluded that *Leu. carnosum* DSM 32756 is GRAS through scientific procedures, in accordance with 21 CFR 170.30 (a) and (b).

1.6 Premarket Approval Status

It is the opinion of Chr. Hansen that *Leu. carnosum* DSM 32756 is not subject to premarket approval requirements of the Federal Food, Drug, and Cosmetics Act based on our conclusion that the notified substance is GRAS under the intended use conditions.

1.7 Availability of Information

The data and information that are the basis for Chr. Hansen's conclusion that *Leu. carnosum* DSM 32756 is GRAS, are available for review and copying by Food and Drug Administration (Food and Drug administration (FDA)) during customary business hours, at the location below, or will be sent to FDA upon request, made to:

Chr. Hansen, Inc.
Arie Carpenter
Senior Regulatory Affairs Specialist
9015 W Maple St., Milwaukee, WI 53214
usarbr@chr-hansen.com

1.8 Freedom of Information Act

It is our opinion that the information contained in this notification is not exempt from disclosure under the Freedom of Information Act.

1.9 Certification

To the best of our knowledge, this GRAS notification is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of *Leu. carnosum* DSM 32756.

1.10 Signature

___ April 20, 2020_____
Arie Carpenter, Senior Regulatory Affairs Specialist Date

___ April 20, 2020_____
Katharine Urbain, Head of Regulatory Affairs - North America Date

1.11 FSIS Authorization

We also request that a copy of the notification be shared with USDA-FSISUSDA-FSIS, regarding the use of *Leu. carnosum* DSM 32756 as a safe and suitable food ingredient used in cured meat products including, but not limited to cured ham and baconcured meat products including, but not limited to cured ham and bacon.

Part 2: Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

In preparing this dossier, Chr. Hansen has consulted and applied the Pariza *et al*. "Decision Tree for Determining the Safety of Microbial Cultures to be Consumed by Humans or Animals" (2015). The decision tree is composed of thirteen questions which, when applied, provide a "comprehensive approach for determining the safety of microbial cultures that lack an established history of safe use for their intended new applications". These questions include criteria related to characterization, antimicrobial substances, genetic engineering, and other relevant topics. Throughout this notification evidence will be given to support those criteria.

2.1 Name of the GRAS Organisms

The subject of this GRAS determination is *Leu. carnosum* DSM 32756, whichcurrently sold commercially as a component of a blend under the name SafePro® B-SF-77.

2.2 Source of the GRAS Organisms

Leu. carnosum DSM 32756 was isolated from the surface of sliced vacuum-packed pork product by the Danish Meat Research Institute (Denmark), as reported in Budde *et al.* (2003). The *Leu. carnosum* strain that is the subject of this notice was deposited in the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) under the accession number DSM 32756 in October of 2018.

2.3 Description of the GRAS Organisms

Leu. carnosum DSM 32756 is part of the broader category of lactic acid bacteria (LAB). LAB belong to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*. The families include *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae*. LAB have been used since ancient times in the preservation and production of fermented foods (Mozzi, 2016). In addition to being abundant in fermented, LAB are also part of the human microflora. Due to the long history of consumption and human exposure, LAB are considered generally safe by the scientific community (Adams, 1999). Leuconostocs are mesophilic, Gram-positive, catalase-negative, cocci. Leuconostoc have been isolated from an array of foods including chill-stored and fermented meats, fermented vegetables, and fermented dairy products (Holland & Liu, 2011). They play an important role in several food fermentation processes including fermented sausages. *Leu. carnosum* is widely present in vacuum-packed meat products (Ogier, Casalta, Farrokh, & Saihi, 2008).

Leuc. carnosum was identified by Chr. Hansen as is discussed in Appendix 1.

Leuc. carnosum, the topic of this notification, used individually or blended, such as in Chr. Hansen's SafePro® B-SF-77, are safe and suitable for human consumption.

2.3.1 Genotypic characteristics

The genome sequence of *Leu. carnosum* DSM32756 obtained in-house at Chr. Hansen was used for the genome safety assessment (Chr. Hansen, 2018). For the assessment, the genome sequence of *Leu. carnosum* strain DSM32756 was subjected to annotation using published methods. The DSM32756 genome size was 1.82 Mb and it contained 2,027 coding sequences (CDS) and 50 RNAs, which was comparable to *Leu. carnosum* genome in the National Center for Biotechnology Information (NCBI) genome database (1.77 Mb in size and 1761 CDSs). Plasmids were detected in the strain.

2.3.1.1 Search Against Antibiotic Resistance Gene Databases

To identify genes with high identity to previously published antibiotic resistance genes, the genomes of this strain was screened against a curated published database of antibiotic resistance genes.

The genome screening of *Leu. carnosum* DSM32756 resulted in one hit with low coverage (65%) and 70% identity to *vat(B)* U19459 (E-value of $1.7x10^{-41}$) an acetyl transferase involved in resistance to virginiamycin-like compounds in *S. aureus*. The protein encoding gene was Rapid Annotations using Subsystems Technology (RAST) annotated as a chloramphenicol acetyl transferase. The gene was also found in the one *Leu. carnosum* genome in the NCBI Non-reductant (NR) database with 100% coverage, 99% identity and annotated as an acetyltransferase. Although annotated by RAST as chloramphenicol acetyl transferase, the gene is not likely to be involved in chloramphenicol resistance as the strain is sensitive to chloramphenicol. The gene is an acetyl transferase, an enzyme involved in transfer of an acetyl group. Acetyl transferase serves various housekeeping functions within the cell and are not antibiotic resistance genes. Two other hits with higher e-value (10^{-18}) and *vanYA* (10^{-13}), respectively revealed housekeeping genes of no safety concern.

Overall, the *in-silico* genome screening for potential acquired antibiotic resistance genes did not reveal any antibiotic resistance genes of safety concern. This was further supported by the strain being sensitive to all relevant antibiotics for which it was tested.

2.3.1.2 Search Against the Virulence Factor Database

The draft genome of *Leu. carnosum* DSM 32756 was screened for virulence and toxicity genes as recommended by the European Food Safety Authority (EFSA) (EFSA FEEDAP, 2018).

The annotated draft genome of *Leu. carnosum* DSM 32756 was analyzed against a published database of virulence factors containing virulence factors from 30 different pathogens including Gram-positive pathogens such as *Enterococcus, Staphylococcus, Streptococcus* and *Listeria*. Most of the hits were associated with stress regulation (Clp), heat shock proteins, biosynthesis, transport proteins, biodegradation, and capsule formation. None of the hits were assessed to be virulence factors and all hits could be regarded as 'niche factors' (Hill, 2012), since they are also found in commensal bacteria. The one gene which had 100% coverage and 99% identity to *Leu. carnosum* in the NCBI NR database, but low coverage in related species, was annotated as a Rhodanese-like domain protein. Such domains are found in various non-pathogenic bacteria such as *Leuconostoc*, *Lactobacillus* and *Fructobacillus* species.

Although the specific function of the gene could not be determined, the gene is not considered a safety concern.

Another gene was annotated as a bacteriocin ABC-transporter, ATP binding and permease component. The gene had closest coverage (100%) and identity (74%) to *Leuconostoc gelidum* and a transporter on a plasmid found in a *Leu. carnosum* strain. The transporter was described as part of a bacteriocin cluster. The putative bacteriocin transporter is probably involved in secretion of bacteriocin as two bacteriocins which inhibit *Listeria* sp. are described within the strain (Budde, Hornbeak, Jacobsen, Barkholt, & Koch, 2003). Bacteriocins and bacteriocin transporters are not involved in pathogenicity and are considered beneficial for the bioprotective effect of the strain. The gene is therefore not considered a safety concern.

Overall, the *in-silico* genome screening of *Leu. carnosum* DSM 32756 for potential virulence factors and other genes related to pathogenicity, virulence or toxicity did not reveal any virulence, toxicity genes or other genes of safety concern.

2.3.2 Phenotypic Characteristics

Table 1 shows the physiological data for *Leu. carnosum*. The following paragraphs in this section describe other phenotypic characteristics related to the safety of the *Leu. carnosum* which is the topic of this notification (Appendix 2).

Culture Composition	***Leuconostoc carnosum***
Growth temperature Opt/max/min	20°C/30°C/2°C (68°F/86°F/36°F)
Salt limit	5% salt-in-water Survives in higher salt-in-water concentrations
Characteristics	Facultative anaerobic D(-) -lactic acid Heterofermentative
Fermentable sugars	
Glucose (dextrose)	+
Fructose	+
Maltose	-
Lactose	-
Saccharose (sucrose)	+
Starch	-

Below minimum temperature for growth the strain will still be alive but will not multiply in the application

***Table 1: Physiological data of Leu. carnosum* DSM 32756**

2.3.3 Biogenic Amines

For testing of biogenic amine (BA) activity no standardized method exists, but several methods have been published in the scientific literature. The most crucial steps are the induction of BA production and the biochemical analysis of the compounds in the induced samples. The occurrence of BA is attributed to the decarboxylase activity in certain bacteria and the BA are mainly synthesized by decarboxylation of the corresponding amino acids (Fernandez, Hudson, Korpela, & de los Reyes-Gavilan, 2015). Histamine and tyramine, along with cadaverine and putrescine, have been identified by the Pariza decision tree as well as EFSA ((BIOHAZ), 2018) to be the BA of most concern related to food safety (Pariza, Gillies, Kraak-Ripple, & Leyer, 2015).

Leu. carnosum DSM 32756 was tested for BA production by use of an in-house standard operation procedure (SOP) modified based on scientific literature (Cid, Miguelez-Arrizado, Becker, Holzapfel, & Vidal-carou, 2008). In brief, the strain was grown in Brain Heart Infusion broth aerobically at 25^{o}C in the presence of the corresponding amino acids (Cid, Miguelez-Arrizado, Becker, Holzapfel, & Vidal-carou, 2008). The corresponding amino acids were added to the Brain Heart Infusion broth to a final concentration of L-histidine (6.4mM), L-tyrosine (5.5 mM), L-lysine (5mM) and L-ornithine (5mM) to induce expression of the BA genes if present.

The presence of the four BA compounds (histamine, tyramine, cadaverine, and putrescine) was tested by use of an in-house validated Gas Chromatography Mass Spectrometry method modified from Smart *et al*. (2010). In both steps, positive and negative controls were included. The results showed that *Leu. carnosum* DSM 32756 tested negative for the four BA of concern.

BA production in DSM 32756 is shown in the table below.

BAFDA compound	Monoamines		Polyamines	
	Histamine	Tyramine	Cadaverine	Putrescine
DSM 32756	Not produced	Not produced	Not produced	Not produced

Table 2: Results of BA production for* Leu. carnosum *DSM 32756

2.3.4 Antibiotic Resistance

In order to measure antimicrobial susceptibility of the *Leu carnosum* DSM 32756, the minimum inhibitory concentration (MIC) was determined according to the CLSI standards M45-A2 and M100-S25 (a Clinical and Laboratory Standards Institute procedure) with minor modifications. In brief, the susceptibility test is a broth microdilution method using growth in LSM[1] medium (International Organization for Standardization (ISO)-sensitest medium (Oxoid) supplemented with 10% MRS[2] medium; (Klare, et al., 2005)) for 24 hours at 25°C under aerobic conditions with two biological replicates. The medium was controlled as recommended by the ISO 10932 | International Dairy Federation (IDF) 223 international standard by the use of *Lactobacillus plantarum* ATCC 14917, which was tested in parallel and had MIC values within the ranges given in the ISO standard. The range of antibiotics tested complies with the EFSA 'Guidance on the characterization of microorganisms used as feed additives or as production organisms' (EFSA FEEDAP, 2018).

The results showed that *Leu. carnosum* DSM 32756 was susceptible to all tested antimicrobial agents except vancomycin as vancomycin is an intrinsic property of *Leuconostoc* due to the composition of the cell wall (Handwerger, Pucci, Volk, Liu, & Lee, 1994).

Antibiotic type	Antibiotic	MIC in µg/mL	EFSA breakpoints in µg/mL[3]
Aminoglycoside	Gentamicin	0.5	16
	Kanamycin	8	16
	Streptomycin	4	64
Tetracycline	Tetracycline	1	8
Macrolide	Erythromycin	0.12	1
Lincosamide	Clindamycin	0.12	1
Chloramphenicol	Chloramphenicol	4	4
B-lactam	Ampicillin	0.5	2
Glycopeptide	Vancomycin	>128	n.r.*

Table 3: MIC values for* Leu. carnosum *DSM 32756

*n.r. – not required to be tested by EFSA

2.3.5 GM Status

Leu. carnosum DSM 32756 is not genetically modified by use of recombinant DNA techniques. The finished culture preparation(s) does not contain genetically modified organisms (GMOs) and does not contain GM labeled raw materials (Appendix 3).

[1] Lymphocyte separation medium
[2] DeMan, Rogosa and Sharpe medium
[3] EFSA epidemiological cut-off values for *Leuconostoc* as listed in EFSA 'Guidance on the characterization of microorganisms used as feed additives or as production organisms' (EFSA FEEDAP, 2018).

2.4 Method of Manufacture

Leu. carnosum DSM 32756 is currently manufactured, in accordance with current good manufacturing practices (cGMP) consistent with 21 CFR Parts 110 and 117, by Chr. Hansen GmbH Giessener Str. 94, Pohlheim, Germany following Chr. Hansen's global protocol for production of meat cultures (Appendix 4). This plant complies with a set of basic GMP-rules, also called Pre-Requisite Program (PRP) according to Chr. Hansen's Quality, GMPs and Food Safety Principles (Appendix 5). This includes allergen control both in the plant and with raw materials including fermentation media (Appendix 6, Appendix 7, and Appendix 8). In addition, each plant has an appointed local OPRP (Operational Pre-Requisite Program) that includes Pre-Requisite Program issues and Critical Control Points, which are documented and are classified as specifically critical for the safety of food ingredients produced in the plant. The Pohlheim plant maintains the following certifications: Food safety system certificate (FSSC) 22000 and ISO 22000.

Leu. carnosum DSM 32756 is sold as freeze-dried powder. It is produced by first inoculating the microorganism into sterilized growth substrate. Anaerobic conditions are maintained during fermentation; pH and temperature are controlled. When the microbiological growth stops, fermentation is stopped by cooling. The microorganisms are then harvested and concentrated by centrifugation. They are then frozen into pellets. For freeze-dried product, the cultures are submersed in liquid nitrogen and lyophilized into granules. Freeze-dried granules are ground to a powder and blended with excipients to a standardized cell count. The individual culture preparations may then be blended together. Finally, the product is filled into aluminum foil bags and labeled (product name, item number, batch number, amount, storage temperature).

Leu. carnosum DSM 32756 is produced using standard fermentation techniques. This includes the use of fermentation and standardizing ingredients that are safe and suitable for use in human food. These ingredients have no technical function in the finished food product and are all permitted for use in food culture preparations and/or foods in general in addition to meeting the specifications of the Food Chemical Codex. In addition, allergens are managed at both the raw material level and the plant level as described in the previous section.

2.5 Specifications

Leu. carnosum DSM 32756 is a freeze-dried product currently sold in combination with *Staphylococcus carnosus* DSM 25010 (*S. carnosus*) commercially as SafePro® B-SF-77. The finished product is an off-white to brownish ground powder. Purity is controlled as described in Table 4 and additionally in Appendix 9: List of analysis.

Leu. carnosum DSM 32756 freeze-dried products have a shelf life is 18 months when stored in tightly closed original container at <-18°C (1°F) in dry conditions and protected against direct sunlight. When stored at 5°C (41°F), shelf-life is 6 weeks (Appendix 2: Product Information). They are transported at either ambient or refrigerated temperatures. The specifications for each batch are shown in Table 4 below and are based on specification guidelines set forth by the European Food and Feed Cultures Association (EFFCA) in "Industry guidelines of quality control for Microbial Food Cultures used in Meat Fermentation" (European Food and Feed Cultures Association, 2015). Methods used for shelf-life determination are available upon request.

	Criteria	Frequency of analysis
Bacillus cereus	< 100 cfu/g	Every batch
Enterobacteriaceae	< 10 cfu/g	Every batch
Enterococci	< 1000 cfu/g	Every batch
Staphylococcus aureus	< 50 cfu/g	Every batch
Yeast and molds	< 100 cfu/g	Every batch
Listeria monocytogenes	Absence in 25 g	Per monitoring program
***Salmonella* spp.**	Absence in 25 g	Per monitoring program

Table 4: SafePro® B-SF-77 Product Specification

2.6 Intended technical effect & amount required

Meat spoilage by microorganisms is a common problem and can be attributed to contamination during and/or after slaughtering by way of water, air, worker, and equipment. It can also occur in the later processing steps when factors such as pH, temperature, and sodium chloride levels select for certain bacteria, allowing colonization of the meat surface by different spoilage-related species and strains (Stellato, et al., 2016). Meat undergoes progressive deterioration from the time of slaughter until consumption due to its unique biological and chemical nature (Olaoye & Ntuen, 2011). Typically, spoilage is identified as an appearance defect such as discoloration or slime. It can also be related to off-flavors or odors (Pothakos, Devlieghere, Villani, Bjokroth, & Ercolini, 2015).

Leu. carnosum DSM 32756 is intended to be applied to raw cured meat products including but not limited to cured ham and bacon at a use-level up to and including 9.0 log CFU/g to enhance the quality of packed bacon throughout shelf-life by inhibiting slime formation due to spoilage organisms.

2.6.1 Hurdle Technology

The addition of *Leu. carnosum* DSM 32756 to cured meats including but not limited to cured ham and bacon creates additional hurdles against slime producing spoilage organisms. The hurdles include competitive exclusion (Jameson effect) and the ability of the strain to produce inhibitory compounds.

The competitive exclusion effect plays a large role in inhibiting the growth of unwanted bacteria in cured meat products inoculated with *Leu. carnosum* DSM 32756. The dominating microbiota, when well adapted inhibits the growth of other bacteria via physical interactions (Marshall, R.J. & McElhatton, A., 2007). Growth of the unwanted bacteria is thus stopped when the dominating microbiota reach their maximum population (Møller C.O.A., et al., 2013). This phenomenon can be explained by the Jameson effect, first observed in 1962. It was described by Mellefont *et al*. (2008) as “a race between species to use the resources of the environment to maximize their growth and population numbers. When those resources are depleted, the race is over, and the growth of each species in the population stops”.

Leu. carnosum also produces inhibitory compounds such as bacteriocins which may assist with the suppression of unwanted bacteria (Jacobsen, Budde, & Koch, 2003).

2.6.2 Experimental Studies

2.6.2.1 Challenge tests with *Leuconostoc mesenteroides* (Summary from "SafePro® B-SF-77 Bacon challenge study")

The bacon was processed and sliced in the Chr. Hansen pilot plant following standard US recipe[4]. Slices were contaminated with a spoilage *Leu. mesenteroides* strain isolated from spoiled bacon (targeted contamination level around 2.0 log CFU/g). *Leu. mesenteroides* was chosen as a characteristic spoilage organism that causes slime formation (Chenoll E., Macián M.C., Elizaquível P., & Aznar R., 2006). Slices of bacon were then portioned into 2 batches. The first batch was used as a control, the second was sprayed on both sides with *Leu. carnosum* DSM 32756 and *S. carnosus* DSM 25010 solution. Initial inoculum level 8.2 log CFU/g for *Leuconostoc spp.* and 8.4 log CFU/g for coagulase negative staphylococci (CNS). Both strains were inoculated because the commercial product contains *Leu. carnosum* as well as *S. carnosus*. Both batches were then vacuum packed and stored at 5±1°C. Evolution of inoculated *Leu. mesenteroides* was studied over the shelf life using 3 replicates per sampling time and per batch (Figure 1).

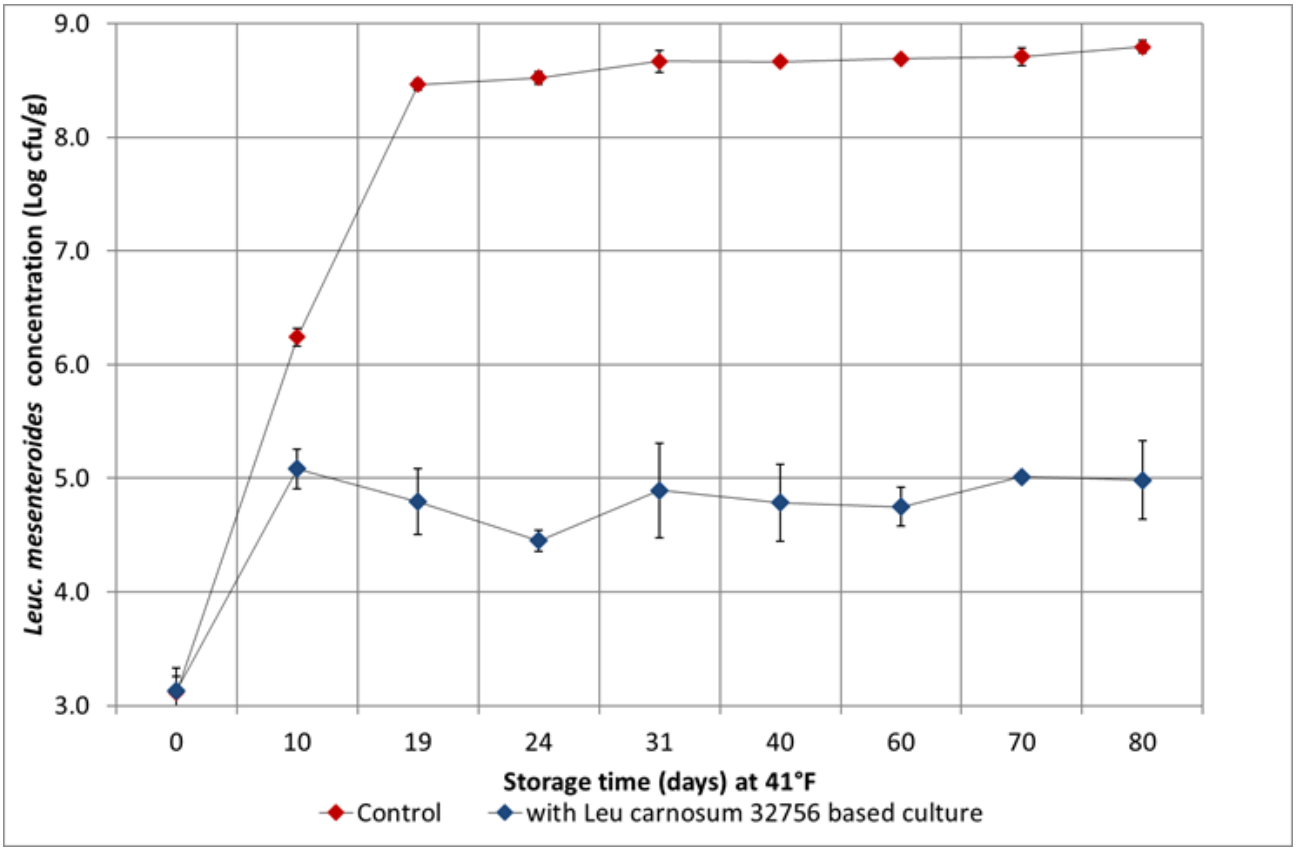

Figure 1: Influence of SafePro® B-SF-77 on the evolution artificially inoculated Leu. mesenteroides on vacuum packed bacon.

2.6.2.2 Shelf life study (Summary of "SafePro® B-SF-77 Bacon shelf-life study")

[4] "Pumped" bacon method. (FSIS, Bacon and Food Safety, 2013)

Freshly produced bacon was halved. One half was sprayed with SafePro® B-SF-77 during slicing. The other half was used as a control. Samples were then vacuum packed and stored at 5±1°C for 120 days. Contamination with *Leuc. mesenteroides* was checked at D0, at the end of the current shelf life (D90) and after extended shelf life (120 days). Initial inoculum level was 7.3 log CFU/g for *Leu. carnosum.* Table 5 shows microbial results of the *Leu. mesenteroides* enumeration. What we see is that *Leu. mesenteroides* counts are significantly lower than the control when SafePro® B-SF-77 is applied to the sliced meat.

Batch / Day	D0	D90	D120
Control	<2	3.6	4.4
With SafePro® B-SF-77	< 2	3.6 ± 0.9	2.2 ± 0.3

Table 5: Influence of SafePro® B-SF-77 on the concentration (Log CFU/g) of naturally present Leu. mesenteroides on vacuum packed bacon.

2.6.3 Application and Production Safety

The food culture SafePro® B-SF-77 may be applied using one of two methods. The first method, which was used in the various experiments described in this notice, requires the freeze-dried product to be diluted in water and sprayed onto bacon during slicing in a closed system and/or clean room. A second possible method is to add the culture into the brine. The targeted final concentration at application is up to and including 9.0 log CFU/g. Good industrial hygiene practices should be followed when handling and storing the product(s). This includes wearing gloves when handling frozen or freeze-dried product and using ventilation if dust or aerosols are present (Appendix 10). There are no known hazards towards workers or inspection personnel. The use of *Leu. carnosum* DSM 32756 when used under the prescribed conditions will not interfere with USDA inspection procedures as its composition and application is similar to that of products already commercially approved and used in the meat industry (FDA, GRN No. 159, 2005) (FDA, GRN No. 305, 2010) (FDA, GRN No. 760, 2019).

Chr. Hansen suggests that culture SafePro® B-SF-77 be labeled on the finished product as “food culture” or “lactic acid bacteria”.

Part 3: Dietary Exposure

It is expected that only a negligible amount of *Leu. carnosum* DSM 32756, if any, will be present in the bacon after cooking since *Leu. carnosum* is heat sensitive and would be eliminated in the cooking process. The USDA gives recommendations on the handling and preparation of bacon on their website. According to the web page ‘*Bacon and Food Safety*’, “Any bacteria that might be present on the surface would be destroyed by cooking” (FSIS, Bacon and Food Safety, 2013). Therefore, the consumption of cooked bacon containing *Leu. carnosum* DSM 32756 would not increase the dietary intake of these microorganisms assuming consumers are cooking their cured meat products.

Inoculation rate targeted for *Leu. carnosum* DSM 32756 is up to and including 9.0 log CFU/g. At the end of the shelf-life, *Leu. carnosum* DSM 32756 counts are not expected to be above 9.0 log CFU/g as was determined through scientific literature review as well as challenge studies.

According to "What We Eat in America" and NHANES data from 2015-2016, the average amount of cured meat consumed by both males and females ages 2 and up, was 0.96 oz or approximately 27.2 g/per person/per day.

Based on calculations of a "worst-case-scenario," and assuming consumers are eating their cured meat raw and that all cured meat consumed contains *Leu. carnosum* DSM 32756 cultures at a level of 9.0 log CFU/g ($1x10^9$ CFU/g), multiplying by 27.2 g/day results in a maximum intake of $1.3x10^{11}$ CFU per person/per day of *Leu. carnosum* DSM 32756. It is unlikely that all cured meat consumed in a day would be inoculated with *Leu. carnosum* DSM 32756, and because of the cooking step of bacon, this level is of no safety concern.

In addition, these organisms are transient in the gut. It is known that the adult microbiome is very stable and only shifts with significant dietary changes or extreme weight loss (Faith, et al., 2013). Therefore, sporadic consumption of *Leu. carnosum* DSM 32756 would not cause an increase in the gut. As bacteriocins are commonly produced by many LAB and some CNS, it should also be mentioned that bacteriocins are easily degraded by proteolytic enzymes in the mammalian gastro-intestinal tract (Zacharof & Lovitt, 2012), thus exerting the effect in the food product to improve food safety without affecting the micro-flora of the intestine. Since the number of microorganisms consumed is not increased compared to normal intake, the consumption of bacteriocins are also not increased and, therefore, need not be calculated.

Part 4: Self-Limiting Levels of Use

The proposed use of *Leu. carnosum* DSM 32756 is as a food ingredient added at manufacturing to enhance the quality of bacon throughout the shelf-life of the product by inhibiting slime formation due to spoilage organisms (*Leu. mesenteroides*). The self-limiting levels of use are:

- cGMP – Following the use level prescribed by Chr. Hansen, *Leu. carnosum* DSM 32756 will only be added to the bacon at levels required to achieve the technical effect in the food. There would be no benefit to the customer to add the product at higher levels due to the following:
    - Increase in cost to the customer
    - Possibility of negative impact on organoleptic properties due to drop in pH caused by lactic acid formation.

- Competitive exclusion –*Leu. carnosum* DSM 32756, when added to the food, is in competition for space and nutrients with endogenous bacterial floraand therefore its growth is limited.

Part 5: Experience Based on Common Use in Food

The basis for the GRAS conclusion for *Leu. carnosum* DSM 32756 is based on scientific procedures and not common use in food before 1958.

Part 6: Narrative

In the following sections, the data and information providing the basis for Chr. Hansen's determination that *Leu. carnosum* DSM 32756 is GRAS, through scientific procedures, under the conditions of its intended use is presented. The information provided below, and elsewhere in this document that is generally available has been properly cited. Chr. Hansen has rigorously applied the decision tree recommended by Pariza *et al.* for the determination of the safety of the food culture preparation of *Leu. carnosum* DSM 32756. Additionally, Chr. Hansen conducted a thorough search of the scientific literature relating to the safety of these species.

6.1 Natural occurrence of *Leu. carnosum*

LAB, such as *Leu. carnosum* are part of the natural microflora of meat. In fresh meat, LAB will go through a mild fermentation process that usually does not cause changes to the sensorial characteristics due to the low carbohydrate content and strong buffering capacity of meat (Hugas M. , 1998). In products such as vacuum-packed meat products, LAB become the dominant microflora during storage (Budde, Hornbeak, Jacobsen, Barkholt, & Koch, 2003).

Leuconostoc has been isolated from meat products in many studies (Hastings, Stiles, & von Holy, 1994) (Vermeiren, Devlieghere, & Debevere, 2004) (Dušková, M., Kameník, J., Lačanin, I., Šedo, O., & Zdráhal, Z., 2016) (Geeraerts W., Pothakos V., De Vuyst L., & Leroy F., 2017). *Leu. carnosum* is consumed as part of the endogenous flora of cooked meat products. In a study by Geeraerts et al. (2017), a total of 702 bacterial isolates were obtained from sliced cooked pork products in Belgium. Of those isolates, the most dominant genus was *Leuconostoc*. Within the subset of *Leuconostoc*, 68% of the isolates were *Leu. carnosum*. A similar study found that *Leu. carnosum* was the dominant species on cooked ham products (Vasilopoulos, et al., 2008).

6.2 *S. Leu. carnosum* in meat and fermented sausages

Simonová *et. al* stated that "The most frequently used starter cultures in meat products are lactic acid bacteria in combination with coagulase-negative staphylococci, such as *Staphylococcus xylosus* and *carnosus"* (2006). Using either natural fermentation or starter cultures, the fermentation of sausages is typically dominated by LAB and CNS (Janssens, Myter, De Vuyst, & Leroy, 2012).

Leuconostocs play a role in several food fermentations. These foods include fermented sausages, fermented vegetables, cereal products, and dairy products. *Leu. carnosum* is also present in vacuum-packed meat products (Ogier, Casalta, Farrokh, & Saihi, 2008). In some instances, the presence of *Leuconostocs* originate from the raw ingredients as in the case of sauerkraut, raw milk cheese, and some

fermented sausages. In other cases, *Leuconostocs* are added intentionally as starter cultures (Hemme & Foucaud-Scheunemann, 2004).

A review of the presence of *Leu. carno*sum in the literature is presented in Table 6.

Species	Origin	Role of organism	Reference
Leu. carnosum	Meat	Endogenous flora and starter culture	(Shaw & Harding, 1989)
Leu. carnosum	Meat	Endogenous flora	(Dainty & Mackey, 1992)
Leu. carnosum	Meat	Starter culture	(Hugas, Garriga, & Monfort, 1992)
Leu. carnosum	Meat	Endogenous flora, starter culture, bioprotectant	(van Laack, Schillinger, & Holzapfel, 1992)
Leu. carnosum	Meat	Endogenous flora, bioprotectant	(Keppler, Geisen, & Holzapfel, 1994)
Leu. carnosum	Meat	Endogenous flora, bioprotectant	(Parente, Moles, & Ricciardi, 1996)
Leu. carnosum	Meat	Endogenous flora	(Parente, Grieco, & Crudele, Phenotypic diversity of lactic acid bacteria isolated from fermented sausages produced in Basilicata (Southern Italy), 2001)
Leu. carnosum	Meat	Bioprotectant culture	(Jacobsen, Budde, & Koch, 2003)
Leu. carnosum	Meat	Endogenous flora	(Baka, Noriega, Mertens, Van Derlinden, & Van Impe, 2014)

Table 6: Selected literature references of Leu. carnosum in food

6.3 Recognition of Safety by an Authoritative Group of Qualified Experts

Leu. carnosum are on the current IDF "Inventory of Microorganisms with a Documented History of Use in Food" (Bourdichon F. , et al., 2012).

In 2002, the European Food and Feed Cultures Association (EFFCA) and the IDF published a non-exhaustive inventory of microorganisms (82 bacterial species) that are traditionally used in food. Updated in 2018, the inventory now covers a wider range of food matrices and includes starter cultures and natural flora (195 bacterial species). This inventory of species was originally published in 2002 "as a result of a joint project between the IDF and EFFCA" and focused mainly on dairy cultures (Bourdichon F. , et al., 2012) (Bourdichon, et al., 495/2018). Later, the inventory was expanded to include species with a history of use in other applications, such as meat, vegetables, cereals, and vinegar. The inclusion of *Leu carnosum* on the updated list for meat application is supported by a search of the scientific literature.

As is mentioned in the Pariza *et al.* publication (2015), experts have asserted that "microorganisms listed on the IDF and EFFCA inventories meet the criteria for GRAS for their traditional uses." *Leu. carnosum* is traditionally used in fermented products and is found as part of the endogenous flora of many ready-to-eat products. It is not novel to think of this strain as ingredient added to bacon.

6.4 Leu. carnosum DSM 32756 is non-pathogenic and non-toxigenic

As discussed in section 2.3, LAB including *Leu. carnosum* species have long been ingested in the human diet. Although rare, there have been a few cases of LAB being implicated in human disease (Stiles & Holzapfel, 1997).

A review of *Leuconostocs* by Handwerger *et al*. (1990) concluded that some of the bacteria should be viewed as "potential pathogens," although the majority of clinical cases were in immunocompromised patients with underlying disease and may have been treated with antibiotics. Hemme & Foucaud-Scheunemann (2004) concluded in their paper that "Considering the wide distribution of *Leuconostoc* in the environment, the large numbers that are ingested daily in the vast range of fermented foods such as dairy products and the relatively few infections they cause, these bacteria have very little, if any, virulence for healthy human beings." In fact, there have been no human infections related to ingesting food products containing *Leuconostoc.* Similarly, Ogier *et. al* (2008) conducted a review of infections in humans due to *Leuconostocs.* They confirmed that some strains belonging to the *Leuconostoc* genus are regarded as "opportunistic pathogens of susceptible health-compromised individuals" although, again, none of the cases described were linked to consumption of the organism in food. Garcia-Granja, Lopez, Ladron, & San Roman (2018) describes three cases of infective endocarditis, one with *Leu.* m*esenteroides*, one with a vancomycin sensitive *Leuconostoc* spp., and one with a vancomycin resistant *Leuconostoc* spp. Immunosuppression, intravascular catheter or previous vancomycin use are risk factors for *Leuconostoc* infection and *Leu. mesenteroides* is the most common cause (Ishiyama, Yamazaki, Nakao, Senda, & Yamauchi, 2011).

6.4.1 Search against virulence factor database and antibiotic gene database

Leu. carnosum DSM 32756 belongs to a species classified as biosafety level class 1 according to the German and US classification systems. *Leu. carnosum* DSM 32756 also has a long history of safe use in fermented meat. To further confirm the safe use of the strain, an in-silico genome screening for potential virulence factors and other genes related to pathogenicity or toxigenicity was performed. Moreover, phenotypic tests for cytotoxicity and hemolysis were done. Overall, the in-silico genome screening for potential virulence factors and other genes related to pathogenicity, virulence or toxicity did not reveal any virulence, toxicity genes or other genes of safety concern. This was further supported by the strain being non-hemolytic and not causing cytotoxic activity in the vero cell assay.

Leu. carnosum DSM 32756 did not contain any virulence factors and were non-hemolytic and non-cytotoxic. In addition, the *in-silico* genome screening for potential antibiotic resistance genes did not reveal any acquired antibiotic resistance genes of safety concern. The absence of both virulence factors and antibiotic resistant genes further concludes the safety of this strain.

6.4.2 biogenic amines

Leu. carnosum DSM 32756 did not produce any of the four BA compounds tested when grown in presence of specific amino acid precursors known to induce production (see Table 2). These results support our conclusion that this strain is safe and suitable for use in food.

6.4.3 susceptible to all antimicrobial agents tested

In a study conducted to test 23 *Leuconostoc* strains isolated from kimchi, “all strains were susceptible to ampicillin, chloramphenicol, erythromycin, and tetracycline (Jeong & Lee, 2015). *Leu. carnosum* DSM 32756 is sensitive to the antibiotics tested with MIC values that are at or below the EFSA 2018 epidemiological cut-off values for *Leuconostoc.* The resistance to vancomycin is an intrinsic property of *Leuconostoc* due to the composition of the cell wall (Handwerger, Pucci, Volk, Liu, & Lee, 1994). Vancomycin resistance cannot be transferred to other organisms.

6.5 Decision Tree Analysis

***Leu. carnosum* DSM 32756**

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology?

   YES (go to 2)

2. Has the strain's genome been sequenced?

   YES (go to 3)

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity?

   YES (go to 4)

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA?

   YES (go to 5)

5. Does the strain produce antimicrobial substances?

   NO (go to 6)

   Note: The text of Pariza's decision tree defines antimicrobial substances as substances that might be useful in human or veterinary medicine. *Leu carnosum* does produce bacteriocins which are naturally occurring antimicrobial substances, but they do not have use in human or veterinary medicine.

6. Has the strain been genetically modified using rDNA techniques?

   NO (go to 8a)

8a. Was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component?

   YES (go to 9a)

9a. Has the species, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for food use by an authoritative group of qualified scientific experts?

   YES (go to 10a)

10a. Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9a continue to support the conclusion that the species, to which the strain belongs, is safe for use in food?

   YES (go to 11a)

11a. Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in "traditional" food(s) in which they typically found?

NO (go to 12a)

12a. Will the intended use of the strain expand intake of the species (for example, increasing the number of foods beyond the traditional foods in which the species typically found, or using the strain as a probiotic rather than as a fermented food starter culture, which may significantly increase the single dose and/or chronic exposure)?

NO (go to 14a)

14a. The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.

6.6 Conclusion of GRAS Status

Chr. Hansen has concluded, through scientific procedures, that *Leu. carnosum* DSM 32756 is GRAS for its intended use in bacon at a use level that will result in a final concentration up to and including 9.0 log cfu/g of the finished food product. This conclusion is based on published, peer-reviewed literature reviews as well as the framework set forth by Pariza *et al*. (2015) which includes strain-specific parameters. Although this notice is not based on history of use before 1958, we have also included information pertaining to the historical use of these cultures in food fermentation. The data presented above supports our conclusion.

Part 7: List of Supporting Data and Information

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