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

April 20, 2020

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OFFICE OF FOOD ADDITIVE SAFETY

Reference: *Staphylococcus carnosus* DSM 25010

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 *Staphylococcus carnosus* (*S. carnosus*) DSM 25010 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 improving color (red) stability. 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 (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 *S. carnosus* DSM 25010 as a safe and suitable ingredient in cured meat products including but not limited to cured ham and 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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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
CPS: Coagulase positive Staphylococci
EFFCA: European Food and Feed Cultures Association
EFSA: European Food Safety Authority
EUCAST: European Committee on Antimicrobial Susceptibility Testing
FDA: Food and Drug Administration
FSIS: Food Safety and Inspection Service
FSSC: Food safety system certification
GMO: Genetically Modified OrganismGMP: Good Manufacturing Practices
GRAS: Generally recognized as safe
IDF: International Dairy Federation
ISO: International Organization for Standardization
LAB: Lactic Acid Bacteria
MFS: Major Facilitator Superfamily
MIC: Minimum Inhibitory Concentration
NCBI: National Center for Biotechnology Information
NR: non-redundant
SE: Staphylococcal enterotoxins
S. (carnosus): *Staphylococcus*
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 (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 *Staphylococcus carnosus* DSM 25010 (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) 607-5959

1.3 Common or Usual Name

Food culture, bacterial culture, *Staphylococcus carnosus, S. carnosus*

(S. carnosus DSM 25010 is currently sold under the tradename SafePro®).

1.4 Conditions of Use

S. carnosus DSM 25010 is intended for use to enhance the quality of packed bacon throughout shelf-life by improving color (red) stability. *S. carnosus* DSM 25010 is applied by diluting in water and injecting or spraying on the food surfaces 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. 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 *S. carnosus* DSM 25010 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 *S. carnosus* DSM 25010 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 *S. carnosus* DSM 25010 is GRAS, is available for review and copying by 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 *S. carnosus* DSM 25010.

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 the USDA-FSIS, regarding the use of *S. carnosus* DSM 25010 as a safe and suitable food ingredient used in the production of 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 *S. carnosus* DSM 25010 currently sold commercially as a component of a blend under the name SafePro® B-SF-77.

2.2 Source of the GRAS Organisms

S. carnosus DSM 25010 originates from strains isolated by the German meat culture producer Rudolf Müller & Co, which was acquired by Chr. Hansen in the early nineteen nineties. The *S. carnosus* 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 25010 on July 12, 2011.

2.3 Description of the GRAS Organisms

S. carnosus DSM 25010 is of the genus *Staphylococcus*. *Staphylococcus* are Gram-positive spherical bacteria belonging to the *Firmicutes* phylum, class *Bacilli*, order *Bacillates*, family *Staphyloccoccaceae*. They are mostly facultative, anaerobic, catalase positive bacteria that display a high tolerance to salt (Even, et al., 2010). Staphylococci are ubiquitous bacteria found on the skin and mucous membranes of warm-blooded animals and humans. They can also be isolated from environmental sources such as soil, air and water and from a wide range of foodstuffs including fermented meat and cheese (Irlinger, 2008). The genus *Staphylococcus* currently includes 52 species, which can be divided into coagulase positive (CPS) and coagulase negative (CNS) staphylococci (Muller, et al., 2016). *S. carnosus* is considered a "non-pathogenic representative of the coagulase negative staphylococci" (Buckle, Kranz, Schmidt, & Weiss, 2017). The species *S. carnosus* form together with *S. piscifarmentans* and *S. condiment*, a phylogenic subgroup considered non-pathogenic within the CNS. This group is phylogenetically different from other CNS that are considered opportunistic pathogens such as *S. epidermidis*.

S. carnosus DSM 25010 was identified by Chr. Hansen as is discussed in Appendix 1.

S. carnosus DSM 25010, the topic of this notification, used individually or blended, such as in Chr. Hansen's SafePro® B-SF-77, is safe and suitable for human consumption.

2.3.1 Genotypic characteristics

The genome sequence of *S. carnosus* DSM 25010 obtained in-house at Chr. Hansen was used for the genome safety assessment (Chr. Hansen, 2018). For the assessment, the genome sequence of *S. carnosus* strain DSM 25010 was subjected to annotation using published methods. The DSM 25010 genome size was 2.54 Mb and it contained 2,553 coding sequences (CDS) and 45 RNAs, which was comparable to *S. carnosus* genomes in the National Center for Biotechnology Information (NCBI) genome database (2.57-2.67 Mb in size and 2504-2635 CDSs). Moreover, no 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 genome of the strain was screened against a curated published database of antibiotic resistance genes.

The genome screening of *S. carnosus* DSM 25010 resulted in one hit (73% identity, 1% gaps and 93% coverage) to *norA,* a gene encoding a multi-efflux pump which mediates fluoroquinolone resistance and is naturally occurring in the chromosome of *S. aureus* strains. The gene had 100% homology and coverage to a gene annotated in the two *S. carnosus* genomes in the (NCBI non-redundant (NR) database as multi-efflux major facilitator superfamily (MFS) transporter. DSM 25010 was susceptible to all antibiotics tested including the fluoroquinolone ciprofloxacin. Therefore, the multi-efflux MFS transporter found in DSM 25010 and in the *S. carnosus* chromosomes in the NCBI NR database is probably a naturally occurring transporter protein present in the chromosome, which is of no safety concern regarding acquired antimicrobial resistance.

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 *S. carnosus* DSM 25010 was screened for virulence and toxicity genes as recommended by the European Food Safety Authority (EFSA) (EFSA FEEDAP, 2018).

The annotated draft genome of *S. carnosus* DSM 25010 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. One gene was annotated as a fibronectin/fibrinogen-binding protein and was found in the two *S. carnosus* genomes (100% coverage and 99% identity) present in the NCBI NR database as well as in the related species *S.condimenti* (100% coverage and 96% identity).

A fibronectin/fibrinogen-binding protein is involved in adhesion to extracellular matrix or to host cell surfaces and is not itself a virulence factor. Another gene was annotated as a bi-functional autolysin with low identity to *ami* autolysin amidase, an adhesin from *Listeria monocytogenes* (20% coverage, 51% identity and 1% gaps). The gene may be an adhesin involved in the cell's ability to adhere. The gene was found in the two *S. carnosus* in the NCBI NR database with 100% coverage and 99-100% identity as well as in the related species *S. condimenti* (91% coverage and 91% identity).

In *S. carnosus* DSM 25010, the ability to adhere could be regarded as an efficacy feature rather than a safety issue. In addition to *in-silico* genome screening, phenotypic tests for cytotoxicity and hemolysis were also performed. Results of these phenotypic tests showed that *S. carnosus* DSM 25010 did not cause cytotoxic activity in a Vero cell assay and the strain is non-hemolytic.

In conclusion, the *in-silico* genome screening for potential virulence factors and other genes related to pathogenicity, virulence or toxicity in *S. carnosus* DSM 25010 did not reveal any virulence or toxicity genes or other genes of safety concern.

2.3.2 Phenotypic Characteristics

Table 1 shows the physiological data for *S. carnosus* DSM 25010. The following paragraphs in this section describe other phenotypic characteristics related to the safety of the strain which is the topic of this notification. Additional information on the commercial product SafePro B-SF-77 can be found in Appendix 2.

Physiological data

Culture Composition	***Staphylococcus carnosus***
Growth temperature Opt/max/min	30°C/44°C/10°C (86°F/111°F/50°F)
Salt limit	20% salt-in water
Characteristics	Facultative anaerobic Catalase positive Nitrate reductase positive Lipolytic Proteolytic
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* S. carnosus *DSM 25010

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 sample. 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 the Scientific Panel on Biological Hazards from the European Food Safety Authority (EFSA) ((BIOHAZ), 2018) to be the BA of most concern related to food safety (Pariza, Gillies, Kraak-Ripple, & Leyer, 2015).

For *S. carnosus* DSM 25010, biogenic amines were tested based on a validated in-house method modified from similar methods in the literature. The induction step was performed by growing the strain aerobically at 30 °C in casein-peptone soymeal-peptone (CASO) broth in the presence if the corresponding amino acids. The CASO was supplemented with the amino acids 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. (Cid, Miguelez-Arrizado, Becker, Holzapfel, & Vidal-carou, 2008).

The presence of the four 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 *S. carnosus* DSM 25010 tested negative for the four BA of concern.

BA production of the DSM 25010 is shown in the table below.

Biogenic amine compound	Monoamines		Polyamines	
	Histamine	Tyramine	Cadaverine	Putrescine
DSM 25010	Not produced	Not produced	Not produced	Not produced

Table 2: Results of BA production for* S. carnosus *DSM 25010 [1]

[1] The threshold for reporting is set to 5 mg/l corresponding to approximately 0.04 mM for the monoamines and approximately 0.05 mM for the polyamines. The two positive control stains produced histamine and tyramine, cadaverine and putrescine as expected.

2.3.4 Antibiotic Resistance

To measure antimicrobial susceptibility of *S. carnosus* DSM 25010, the minimum inhibitory concentration (MIC) was determined using the standardized methods recommended by Clinical and Laboratory Standards Institute (CLSI) for *Staphylococcus* sp. The strain was tested for nine antibiotics (ampicillin, vancomycin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol) as recommended by EFSA as well as for ciprofloxacin. The result was interpreted using epidemiological cut-off values also recommended by EFSA (EFSA, 2018) and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) cut-off values for *S. aureus*. The results showed that
S. carnosus DSM 25010 was susceptible to all tested antimicrobial agents (Table 3).

Antibiotic type	Antibiotic	MIC in µg/mL	EFSA cut-off values in µg/mL[2]
Aminoglycoside	Gentamicin	0.5	4 (2)
	Kanamycin	2	16 (8)
	Streptomycin	1	8 (16)
Tetracycline	Tetracycline	0.025	2 (2)
Macrolide	Erythromycin	0.12	0.5 (1)
Lincosamide	Clindamycin	0.12	0.25 (0.25)
Chloramphenicol	Chloramphenicol	4	4 (16)
B-lactam	Ampicillin	0.06	1 (ND)
Glycopeptide	Vancomycin	0.5	2 (2)
Fluoroquinolone	Ciprofloxacin	1	ND (1)

Table 3: MIC values for* S. carnosus *DSM 25010; ND: "not determined"

2.3.5 GM Status

S. carnosus DSM 25010 is not genetically modified by use of recombinant DNA techniques. The finished culture preparation(s) do not contain genetically modified organisms (GMOs) and do not contain genetically modified labeled raw materials (Appendix 3).

2.4 Method of Manufacture

S. carnosus DSM 25010 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)

[2] EFSA cut-off values for 'Corynebacterium and other Gram-positive bacteria' as listed in 'Guidance on the characterization of microorganisms used as feed additives or as production organisms' (EFSA Journal 2018,16:5206). In brackets the EUCAST cut-off values for S. aureus are listed (www.EUCAST.org, June 18th, 2018)

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 International Organization for Standardization (ISO) 22000.

S. carnosus DSM 25010 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. The culture is then 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 culture preparation may then be blended together with other culture preparations. Finally, the product is filled into aluminum foil bags and labeled (product name, item number, batch number, amount, storage temperature).

S. carnosus DSM 25010 is produced using standard fermentation techniques. This includes the use of fermentation and standardized 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

The preparation of *S. carnosus* DSM 25010 is currently blended with another bacterial preparation (*Leu. carnosum* DSM 32756) and is sold commercially as SafePro® B-SF-77. The finished product is an off-white to brownish ground powder. It is recommended to be stored at < 1˚F and has a shelf-life of 18 months when stored under these conditions. The specifications for each batch are shown in Table 4 below. Methods used for shelf-life determination are available upon request.

Performance	Specification
Total cell count cfu/g	>5.2E+10
Purity	**Specification**
Bacillus cereus cfu/g	<100
Enterobacteriaceae cfu/g	<10
Enterococci cfu/g	<1000
S. aureus cfu/g	<50
Yeasts and moulds cfu/g	<100
Listeria monocytogenes *	Absent in 25 g
Salmonella spp. *	Absent in 25 g

* Environmental and statistically based product testing is carried out on an ongoing basis, details can be supplied on request.

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

2.6 Intended technical effect & amount required

Meat undergoes progressive deterioration from the time of slaughter until consumption due to its unique biological and chemical nature (Olaoye & Ntuen, 2011). *S. carnosus* DSM 25010 is intended to be applied to cured meat at a use-level up to and including 9.0 log CFU/g to enhance the quality of packed raw cured meats including but not limited to cured ham and bacon throughout shelf-life by improving color (red) stability.

Barrière *et al.* (2001) and Mainar *et al.* (2017) suggested that meat-associated CNS can neutralize pro-oxidant molecules, limiting the oxidative processes based on their superoxide dismutase and catalase activities and thus contribute to color stability of meat products. CNS have been implicated in stabilizing the color of meat in many studies (Leroy, Talon, & Vermassen, 2016); (Johansson, Berdagué, Larsson, Tran, & Borch, 1994); (Janssens, Myter, De Vuyst, & Leroy, 2012); (Stavropoulou, De Vuyst, & Leroy, 2018). Martin *et al.* (2006) cited nitrite and nitrate reductase activity promoting the desired red color in fermented sausages, as one of the most important technical functions of Gram-positive catalase-positive cocci including CNS. Nitrosative effects emerge from the nitrate and nitrite reductase activities in most CNS species, being particularly pronounced within *S. carnosus* and *S. xylosus*. This partially explains the conventional use of the latter two CNS species as starter cultures for meat fermentation where they generate the bright red color of nitroso myoglobin (Stavropoulou, De Vuyst, & Leroy, 2018).

2.6.1 Experimental Studies on Color Stability

(Summary of "SafePro® B-SF-77 Bacon color stability study")

Freshly sliced bacon was portioned into 2 batches. One was used as a control, one was sprayed on both sides with *S. carnosus* DSM 25010 and *Leu. carnosum* DSM 32756 solution. Initial inoculum level was 7.4 log CFU/g for CNS and 7.9 log CFU/g for *Leuconostoc spp.* Both batches were then vacuum packed and stored at 5±1°C continually under the light (fluorescent lighting to produce 70-80 foot-candles) to mimic storage at retail. During the shelf life, red color intensity (a*) was measured on 3 replicates of each batch using Minolta CM-3700d Spectrophotometer. Figure 1 shows clearly that when SafePro® B-SF-77 is applied then the red intensity of the bacon is significantly higher compared with the control.

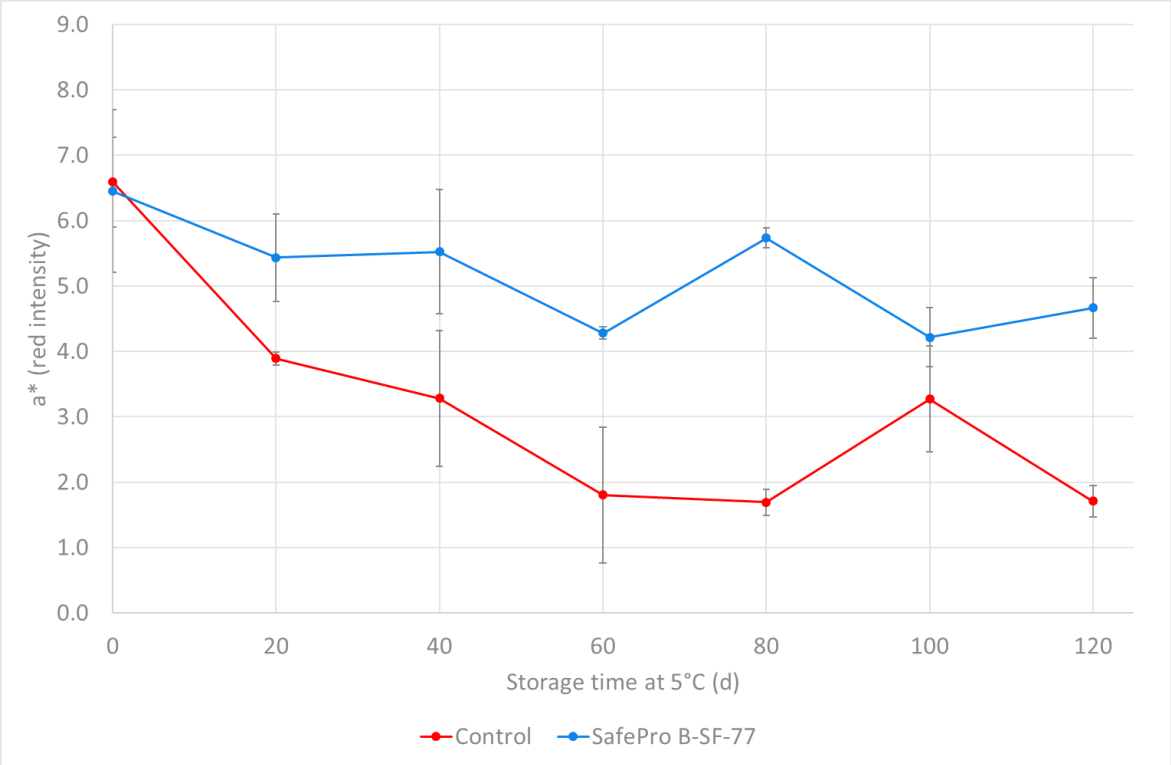

Figure 1: Influence of SafePro® B-SF-77 on the evolution of the red intensity (a*) of vacuum-packed bacon

2.6.2 Application and Production Safety

The food culture SafePro® B-SF-77 containing *S. carnosus* DSM 25010 may be applied using one of two methods. The first method, which was used in the experiment 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. 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. In addition, the use of *S. carnosus* DSM 25010 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 *S. carnosus* DSM 25010, if any, will be present in the meat after cooking since *S. 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, 2013). Therefore, the consumption of cooked bacon containing *S. carnosus* DSM 25010 would not increase the dietary intake of this microorganism.

The inoculation rate targeted for *S. carnosus* DSM 25010. At the end of the shelf-life, *S. carnosus* DSM 25010 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", that all cured meat consumed contains *S. carnosus* DSM 25010 cultures at a level of 9.0 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 *S. carnosus* DSM 25010. It is unlikely that all cured meat consumed in a day would be inoculated with *S. carnosus* DSM 25010 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 *S. carnosus* DSM 25010 would not cause an increase in the gut. As bacteriocins are commonly produced by many lactic acid bacteria (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. Due to the fact that 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 *S. carnosus* DSM 25010 is as a food ingredient added at manufacturing to enhance the quality of packed bacon throughout shelf-life by improving color (red) stability. The self-limiting levels of use are:

- Current GMP – Following the use level prescribed by Chr. Hansen, *S. carnosus* DSM 25010 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 – *S. carnosus* DSM 25010, when added to the food, is in competition for space and nutrients with endogenous flora and therefore its growth is limited.

Part 5: Experience Based on Common Use in Food

The basis for the GRAS conclusion for *S. carnosus* DSM 25010 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 *S. carnosus* DSM 25010 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 *S. carnosus* DSM 25010. Additionally, Chr. Hansen conducted a thorough search of the scientific literature relating to the safety of these species.

6.1 Natural occurrence of *S. carnosus*

Staphylococci are ubiquitous bacteria found on the skin and mucous membranes of warm-blooded animals and humans. They can also be isolated from environmental sources such as soil, air and water and from a wide range of foodstuffs including fermented meat and cheese (Irlinger, 2008). The ubiquity of staphylococci might be explained by their ability to adapt to different environments and their ability to form biofilms (Dordet-Frisoni, Dorchies, De Araujo, Talon, & Leroy, 2007). Commonly CNS such as *S. carnosus* are found in foods of animal origin. Specifically, they are abundant in fermented foods such as cheese and dry fermented sausage where they can be present at levels exceeding 6 log CFU/g (Leroy, Talon, & Vermassen, 2016).

6.2 *S. carnosus* 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).

S. carnosus strains are sold commercially as starter cultures for fermented sausages and have been used traditionally for meat fermentations due to their contribution of flavor and aroma formation (Even, et al., 2010) (Janssens, Myter, De Vuyst, & Leroy, 2012).

A review of the presence of *S. carnosus* in the literature is presented in Table 5.

Species	Origin	Role of organism	Reference
S. carnosus	Meat	Starter culture	(Nychas & Arkoudelos, 1990)
S. carnosus	Meat	Starter culture	(Marchesini, Bruttin, Romailler, & Moreton, 1992)
S. carnosus	Meat	Starter culture	(Hammes & Knauf, 1994)
S. carnosus	Meat	Starter culture	(Lucke, 1994)
S. carnosus	Meat	Starter culture	(Cocolin, Manzano, Cantoni, & Comi, 2001)
S. carnosus	Meat	Endogenous flora	(Papamanoli, Kotzekidou, Tzanetakis, & Litopoulou-Tzanetaki, 2002)
S. carnosus	Meat	Endogenous flora	(Blaiotta, et al., 2004)
S. carnosus	Meat	Endogenous flora and starter culture	(Even, et al., 2010)

Table 5: Selected literature references of S. carnosus in food

6.3 Recognition of Safety by an Authoritative Group of Qualified Experts

S. carnosus is on the current International Dairy Federation (IDF) list "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 2012 and 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 the two species 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". As *S. carnosus* is traditionally used in fermented products and found as part of the endogenous flora of many ready-to-eat products, it is not novel to think of this strain as an ingredient added to bacon.

6.4 *S. carnosus* DSM 25010 is non-pathogenic and non-toxigenic

CNS, such as *S. carnosus*, are mostly considered beneficial and are not associated with pathogenic risk from consumption. In fact, of all *Staphylococcus* species, only the CPS *S. aureus* is considered a foodborne pathogen (Leroy, Talon, & Vermassen, 2016). However, within CNS, beneficial opportunistically pathogenic and pathogenic members can be found. *S. carnosus* form together with *S. piscifarmentans* and *S. condimenti* a phylogenic subgroup within the CNS considered non-pathogenic. Although there have been a few cases reported of catheter-related bacteremia and soft tissue infections with *S. condimenti*, to our knowledge no reports on infections with *S. carnosus* exists (Misawa, Yoshida, Okugawa, & Moriya, 2014); (Gabrielsen, Kols, Oye, Bergh, & Afset, 2017). Most isolates of staphylococci are considered as Class II biohazards by the American Type Culture Collection (ATCC), while the remaining species are not known to cause any disease in humans and are listed as Class I organisms (Gillaspy & Iandolo, 2014). CNS species are often considered valuable for the production of fermented foods from animal origin.

Staphylococcal enterotoxins (SE) are another topic to be addressed when determining the safety of CNS strains. For species commonly associated with (fermented) foods, SE determinants are usually absent or rare, as has been demonstrated for strains of *S. carnosus*, *S. equorum*, *S. saprophyticus* and *S. xylosus* (Even, et al., 2010); (Jeong & Lee, 2015); (Møller C.O.A., et al., 2013).

6.4.1 Search against virulence factor database and antibiotic gene database

S. carnosus DSM 25010 belongs to a species classified as biosafety level class 1 according to the German and US classification system. DSM 25010 also has a long history of safe use. 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.

S. carnosus DSM 25010 did not contain any virulence factors and is 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 these strains.

6.4.2 biogenic amines

S. carnosus DSM 25010 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 these strains are safe and suitable for use in food.

6.4.3 susceptible to all antimicrobial agents tested

S. carnosus DSM 25010 strain is sensitive to all antibiotics tested with MIC values that are less than or at EFSA 2018 cut-off values for '*Corynebacterium* and other Gram-positive bacteria' and less than the EUCAST cut-off values for *S. aureus*.

6.5 Decision Tree Analysis

***S. carnosus* DSM 25010**

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)

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 *S. carnosus* DSM 25010 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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