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Improving food & health

GRN 000760

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
Dr. Rachel Morissette
5100 Campus Drive, College Park, MD 20740

January 31, 2018

Reference: Chr. Hansen GRAS Notification for
Lactobacillus curvatus DSM 18775

Dear Dr. Rachel Morissette:

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, the organism *Lactobacillus curvatus* DSM 18775, is generally recognized as safe and is not subject to the pre-market approval requirements for the use in ready-to-eat cooked meat and poultry products including but not limited to cooked ham, beef, turkey, chicken, emulsified lunch meats, and emulsified sausages to help suppress the growth of *Listeria monocytogenes*.

We also request that a copy of the notification be shared with the United States Department of Agriculture's Food Safety and Inspection Service, regarding the use of *Lactobacillus curvatus* DSM 18775 as a safe and suitable ingredient in ready-to-eat cooked meat and poultry products.

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

Yours sincerely,

(b) (6)

Emily Gregoire
Regulatory Affairs Specialist

(b) (6)

Katharine Urbain
Regional Regulatory Affairs Manager North America

CHR. HANSEN, INC.

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ABBREVIATIONS

EFFCA: European Food and Feed Cultures Association
EFSA: European Food Safety Authority
FDA: Food and Drug Administration
USDA: United States Department of Agriculture
FSIS: Food Safety and Inspection Service
GRAS: Generally Recognized As Safe
IDF: International Dairy Federation
LAB: Lactic Acid Bacteria
L. curvatus: *Lactobacillus curvatus*
L. monocytogenes: *Listeria monocytogenes*
MIC: Minimum Inhibitory Concentration
BA: Biogenic Amine
QPS: Qualified Presumption of Safety

Part 1: Signed Statements and Certification

1.1 Statement of Intent

In accordance with the 21 CFR 170 Subpart E, regulations for Generally Recognized as Safe (GRAS) notifications, Chr. Hansen, Inc. is pleased to submit a notice that we have concluded, through scientific procedures, that *Lactobacillus curvatus* DSM 18775 is GRAS and is not subject to the premarket approval requirements for use in ready-to-eat cooked meat and poultry products including but not limited to cooked ham, beef, turkey, chicken, emulsified lunch meats, and emulsified sausages 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 / Lactic acid bacteria (LAB) / *Lactobacillus curvatus* DSM 18775

1.4 Conditions of Use

L. curvatus DSM 18775 is intended for use as an additional hurdle to assist in suppressing the growth of *Listeria monocytogenes*. *L. curvatus* DSM 18775 is applied by diluting in water and spraying on the food surfaces at a use level that will result in a final concentration between 6.4 and 7.4 log cfu/g of the finished food product. The applications covered in this GRAS notice are ready-to-eat cooked meat and poultry products including but not limited to cooked ham, beef, turkey, chicken, emulsified lunch meats, and emulsified sausages.

1.5 Basis for GRAS Determination

Pursuant to the GRAS rule [81 Fed. Reg. 159 (17 August 2016)], Chr. Hansen has concluded that *Lactobacillus curvatus* DSM 18775 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 *L. curvatus* DSM 18775 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 *L. curvatus* DSM 18775 is GRAS are available for review and copying by FDA during customary business hours, at the location below, or will be sent to FDA upon request, made to:

Chr. Hansen, Inc.
Emily Gregoire
Regulatory Affairs Specialist
9015 W Maple St., Milwaukee, WI 53214
usemgr@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 the use of *L. curvatus* DSM 18775.

1.10 Signature

(b) (6)

January 31, 2018

_____ _____

Katharine Urbain, Regional Regulatory Affairs Manager North America — Date

(b) (6)

January 31, 2018

_____ _____

Emily Gregoire, Regulatory Affairs Specialist — Date

1.11 FSIS Authorization

We also request that a copy of the notification be shared with the United States Department of Agriculture's Food Safety and Inspection Service (FSIS), regarding the use of *L. curvatus* DSM 18775 as a safe and suitable food ingredient used in the production of ready-to-eat cooked meat and poultry products including but not limited to cooked ham, beef, turkey, chicken, emulsified lunch meats, and emulsified sausages.

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. The sections in Part 2 satisfy much of those criteria. The criteria will be further discussed and met in sections 3 and 6.

2.1 Name of the GRAS Organism

The subject of this GRAS determination is a strain of the bacterial species *L. curvatus* designated as DSM 18775.

2.2 Source of the GRAS Organism

L. curvatus DSM 18775 was isolated from a Danish fermented sourdough in 1993. The *L. curvatus* strain was deposited in the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) under the accession number DSM 18775 on November 9, 2006.

2.3 Description of the GRAS Organism

L. curvatus is part of the broader category of LAB. This classification of bacteria, when supplied with suitable carbohydrates, produces lactic acid as their main fermentation product (Teuber, 2008). *Lactobacillus* are Gram positive, catalase negative, non-spore-forming, rod shaped bacteria producing lactic acid. They have complex nutritional requirements (De Angelis & Gobbetti, 2016) and are facultative anaerobes (Batt, 2014). *Lactobacillus* are native to many different habitats such as plants, silage, meat, milk, and soil. They can also be found in abundance in the human oral cavity, intestinal tract, and vagina (Stiles & Holzapfel, 1997).

Lactobacillus is a non-pathogenic genus that consists of over a hundred species (Bernardeau, Vernoux, Henri-Dubernet, & Gueguen, 2008). A report by EFSA in November 2007 (2007) identified 112 species, while Bernardeau *et al*. (2008), writing the following year, suggested that the genus contains some 135 species and 27 subspecies. *Lactobacillus* can be categorized into three sup-groups based on phenotypic characteristics. *L. curvatus* is a Group 2 facultative heterofermenter. *L. curvatus* is a species known to be important to the microflora of refrigerated packaged meats. Along with L. *sakei*, *L. curvatus* typically dominates the microbial population of these products (Stiles & Holzapfel, 1997). In a study conducted by Duskova, M. *et al*., *L. curvatus* was found in all samples of cooked ham after one week storage at 2° C and up to 4 weeks thereafter at the same storage temperature. The final LAB count which included only three species (*L. curvatus*, *L. sakei*, and *Weisella viridescens*) was 8.4 log cfu/g (Dušková, M., Kameník, J., Lačanin, I., Šedo, O., & Zdráhal, Z., 2016).

The *Lactobacillus* spp. are classified as risk group 1 (Public Health Agency of Canada, 2011), with no specific special hazards identified. The genus *Lactobacillus* is rarely pathogenic and found "widely distributed in the environment, especially in animal and vegetable food products" (Bergey & Holt, 1994).

Many examples of these products will be given in section 6.1 of this dossier. *L. curvatus* has been listed on the EFSA Qualified Presumption of Safety (QPS) list since 2007. *L. curvatus* is also on the International Dairy Federation (IDF) list of safe food cultures with a documented history of use in food (Mogensen, et al., 2002).

2.3.1 Phenotypic Characteristics

L. curvatus DSM 18775 has been characterized using comparative small subunit (SSU) rRNA gene (also called 16S rDNA) sequence analysis (Appendix 1: DSM 18775 IDENTIFICATION CERTIFICATE). 16S rDNA sequences are used for species identification as they occur in all prokaryotic genomes and contain both variable and conserved sequence stretches. The principle is that ribosomal RNA genes of a bacterial isolate are amplified by the polymerase chain reaction (PCR) with oligonucleotide primers directed against conserved regions of the rRNA genes. The sequence of the obtained PCR-product is determined by the dideoxy-chain-terminating method (Sanger, Nicklen, & Coulson, 1977). The resulting rRNA gene sequence is compared to rRNA gene sequences from type strains. Comparative sequence analysis of 16S rDNA is established as a powerful standard method for the identification of microorganisms (Ludwig & Klenk, 2005). However it is also well known that closely related species can share identical 16S rDNA sequences. In these cases comparative sequence analysis of housekeeping genes has proven valuable for further differentiation. In the genus *Lactobacillus* the suitability of rpoA and pheS gene sequences for reliable species identification of species undistinguishable by 16S rDNA sequence analysis has been demonstrated by Naser *et al.* (Naser, et al., 2007). The strain has therefore been species identified by use of 16S rDNA sequencing and partial sequencing of *pheS* (Appendix 1).

Enumeration of *L. curvatus* DSM 18775 is performed following ISO 15214 standards (de Man, Rogosa and Sharpe (MRS) agar) under anaerobic conditions. Plates are inoculated and then incubated for 72 hours at 30°C (86°F).

L. curvatus DSM 18775 is a facultative anaerobe, able to ferment several carbohydrates such as glucose (dextrose), fructose, maltose and saccharose (sucrose) as determined by use of Api50 CHL method (Appendix 2: *L. CURVATUS* DSM18775 PASSPORT).

Fermentable Sugars	
Glucose (dextrose)	+
Fructose	+
Maltose	+
Lactose	-
Saccharose (sucrose)	+
Starch	-

TABLE 1: ABILITY OF L. CURVATUS DSM 18775 TO USE SUGARS AS A CARBON SOURCE (API50 CHL METHOD) (CHR. HANSEN)

2.3.2 Biogenic Amines

For testing of biogenic amine activity no standardized method exists, but several methods have been published in the scientific literature. The most crucial steps are the induction of biogenic amine production and the biochemical analysis of the compounds in the induced samples. The occurrence of biogenic amines is attributed to the decarboxylase activity in certain bacteria and the biogenic amines 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), Scientific Opinion on risk based control of biogenic amine formation in fermented foods, 2011) to be the biogenic amines of most concern related to food safety (Pariza, Gillies, Kraak-Ripple, & Leyer, 2015). For the present strain the test was done based on a validated in-house method modified from similar methods in the literature. The induction step was performed by growing the strain in MRS broth anaerobically at 30 °C in the presence of the corresponding amino acids (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 GS-MS method modified from Smart *et al*. (2010). In both steps positive and negative controls were included. The results showed that *L. curvatus* DSM 18775 tested negative for the four biogenic amines of concern (Appendix 3: DSM18775 BIOGENIC AMINE STATEMENT).

2.3.3 Antibiotic Resistance

In order to measure antimicrobial susceptibility of the strain, the minimal inhibitory concentration (MIC) was determined using the standardized methods recommended by ISO 10932 | IDF 223 international standard (Appendix 4: DSM18775 ANTIBIOTIC SUSCEPTIBILITY STATEMENT). The strain was tested for nine antibiotics (ampicillin, vancomycin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol) as recommended by EFSA, and the result was interpreted using epidemiological cut-off values also recommended by EFSA (EFSA, 2012)The results showed that *L. curvatus* DSM 18775 was susceptible to all tested antimicrobial agents except vancomycin for which the *L. curvatus* species is intrinsically resistant (Appendix 4). *L. curvatus* cannot transfer this vancomycin characteristic to other microorganisms; including pathogenic species (Danielsen & Wind, 2003). Therefore antibiotic resistance in not considered a safety concern.

2.3.4 GM Status

L. curvatus DSM 18775 is not genetically modified by use of recombinant DNA techniques (Appendix 5: PRODUCT INFORMATION SHEET 690607).

2.4 Method of Manufacture

Viable *L. curvatus* DSM 18775 is manufactured globally following Chr. Hansen's global protocol for production of meat cultures. Currently it is being manufactured by Chr. Hansen GmbH Giessener Str. 94, Pohlheim, Germany as well as Chr. Hansen, Inc., 9015 W Maple St., Milwaukee, WI USA in accordance with current good manufacturing practices consistent with 21 CFR Parts 110 and 117. Both plants comply with a set of basic GMP-rules, also called Pre-Requisite Program (PRP) according to Chr. Hansen's Quality, GMPs and Food Safety Principles, which are available from our website: www.chr-hansen.com. In addition, each plant has an appointed local OPRP (Operational Pre-Requisite Program) that includes PRP issues and CCPs (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: FSSC 22000 and ISO 22000 (Appendix 6: FSSC 22000 CERTIFICATE POHLHEIM and Appendix 7: ISO 22000 CERTIFICATE POHLHEIM). The Milwaukee plant maintains the following certifications: FSSC 22000 (Appendix 8: FSSC 22000 CERTIFICATE MILWAUKEE).

Chr. Hansen's *L. curvatus* DSM 18775 product is sold both as frozen pellets as well as freeze dried powder. Both *L. curvatus* DSM 18775 products are produced by inoculating the microorganism into the

sterilized growth substrate. Anaerobic conditions are maintained during the 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 culture is 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. Finally, the product is filled into aluminum foil bags and labeled (product name, item number, batch number, amount, storage temperature). The process flow for both the frozen and freeze dried products, including critical control points, is shown in Appendix 9: HACCP FLOW SHEET GLOBAL - MEAT CULTURES.

2.4.1 Raw Materials and Processing Aids

L. curvatus DSM 18775 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 this application in addition to meeting the specifications of the Food Chemical Codex.

2.5 Specifications

L. curvatus DSM 18775 frozen pellets are off-white to brownish in color. The freeze-dried product is an off-white to brownish ground powder. The suspension is water soluble and has a pH between 5 and 7. A single culture containing *L. curvatus* DSM 18775 has a total cell count higher than 3.2E+10 cfu/g (10.5 log cfu/g) for freeze-dried product, and 4.0E+10 cfu/g (10.6 log cfu/g) for frozen product. Purity is controlled as described in Table 2 and additionally in Appendix 10: 10 PRODUCT SPECIFICATION SHEET 690607 and Appendix 11: PRODUCT SPECIFICATION SHEET 713185. A complete list of analyses along with methods used can be found in Appendix 12: LIST OF ANALYSIS – MEAT CULTURES. In addition to the analyses mentioned, all intermediate batches of products are analyzed for sulfite-reducing *Clostridia*. Absence of chemical contamination of the batches is also controlled for heavy metals, arsenic and melamine (Table 3).

Microorganisms	Criteria	Frequency of analysis
Purity:		
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 2: MICROBIOLOGICAL CRITERIA AND FREQUENCY OF ANALYSES OF FREEZE-DRIED AND FROZEN MEAT CULTURES

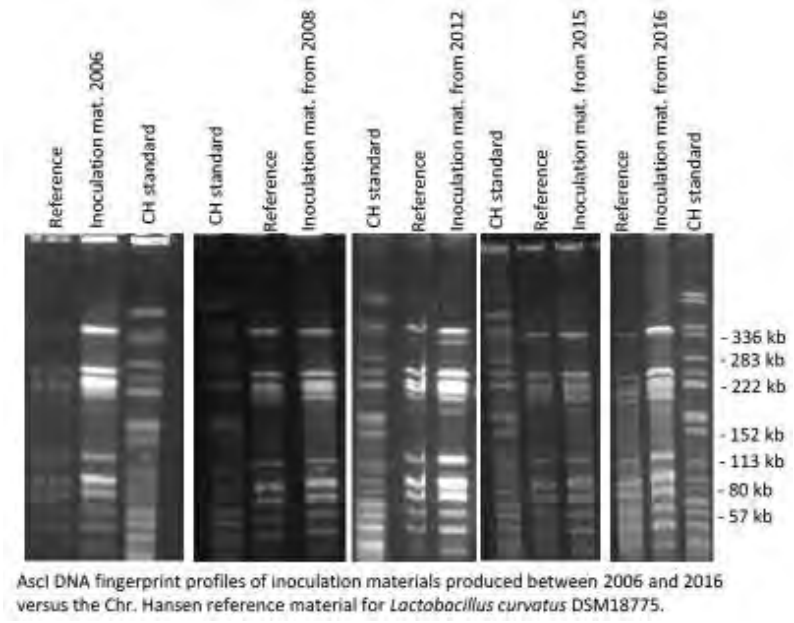
Contaminants tested	Results of our latest screening of culture as ingredient	Maximum contribution from culture in a meat application**	EU limits (except arsenic) as stated in the Commission Regulation (EC) No. 1881/2006 for final food products
Arsenic (As)	<0.1 mg/kg	<0.00002 mg/kg	<3.0 mg/kg*
Lead (Pb)	<0.05 mg/kg	<0.00001 mg/kg	0.1 mg/kg
Cadmium (Cd)	<0.01 mg/kg	<0.000002 mg/kg	<0.05 mg/kg
Mercury (Hg)	<0.005 mg/kg	<0.000001 mg/kg	-
Melamine	<0.5 mg/kg	<0.0001 mg/kg	2.5 mg/kg

TABLE 3: CRITERIA REGARDING THE PRESENCE OF CHEMICAL CONTAMINANTS IN FREEZE-DRIED MEAT CULTURES

**ARSENIC IS NOT REGULATED FOR CULTURES, BUT CHR. HANSEN USES THE LIMIT VALID FOR ENZYMES, REGULATED BY FRENCH LEGISLATION: ARRETE DU 19 OCTOBRE 2006 RELATIF A L'EMPLOI D'AUXILLIARIES TECHNOLOGIQUES DANS LA FABRICATION DE CERTAINS DENREES ALIMENTAIRS ARTICLE ANNEXE II*
***BASED ON RESULTS, AND WITH AN INOCULATION RATE OF 0.02%*

L. curvatus DSM 18775 freeze-dried products have a shelf life of 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 13: GENERAL HANDLING OF FREEZE-DRIED CULTURES). They are transported at either ambient or refrigerated temperatures. When sold as frozen pellets, shelf life is 12 months stored < -45°C (<49°F) in dry conditions. Frozen pellets at transported in dry ice (Appendix 14: PRODUCT INFORMATION SHEET 713185).

Genetic stability is monitored during storage and production for all pre-inoculation material (PIM) and direct inoculation material (DIM) via DNA fingerprinting and plasmid profiling. The results are compared with the reference material for *L. curvatus* DSM 18775 as seen below. Chr. Hansen's reference stock material and inoculation materials produced since 2006 show identical fingerprints and plasmid profiles.



AscI DNA fingerprint profiles of inoculation materials produced between 2006 and 2016 versus the Chr. Hansen reference material for *Lactobacillus curvatus* DSM18775.

FIGURE 1 GENETIC STABILITY DURING STORAGE AND PRODUCTION

2.6 Intended technical effect & amount required

Food-borne pathogenic and spoilage bacteria can be aerobic, anaerobic or facultative anaerobic, and thus, the elimination of oxygen alone from a food package or from a food storage environment will not effectively eliminate all types of undesired bacteria. Moreover, control of the temperature in the storage of food is not totally effective to preclude the growth of such bacteria because several types of pathogenic and spoilage bacteria are able to grow at various low temperatures. On the other hand, there are pathogenic bacteria, which, due to their tolerance to refrigerated temperatures, relatively high concentrations of NaCl and anaerobic conditions, are of particular concern in ready-to-eat food products (WO 2008/113781, 2008). Thus the microbial stability and safety of the food is based on a combination of several hurdle factors that unwanted organisms, such as *L. monocytogenes*, are unable to overcome.

L. curvatus DSM 18775 is intended to be sprayed onto the surface of ready-to-eat cooked meat and poultry products. The addition of *L. curvatus* DSM 18775 to the surface of ready-to-eat cooked meat and poultry products is an additional hurdle that assists in limiting the growth of *L. monocytogenes* at the manufacturing step as well as throughout shelf-life of the product. The optimal effective use level is 6.4 and 7.4 log cfu/g at time of inoculation. Chr. Hansen has validated this use level through challenge studies conducted both internally and by third party laboratories (Appendices 15, 16, 17). These challenge studies were conducted following parameters set forth by EFSA (EFSA, 2014) as well as the National Advisory Committee on Microbiological Criteria for Foods (NACMCF Executive Secretariat, 2010). In these studies, the effect of *L. curvatus* DSM 18775 was tested using products that are representative of products typically sold in the US. It is important to note that the use of *L. curvatus* DSM 18775 is meant for cooked, ready-to-eat products that typically contain added nitrites or other “curing” ingredients that naturally contain nitrites or nitrates (such as celery juice) (Sullivan, 2013). As such, the products used in these challenge studies all contain added nitrates at levels less than

120 ppm, which is below the limits set by USDA (156 ppm for frankfurters; 200 ppm for brine cured or injected products) (Sullivan, 2013). The studies confirmed that *L. monocytogenes* growth is suppressed when the product is inoculated with *L. curvatus* DSM 18775. The studies also show that the final concentration of LAB at the end of shelf-life in the inoculated samples is comparable to that in the control.

2.6.1 Hurdle Technology

Inoculating cooked meat and/or poultry products with *L. curvatus* DSM 18775 creates additional hurdles against *L. monocytogenes* growth. These hurdles include bacitracin production and competition for space and substrate (the Jameson effect). More detail on these hurdle technologies will be discussed in the following paragraphs.

L. curvatus may produce various bacteriocins such as curvacin, sakacin or lactocin, or no bacteriocin. Bacteriocins are bioactive bacterial peptides or protein molecules ribosomally synthetized by various bacteria during their growth, and displaying antimicrobial activity against closely related bacteria. Proteolytic enzymes, such as the proteases of the mammalian gastrointestinal tract, can easily degrade the bacteriocins, making them safe for human consumption (Zacharof & Lovitt, 2012). Various bacteriocins produced by *L. curvatus* strains have already been used in commercially available meat products by direct addition. Strains are either used as starter cultures in the case of fermented products, or as “protective cultures” on non-traditionally fermented products. These uses were documented in fermented sausage, raw beef, cooked meat, and salami (Woraprayote, Malila, Sorapukdee, Swetwiwathana, & Visessanguan, 2016). Use of nisin, a bacteriocin produced by *Lactococcus lactis* strains, was approved in food products by the FDA in 1998 (FDA Federal Register, 1998).

In addition to bacteriocin production, the competition between species for resources (substrate) and space plays a large role in suppressing the growth of *L. monocytogenes* in cooked meat and poultry products inoculated with *L. curvatus* DSM 18775. The dominating microbiota, when well adapted, inhibits the growth of other bacteria via physical interactions (Marshall, R.J. & McElhatton, A., 2007). Growth of the pathogen is thus stopped when the dominating microbiota reach their maximum population density (Møller C.O.A., Ilg Y., Aabo S., Christensen B.B., Dalgaard P., & Hansen T.B., 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”. Thus, according to Patent WO 2008/113781 (Appendix 18: PATENT SPECIFICATION), growth of *L. monocytogenes* is naturally stopped as soon as the LAB reach the stationary phase. When products are inoculated with a high density of *L. curvatus* DSM 18775 (around 6.4 log cfu/g), stationary phase is reached earlier. Growth of *L. monocytogenes* is thus naturally stopped, (i.e. stationary phase is reached) and at an earlier time and lower level than in products which are not inoculated (Figure 2). The Jameson Effect has also been mentioned in EFSA’s most recent report “Listeria monocytogenes contamination of ready-to-eat foods and the risk for human health in the EU” ((BIOHAZ), 2018). The report points to competitive microflora as an additional factor that can affect growth of L. monocytogenes thus confirming that “the growth of *L. monocytogenes* is known to be negatively affected by the competitive growth of lactic acid bacteria…” The report also points to several studies that have successfully applied the Jameson Effect model predict to *L. monocytogenes* growth

supression in applications including processed seafood, mayonnaise-based seafood salads, pork products, and cottage cheese ((BIOHAZ), 2018).

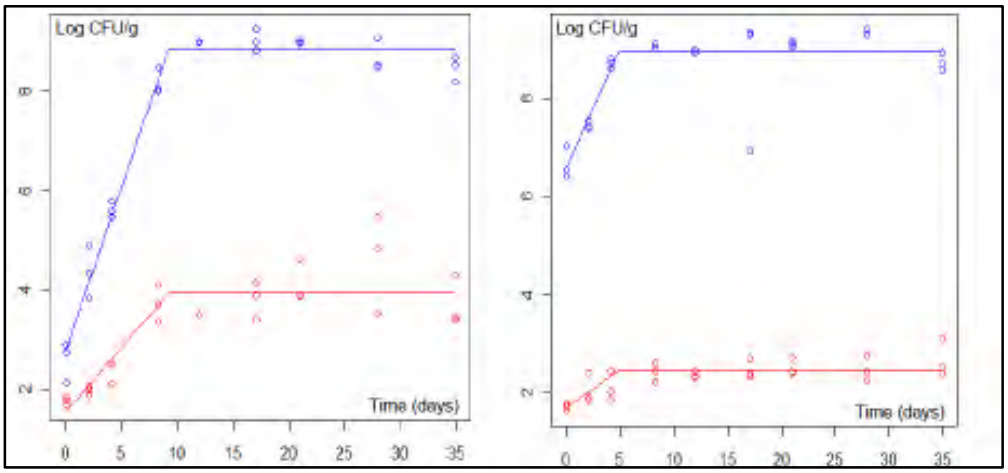


FIGURE 2: SIMULTANEOUS EVOLUTION OF OBSERVED AND SIMULATED CONCENTRATIONS IN LOG CFU/G OF LAB (BLUE) AND *L. MONOCYTOGENES* (RED) IN COOKED HAM SAMPLES WITHOUT PROTECTIVE FLORA (LEFT) AND WITH ADDITION OF *L. CURVATUS* DSM 18775 (RIGHT) STORED AT 7°C FOR 35 DAYS

2.6.2 Application and Plant Safety

L. curvatus DSM 18775 is intended to be sprayed onto the product in a closed system and/or clean room. Good industrial hygiene practices should be followed when handling and storing the product. This includes wearing gloves when handling frozen or freeze-dried product and using ventilation if dust or aerosols are present (Appendix 19: SAFETY DATA SHEET). There are no known hazards towards workers or inspection personnel. In addition, the use of *L. curvatus* DSM 18775, 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 used on meat and poultry (GRN No. 159, 2004).

Chr. Hansen suggests that *L. curvatus* DSM 18775 be labeled on the finished product as “food culture” or “lactic acid bacteria”.

Part 3: Dietary Exposure

Inoculation rate targeted for *L. curvatus* DSM 18775 is between 6.4 and 7.4 log cfu/g. At the end of the shelf-life, *L. curvatus* DSM 18775 count is not above 9.0 log cfu/g. This concentration is similar to the LAB count at the end of the storage of currently sold cooked meat and poultry products (count of the endogenous flora only) (Kotzekidou, P. & Bloukas, J.G., 1996). In addition, it is known that the adult microbiome is very stable and only shifts with significant dietary changes or extreme weight loss (Faith, Guruge, Charbonneau, Subramanian, Seedorf, & Goodman, 2013). The addition of *L. curvatus* DSM 18775 to cooked meat and poultry products would, therefore, not cause a significant increase in the gut. As bacteriocins are commonly produced by many lactic acid bacteria, 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. Because of the fact that the amount of LAB consumed using *L. curvatus* DSM 18775 as a *L. monocytogenes* inhibitory ingredient 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 *L. curvatus* DSM 18775 is as a food ingredient added at manufacturing to inhibit the growth of *L. monocytogenes* in cooked ready-to-eat meat and poultry products. The self-limiting levels of use are:

- cGMP – Following the use level prescribed by Chr. Hansen, *L. curvatus* DSM 18775 will only be added to the product 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.

- Competitive exclusion – *L. curvatus* DSM 18775, when added to the food, is in competition for space and nutrients with endogenous LAB and therefore its growth is limited.

Part 5: Experience Based on Common Use in Food

The basis for the GRAS conclusion for *L. curvatus* DSM 18775 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 our conclusion that *L. curvatus* DSM 18775 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.* as well as the risk assessment conducted by EFSA per the Qualified Presumption of Safety (QPS) approach for the determination of the safety of *L. curvatus* DSM 18775.

6.1 *L. curvatus* is part of the endogenous flora of cooked meat and poultry

Fermentation, one of the oldest food processing technologies, is a biochemical reaction which occurs under the action of microorganisms. Called starter cultures, these microbial cultures may be part of the endogenous flora of the food or may be intentionally added, in case of industrial food fermentation process (Herody, C., Soyeux, Y., Bech Hansen, E., & Gillies, K., 2010). The consumption of *Lactobacillus* has occurred since humans first began eating fermented foods, especially fermented milk products. They are abundant in in the human diet and colonize the gastro-intestinal tract soon after birth (Bernardeau, Vernoux, Henri-Dubernet, & Gueguen, 2008). *Lactobacillus* are also found naturally in a variety of habitats such as human and animal mucosal membranes, on plants and plant materials, and in man-made habitats such as sewage.

L. curvatus is part of the endogenous flora of cooked meat and poultry products, and is also one of the main strains used as a starter culture for the fermentation of sausages (Casaburi,A., Di Martino, V., Ferranti, P., Picariello, L., & Villani, F., 2016). LAB, especially *L. sakei* and *L. curvatus*, are also the main bacteria able to grow on vacuum-packed cooked ham during its storage at chilled temperature (28 days at 4°C), reaching a concentration above 8.0 log cfu/g (Kotzekidou, P. & Bloukas, J.G., 1996); generally between 7.9 and 8.9 log cfu/g (Dušková, M., Kameník, J., Lačanin, I., Šedo, O., & Zdráhal, Z., 2016). *L. curvatus* is widely occurring in a variety of food products that have been consumed for many years. Table 4 shows a summary of some of the literature referring to the naturally occurring presence of *L. curvatus* in meat and poultry products since 1987.

Product	Country	Origin of the samples	Publication
Raw meat			
Ground beef	US	Retail supermarkets	(Garver, K.I. & Muriana, P.M. , 1993)
Ground pork			
Vacuum-packaged beef	Germany	Unknown	(Schillinger, U. & Lücke, F.K., 1987)
Vacuum-packaged pork			
Fermented sausages			
Fermented sausages	Germany	unknown	(Schillinger, U. & Lücke, F.K., 1987)
Naturally fermented dry salami	Greece	Local meat manufacturer	(Samelis, J., Maurogenakis, F., & Metaxopoulos, J., 1994)
Salami	Italy	Unknown	(Böhme, H.M., Mellett, F.D., Dick L.M.T., & Basson, D.S., 1996)
Chorizo (*L. curvatus* is one of the most dominant species present during the ripening)	Spain	Industrial plants	(Santos, E.M., González-Fernández, C., Jaime, I., & Rovira, J., 1998)
Artisanal dry sausages	Argentina	Local meat manufacturer	(Rivas, F.P., Castro, M.P., Vallejo, M., Marguet, E., & Campos, C.A., 2014)
Traditional fermented sausages (starter culture)	Italy	Unknown	(Casaburi,A., Di Martino, V., Ferranti, P., Picariello, L., & Villani, F., 2016)
Cooked products			
Mortadella	Greece	Industrial plant	(Samelis, J., Kakouri, A., & Rementzis, J., 2000))
Pariza (emulsion sausage)			
Sliced pork ham *L. curvatus* dominates the endogenous flora characterized on sell-by day	Belgium	Unknown	(Audenaert K., D'Haene K., Messens K., Ruyssen T., Vandamme P., & Huys G., 2010)
Sliced turkey *L. curvatus* dominates the endogenous flora characterized on sell-by day			
Sliced chicken breast *L. curvatus* dominates the endogenous flora characterized on sell-by day			
Sliced cooked ham	Belgium	Supermarkets	(Geeraerts W., Pothakos V., De Vuyst L., & Leroy F., 2017)
Sliced cooked ham	France	Industrial plants	(Chr. Hansen, Unimore, Unito, & Fisabio, 2017).
Sliced cooked ham	Macedonia	Industrial plants	

TABLE 4: SUMMARY OF LITERATURE REFERRING TO THE PRESENCE OF L. CURVATUS IN COOKED MEAT AND POULTRY PRODUCTS

L. curvatus has been on the QPS (Qualified presumption of safety) list since 2007 and is also on the IDF (International Dairy Federation) list of microorganisms traditionally used in foods (Bourdichon, et al., 2011). Through the framework laid out in the Pariza *et al.* decision tree (Pariza, Gillies, Kraak-Ripple, & Leyer, 2015), as well as the general recognition of safety of the species in public literature, it has been concluded that *L. curvatus* DSM 18775 is safe and suitable for human consumption under the intended uses described in this GRAS notification.

6.2 Recognition of Safety by an Authoritative Group of Qualified Experts

The QPS approach was developed by the Scientific Committee of EFSA in 2007 to provide a generic concept to prioritize and to harmonize risk assessment of microorganisms to be added to foods. The list

of QPS recommended biological agents is updated annually, with the latest version being released in March 2017. QPS status is given if the taxonomic group does not raise safety concerns or, if safety concerns exist, can be defined and excluded. *L. curvatus* was placed on the initial list in 2007, and has remained valid up to and including the latest 2017 list (EFSA, 2017).

Taxonomic units (TUs) (usually species for bacteria and yeasts, families for viruses) were included in the QPS list either following notifications to EFSA or proposals made by stakeholders during a public consultation in 2005, even if they were not yet notified to EFSA. Since then and currently, the QPS assessment is only triggered when a microorganism is notified to EFSA through an application for market authorization of regulated products (such as feed additives, food enzymes, novel foods and plant protection products). Absence of specific characteristics such as transmissible antibiotic resistance, food poisoning toxins, surfactant activity and enterotoxic activity are required for the QPS status. The inclusion of the species on this list provides a strong foundation of support that any strains of *L. curvatus* are safe for human consumption.

In 2002, the European Food and Feed Cultures Association (EFFCA) and IDF published a non-exhaustive inventory of microorganisms (82 bacterial species) that are traditionally used in food. Updated in 2012, the inventory now covers a wider range of food matrices and includes starter cultures but also natural flora (195 bacterial species). *L. curvatus* was included in the 2002 list with a documented usage since the 1990's for meat fermentation. Because of this history of use in meat, the culture can be legally sold in Europe as a meat culture.

As is mentioned in the Pariza *et al.* publication (2015), experts have asserted that "microorganisms listed on the IDF and EFFCA/QPS inventories meet the criteria for GRAS for their traditional uses". As *L. curvatus* traditionally is used in fermented meat products and found as part of the endogenous flora of cooked meat products, it is not novel to think of it as an ingredient added to non-fermented meat (and poultry). Using the decision tree proposed by Pariza *et al*., it can be concluded that *L. curvatus* DSM 18775 is safe for use in the applications presented in this GRAS notice.

6.3 *L. curvatus* DSM 18775 is non-pathogenic and non-toxigenic

Infection due to the genus *Lactobacillus* is extremely rare, even despite their very widespread presence in food. Salminen *et al*. reviewed 89 cases of patients with *Lactobacillus* bacteremia in which they stated that bacteremia caused by *Lactobacillus* is rare. The majority of cases involved patients who had rapidly fatal major underlying diseases. The majority of patients had also undergone a surgical intervention.
L. curvatus was not the source of any of the bacteremia cases, further indicating the safety of the species and its subsequent strains (Salminen, et al., 2004). Bernerdeau et al. (2008) suggested that *Lactobacillus* poses no hazards and that instances of lactobacillemia is extremely rare, occurring only in predisposed patients.

6.3.1 *L. curvatus* DSM 18775 does not produce biogenic amines

As mentioned in section 2.3.2, biogenic amines in foods are known to be toxic and can cause an allergic reaction, especially in susceptible individuals. The aromatic amines histamine and tyramine are considered the most toxic and relevant to food safety, and fermented foods are of particular biogenic amine concern due to associated intensive microbial activity and potential for biogenic amine formation

(EFSA, 2011). The biogenic amines diamine, cadaverine, and putrescine are not as toxic, but they may enhance the toxicity of the aromatic amines by blocking their metabolism or increasing their absorption (Cid, Miguelez-Arrizado, Becker, Holzapfel, & Vidal-carou, 2008). Cid *et al.* (2008), as well as Pariza *et al*. (2015) in the decision tree for determining the safety of microorganisms for food stated that the potential for biogenic amine production must be taken into consideration in the selection and implementation of starter and protective cultures to reduce hygienic and toxicological risks. *L. curvatus* DSM 18775 was tested for all mentioned biogenic amines of which none were detected (see section 2.3.2). Therefore *L. curvatus* DSM 18775 meets the safety criteria for absence of biogenic amine production.

6.3.2 *L. curvatus* DSM 18775 is susceptible to all antimicrobial agents tested

It is important to verify that culture strains used as starter or protective cultures do not present transferable antimicrobial resistance. This is also criterion number 4 of the Pariza *et al*. decision tree (Pariza, Gillies, Kraak-Ripple, & Leyer, 2015). Minimum inhibitory concentrations (MIC) of nine antibiotics were determined for *L. curvatus* DSM18775 according to the ISO 10932/IDF 223 international standards. *L. curvatus* DSM 18775 was found to be susceptible to the antibiotics with the exception of vancomycin. This resistance to vancomycin is intrinsic to many *Lactobacillus* species, including *L. curvatus* (Billot-Klein, Gutmann, Sable, Guittet, & van Heijenoort, 1994) and thereby non-transferable to other bacteria. This further shows the safety of *L. curvatus* DSM 18775 for use in food.

6.5 Conclusion of GRAS Status

In summary, Chr. Hansen has applied the framework of the Pariza *et al.* decision tree, and the data presented in this document fully supports the conclusion that *Lactobacillus curvatus* DSM 18775 is GRAS for the intended use described. The basis for this conclusion can be summed up in five main points. First, the publicly available scientific literature documents the use of this microorganism in fermented food, as well as part of the endogenous flora of food matrices in which Chr. Hansen intends it to be used in. Second, *L. curvatus* DSM 18775 in not genetically modified, is not able to produce biogenic amines, and does not carry any transferrable gene coding for antibiotic resistance. Third, Chr. Hansen's manufacturing and quality control programs ensure the safety and quality of the final *L. curvatus* DSM 18775 product. Fourth, the estimated daily intake of *L. curvatus* DSM 18775 does not increase the overall intake of lactic acid bacteria in the diet. And, fifth, *L. curvatus* has been evaluated and deemed safe and nonpathogenic by EFSA per the QPS approach and has been included in the IDF list since 2002. Based on this information, it is apparent that *L. curvatus* DSM 18775 is GRAS.

Part 7: List of Supporting Data and Information

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Identification Certificate

October 2017
Valid two years from date of issue

Lactobacillus curvatus DSM 18775

Scientific Ref.: *Lactobacillus curvatus* (Abo-Elnaga and Kandler 1965)

Method

Comparing sequence analysis of the strain's 16S rDNA and partial rpoA sequence to a database of 16S rDNA sequences of type strains resp. rpoA reference sequences.

Results

The 16S rDNA sequence (*E. coli* pos. 1-1542, 1570 basepairs) of strain DSM 18775 places it into the *Lactobacillus sakei* group of species (Appendix, table 1). Ambiguous sequence positions due to operon heterogeneity (E. coli pos. 114, 184, 211, 648, 1031, 1038 and 1108) were omitted from the calculation. Sequence comparison of the partial rpoA gene sequence (417 bp) of the DSM 18775 strain to an rpoA sequence database of strains of the *Lactobacillus sakei* group (Naser et al., 2007) places the DSM 18775 strain into the species *Lactobacillus curvatus* (Appendix, table 2).

Conclusion

The DSM 18775 strain was identified as *Lactobacillus curvatus*.

Identification Department
R&D Microbial Platform

Elke Brockmann (Dr. rer. nat.)
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Appendix

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Tables

Table 1: Results of comparing 16S rDNA sequence analysis. Since 16S rDNA sequence comparison does not resolve between the species *Lactobacilus curvatus* and *Lactobacillus graminis* only the three species with the highest values are shown.

closest relative (species)	acc No of closest relative	identity %
Lactobacillus curvatus	AM113777	99,6
Lactobacillus graminis	AM113778	99,6
Lactobacillus sakei subsp. sakei	AM113784	99,4

Table 2: Results of comparing partial rpoA sequence analysis (% identity values of DSM 18775 to a set of reference strain sequences (Naser et al., 2007)).

Lactobacillus curvatus	Lactobacillus sakei	Lactobacillus graminis
99,8-100	95,9-96,4	97,6

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Sequences

16S rRNA sequence of DSM 18775

TTAATCGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGCACTCTCGT
TAGATTGAAGAAGCTTGCTTCTGATTGATAACATTTGAGTGAGTGGCGGACGGGKGAGTAACACGTGGGTAACCTGC
CCTAAAGTGGGGGATAACATTTGGAAACAGATGCTAATACCGCATAAAACMTAGCACCGCATGGTGCAAGGTTGAAA
GATGGTTTCKGCTATCACTTTAGGATGGACCCGCGGTGCATTAGTTAGTTGGTGAGGTAAAGGCTCACCAAGACCGT
GATGCATAGCCGACCTGAGAGGGTAATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAAC
TCTGTTGTTGGAGAAGAACGTATTTGATAGTAACTGATCAGGTAGTGACGGTATCCAACCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGG
TTTCTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGRAACTTGAGTGCAGAAGA
GGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGG
TCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGA
TGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGA
CCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGC
GAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCTTYGGGGACRAAGTGACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCKCAACCCTTATTACTAGTT
GCCAGCATTTAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAATCATCA
TGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACCGCGAGGTTTAGCTAAT
CTCTTAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGAT
CAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAG
CCGGTGAGGTAACCTTCGGGAGCCAGCCGTCTAAGGTGGGACAGATGATTAGGGTGAAGTCGTAACAAGGTAGCCGT
AGGAGAACCTGCGGCTGGATCACCTCCTTT

Partial rpoA sequence of DSM 18775

AATTGATGGCGTCCTAGAAGACGTAACACAAATCATCTTGAATATTAAAAAATTAGCACTTAAATTGCATGTTGAAG
AAGACAAGACAATTGAAATCGATGTTAAGGGTCCGGCAACAGTTACTGCTGCTGATATCATTTCTGATGATGACGTT
GAAGTCTTAAATACTGATCAATATATTTGTACAGTAGCTGAAGGCGGCAATTTCCACGTGCGAATGACAGTTAAAAA
AGGCCGTGGTTATGTTGCTGCTGATCAAAACAAGTCAGACGATATGCCAATTGGTGTTTTGCCAATCGACTCAATTT
ATACCCCAATCAGTCGTGTCAACTATCAAGTAGAAAGTACACGTGTTGGTCGTCGTAACGATTTCGACAAGTTAACA
CTTGATGTTTGGACAAACGGTTCCATCAGTCC

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Strain Passport

Valid two years from date of issue

Lactobacillus curvatus DSM18775

Scientific ref.: *Lactobacillus curvatus* (Troili-Petersson 1903) Abo-Elnaga and Kandler 1965

Taxonomy	*Lactobacillus curvatus* (DSM18775). ABO-ELNAGA (I.G.) and KANDLER (O.): Zur Taxonomie der Gattung *Lactobacillus* Beijerinck. I. Das Subgenus *Streptobacterium* Orla-Jensen. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Abteilung II*, 1965, **119**, 1-36
DSMZ code	DSM18775 (deposit according to the Budapest Treaty)
Colony morphology	Form: Round with regular edge; Elevation: convex; Surface: Smooth, shiny; Consistency: soft; Appearance: white, non-transparent (on MRS, after 3 days at 30°C, anaerobic incubation)
Cell morphology	Bean-shaped rods with rounded ends, pairs or short chains, non-motile
Gram stain	Positive
Catalase reaction	Negative
Carbohydrate fermentation (API 50 CHL)	Please see Table 1
Configuration of lactic acid	DL
Source material	Sour dough from Denmark.
GMO status	No gene modifications have been performed for the strain.
Safety of strain	The species *Lactobacillus curvatus* has QPS (qualified presumption of safety) status by European Food Safety Authority (EFSA). Ref.: EFSA Journal 2017;15(3):4664
Plasmid status	Contains plasmids
Culture Collection	Chr. Hansen Culture Collection (CHCC) Bøge Allé 10-12, DK – 2970 Hørsholm, Denmark

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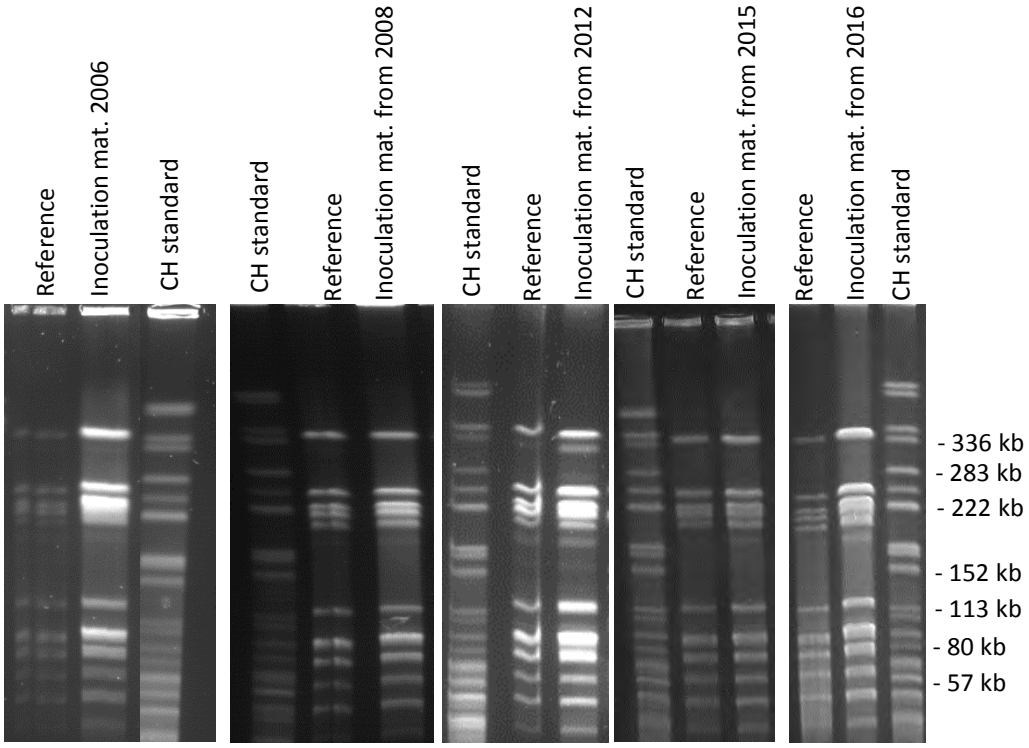
Cultivation	MRS, anaerobic incubation at 30°C
Long term storage in CHCC	MRS culture supplemented with glycerol to 15-20% stored at -80°C
Genetic stability during storage and production	DNA fingerprinting and plasmid profiling of Chr. Hansen's reference stock material and inoculation materials produced since 2006 show identical fingerprints and plasmid profiles. Please see pictures below.



AscI DNA fingerprint profiles of inoculation materials produced between 2006 and 2016 versus the Chr. Hansen reference material for *Lactobacillus curvatus* DSM18775.

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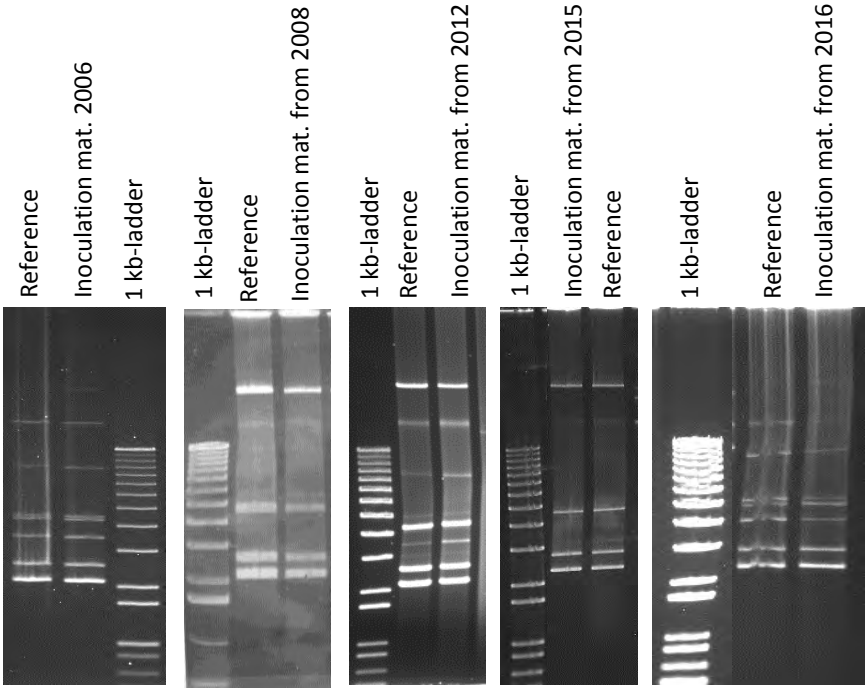

Native plasmid profiles of inoculation materials produced between 2006 and 2016 versus the Chr. Hansen reference material for *Lactobacillus curvatus* DSM18775. It is well-known that native plasmid profiles vary from preparation to preparation due to different amounts of supercoil forms of plasmids in the cultures used for plasmid DNA extraction.

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Table 1: Carbohydrate fermentation (API 50 CHL)

Control	-	Esculine	-
Glycerol	-	Salicine	-
Erythritol	-	Cellobiose	+/-
D-Arabinose	-	Maltose	+
L-Arabinose	-	Lactose	-
Ribose	+	Melibiose	-
D-Xylose	-	Saccharose	-
L-Xylose	-	Trehalose	+
Adonitol	-	Inuline	-
β-Methyl-xyloside	-	Melezitose	-
Galactose	+	D-Raffinose	-
D-Glucose	+	Amidon	-
D-Fructose	+	Glycogen	-
D-Mannose	+	Xylitol	-
L-Sorbose	-	β-Gentiobiose	-
Rhamnose	-	D-Turanose	-
Dulcitol	-	D-Lyxose	-
Inositol	-	D-Tagatose	-
Mannitol	-	D-Fucose	-
Sorbitol	-	L-Fucose	-
α-Methyl-D-mannoside	-	D-Arabitol	-
α-Methyl-D-glucoside	-	L-Arabitol	-
N-acetyl glucosamine	+	Gluconate	-
Amygdaline	-	2-keto-gluconate	-
Arbutine	-	5-keto-gluconate	-

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Hørsholm, November 2017

Identification department
R&D Microbial Platform

Anette Wind
Senior principal scientist, Strain documentation specialist

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Biogenic amine Statement

October 2017

Valid two years from date of issue

Lactobacillus curvatus DSM18775

Scientific ref.: *Lactobacillus curvatus* (Abo-Elnaga and Kandler 1965)

Method

The DSM 18775 strain was tested for biogenic amine production by use of an in-house standard operation procedure (SOP) modified based on scientific literature (e.g. Cid et al. 2008, Food Microbiology 25:269). In brief, the strain was grown in MRS broth supplemented 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 biogenic amine genes if present. The strain was incubated anaerobically at 30°C for 48 hours. A negative control (MRS supplemented broth) and two positive controls (strains known to produce tyramine and histamine, cadeverine and putrescine, respectively) were included.

Detection of histamine, tyramine, cadaverine and putrescine was done by use of a gas chromatography-mass spectrometry (GS-MS) based in-house SOP modified from Smart et al. 2010 (Nature Protocols 5:1709). The method was optimized and validated for both qualitative and quantitative detection of the four biogenic amines. Positive and negative controls as well as an internal standard were included.

Results

Biogenic amine production of the DSM 18775 is shown in the table below.

Biogenic amine compound	Histamine	Tyramine	Cadaverine	Putrescine
DSM 18775	Not Detected	Not Detected	Not Detected	Not Detected

Limit of detection (LOD) vary between 0.001-0.007 mM depending on the compound. Due to this variation in accuracy of the detection method LOD is set to >0.01mM for all four compounds. The two positive control stains produced histamine and tyramine, cadaverine and putrescine as expected. None of the four compounds were detected in the negative control (MRS supplemented broth).

Conclusion

The DSM18775 strain did not produce any of the four biogenic amine compounds tested when grown in presence of specific amino acid precursors known to induce production.

Identification Department
R&D Microbial Platform

Yvonne Agersø
Strain safety specialist

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Antibiotic Susceptibility Statement

September 2017
Valid two years from date of issue

Lactobacillus curvatus DSM 18775

Scientific ref.: *Lactobacillus curvatus* (Abo-Elnaga and Kandler 1965)

Method
Minimum inhibitory concentrations (MICs) of 9 antibiotics were determined for the DSM18775 strain according to the ISO 10932 | IDF 223 international standard. In brief, the susceptibility test performed is a broth microdilution method using VetMIC Lact-1 and Lact-2 panels (National Veterinary Institute of Sweden, Uppsala, Sweden) and growth in LSM medium (ISO-sensitest medium (Oxoid) supplemented with 10% MRS medium; Klare et al. 2005, Applied and Environmental Microbiology, 71:8982) for 48 hours at 30°C under anaerobic conditions with two biological replicates. The medium was controlled as recommended in the ISO 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 assessment of bacterial susceptibility to antimicrobials of human or veterinary importance' (EFSA Journal 2012,10:2740).

Results
MIC values for the DSM 18775 strain are shown in the table below.

Antibiotic type	Antibiotic	MIC in µg/ml	EFSA cut-off values in µg/ml[a]
Aminoglycoside	Gentamicin	2	16
	Kanamycin	8	64
	Streptomycin	16-32	64
Tetracycline	Tetracycline	2	8
Macrolide	Erythromycin	0.25	1
Lincosamide	Clindamycin	0.03	1
Chloramphenicol	Chloramphenicol	4	4
ß-lactam	Ampicillin	0.25-0.5	4
Glycopeptide	Vancomycin	32-64	n.r.

n.r.: not required to be tested by EFSA
a: EFSA cut-off values for *Lactobacillus* facultative heterofermentative *group* as listed in 'Guidance on the assessment of bacterial susceptibility to antimicrobials of human or veterinary importance', EFSA Journal 2012, 10:2740

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Antibiotic Susceptibility Statement

September 2017
Valid two years from date of issue

Conclusion
The DSM 18775 strain is sensitive to most of the antibiotics tested with MIC values that are less than or equal to EFSA 2012 cut-off values for *Lactobacillus* facultative heterofermentative group. The resistance to vancomycin is intrinsic to many *Lactobacillus* species, including *L. curvatus* (Billot-Klein et al. 1994, Journal of Bacteriology, 176:2398; Kirtzalidou et al. 2011, Anaerobe, 17:440; Solieri et al. 2014, Food Microbiology, 38:240).

Hørsholm, September 2017

Identification Department
R&D Microbial Platform

Yvonne Agersø (PhD)
Strain Safety Specialist

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SafePro® B-LC-48

Product Information

Version: 3 PI GLOB EN 10-05-2016

Range

The SafePro ® range covers a series of specially developed cultures for application in a variety of food products. These cultures help develop a positive eco-system that will improve food safety and/or potentially extend shelf life.

Description

SafePro® B-LC-48 is a single strain meat culture for cooked or cured meat products. The strain in the culture is able to suppress growth of spoilage and pathogenic bacteria such as indigenous lactic acid bacteria and *Listeria monocytogenes* . The strain in the culture grows within a wide temperature range down to 4°C (39°F) and survives freezing.

Culture composition:
Lactobacillus curvatus

Application

Usage
B-LC-48 is recommended for different types of Ready-To-Eat food products which are packed under vacuum or modified atmosphere and cold-stored.
The culture works particularly well in cooked and sliced meat products and Wiener sausages .

The culture does not ferment lactose and consequently, if lactose is used as filler, the acid formation will be limited. The producer obtains the advantages of maintaining both product safety and sensory quality during shelf life.

Dosage
25g for 200kg

Directions for use
Sliced cooked ham and emulsion sausages: The culture is applied by dipping, dripping or spraying a culture suspension onto the surface after cooking. Please read our suggested recipes for Cooked ham, Wiener sausages and Mortadella.

Fresh cured sausages and spreadable sausages: The culture is added directly to the chopper together with the dry ingredients but could also be applied onto the finished product as mentioned above. Please read our suggested recipes for Fresh Chorizo sausages and Spreadable sausages.

Raw cured meat products: The culture is added directly to the brine that is injected into the product but could also be applied onto the finished product as mentioned above. Please read our suggested recipe for Bacon.

Physical Properties

Color:	Off-white to brownish	Form:	Powder, ground
Solubility:	Water soluble suspension		

Packaging

Material No:	Size	Type
690607	50X25 g	Pouch(es) in box

Storage and handling

Temperature:	< -17 °C / < 1 °F
Conditions:	Dry

Transport condition
Shipment at ambient temperature.

Ingredients
Sucrose, Culture, Silicon dioxide E551

Shelf life
For freeze-dried cultures at least 18 months when stored according to recommendations.
When stored at +5°C/ 41°F the shelf life is max. 6 weeks.

Technical Data

Physiological data

Culture composition	*Lactobacillus curvatus*
Growth temperature	
Opt/max/min	37°C/40°C/4°C (98°F/104°F/39°F)
Salt limit	10% salt-in-water
Characteristics	Facultative anaerobic L(+)-lactic acid
Fermentable sugars	
Glucose (dextrose)	+
Fructose	+
Maltose	+
Lactose	-
Saccharose (sucrose)	+
Starch	-

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

Analytical methods
References and analytical methods are available on request.

Legislation
Chr. Hansen's cultures comply with the general requirements on food safety laid down in Regulation 178/2002/EC. Lactic acid bacteria are generally recognized as safe and can be used in food, however, for specific applications we recommend to consult national legislation.

The product is intended for food use.

Food Safety
No guarantee of food safety is implied or inferred should this product be used in applications other than those stated above. Should you wish to use this product in another application, please contact your Chr. Hansen representative for assistance.

Labeling
Suggested labeling “starter culture” or "culture", however as legislation may vary, please consult local legislation.

Trademarks

Product names, names of concepts, logos, brands and other trademarks referred to in this document, whether or not appearing in large print, bold or with the ® or TM symbol are the property of Chr. Hansen A/S or used under license. Trademarks appearing in this document may not be registered in your country, even if they are marked with an ®.

Dietary information

Kosher:	Kosher Meat Excl. Passover
Halal:	Certified

Technical support

Chr. Hansen's Application and Product Development Laboratories and personnel are available if you need further information.

GMO Information

In accordance with the legislation in the European Union* SafePro® B-LC-48 does not contain GMOs and does not contain GM labeled raw materials**. In accordance with European legislation on labeling of final food products** we can inform that the use of SafePro® B-LC-48 does not trigger a GM labeling of the final food product. Chr. Hansen's position on GMO can be found on: www.chr-hansen.com/About us/Policies and positions/Quality and product safety.

** Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms with later amendments, and repealing Council Directive 90/220/EEC.*
*** Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed with later amendments.*
Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labeling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms amending Directive 2001/18/EC, and with later amendments.

Allergen Information

List of common allergens in accordance with the US Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) and EU Regulation 1169/2011/EC with later amendments	Present as an ingredient in the product
Cereals containing gluten* and products thereof	No
Crustaceans and products thereof	No
Eggs and products thereof	No
Fish and products thereof	No
Peanuts and products thereof	No
Soybeans and products thereof	No
Milk and products thereof (including lactose)	No
Nuts* and products thereof	No
List of allergens in accordance with EU Regulation 1169/2011/EC only	
Celery and products thereof	No
Mustard and products thereof	No
Sesame seeds and products thereof	No
Lupine and products thereof	No
Mollusks and products thereof	No
Sulphur dioxide and sulphites (added) at concentrations of more than 10 mg/kg or 10 mg/litre expressed as SO_2	No

** Please consult the EU Regulation 1169/2011 Annex II for a legal definition of common allergens, see European Union law at: www.eur-lex.europa.eu*

BUREAU VERITAS
Certification

Certification

Awarded to

Chr.Hansen GmbH

Giessener Strasse 94, 35415 Pohlheim, Germany

Bureau Veritas Certification Holding SAS – UK Branch certify that the Management System of the above organisation has been audited and found to be in accordance with the requirements of the management system standards detailed below.

STANDARD

FSSC 22000
Certification scheme for food safety systems including ISO 22000:2005, ISO/TS 22002-1:2009 and additional FSSC 22000 requirements

SCOPE OF SUPPLY

Research, development, production, applied technology and shipment of bio technological, functional and natural ingredients and processing aids for the food industry.

Product category: L1 Bio Chemical Manufacturing

This certificate is provided on the base of the FSSC 22000 certification scheme, version 3 published 10 April, 2013.
The certification system consists of an annual audit of the food safety management systems and an annual verification of the PRP elements and additional requirements as included in the scheme and the ISO/TS 22002-1:2009.

Original approval date: 22-09-2006
Date of certification decision: 20-04-2015

Subject to the continued satisfactory operation of the organisation's Management System, this certificate is valid until: 23-04-2018

To check the validity of this certificate, please call: (+45) 77 311 000.
Further clarification regarding the scope of this certificate and the applicability of the system requirements may be obtained by consulting the organisation.

Certificate Number: DK005256-1 *Date:* 21-04-2015

(b) (6)

UKAS MANAGEMENT SYSTEMS
008

Signed on behalf of BVCH SAS UK Branch

Certification body address: 66 Prescot Street, London E1 8HG, United Kingdom
Certification office: Oldenborggade 1B, DK-7000 Fredericia, Denmark
Email: fooddkmail@dk.bureauveritas.com

Food Safety System Certification 22000

BUREAU VERITAS
Certification

Certification

Awarded to

Chr. Hansen GmbH

Giessener Strasse 94, 35415 Pohlheim, Germany

Bureau Veritas Certification Holding SAS – UK Branch certify that the Management System of the above organisation has been audited and found to be in accordance with the requirements of the management system standards detailed below.

STANDARD

ISO 22000:2005

SCOPE OF SUPPLY

Research, development, production, applied technology and shipment of biotechnological, functional and natural ingredients and processing aids for the food industry and animal nutrition.

Original approval date: 22-09-2006

Certification cycle start date: 20-04-2015

Subject to the continued satisfactory operation of the organisation's Management System, this certificate is valid until: 23-04-2018

To check the validity of this certificate, please call: (+45) 77 311 000.
Further clarification regarding the scope of this certificate and the applicability of the system requirements may be obtained by consulting the organisation.
Certificate Number: DK005256-1-1 *Date:* 20-04-2015

(b) (6)

Signed on behalf of BVCH SAS UK Branch

Certification body address: 66 Prescot Street, London E1 8HG, United Kingdom
Certification office: Oldenborggade 1B, DK-7000 Fredericia, Denmark
Email: fooddkmail@dk.bureauveritas.com

UKAS MANAGEMENT SYSTEMS
008

BUREAU VERITAS
Certification

Certification

Awarded to

Chr. Hansen Inc., USA

West Allis (WA) , 9015 West Maple Street, Milwaukee, WI 53214,USA

Bureau Veritas Certification Holding SAS – UK Branch certify that the Management System of the above organisation has been audited and found to be in accordance with the requirements of the management system standards detailed below.

STANDARD

FSSC 22000
Certification scheme for food safety systems including ISO 22000:2005, ISO/TS 22002-1:2009 and additional FSSC 22000 requirements

SCOPE OF SUPPLY

Manufacturing, development, blending, packaging, warehousing and shipment of food ingredients (Cultures and Colors).
Product category: L

This certificate is provided on the base of the FSSC 22000 certification scheme, version 3.2, published on 26 February 2015. The certification system consists of an annual audit of the food safety management systems and an annual verification of the PRP elements and additional requirements as included in the scheme and the ISO/TS 22002-1:2009.

Original approval date: 17-08-2010
Date of certification decision: 17-08-2016

Subject to the continued satisfactory operation of the organisation's Management System, this certificate is valid until: 16-08-2019

To check the validity of this certificate, please call: (+45) 77 311 000.
Further clarification regarding the scope of this certificate and the applicability of the system requirements may be obtained by consulting the organisation.

Certificate Number: DK006771-1 *Date:* 25-07-2016

UKAS MANAGEMENT SYSTEMS
008

(b) (6)

Certification body address: 66 Prescot Street, London E1 8HG, United Kingdom
Certification office: Oldenborggade 1B, DK-7000 Fredericia, Denmark
Email: fooddkmail@dk.bureauveritas.com

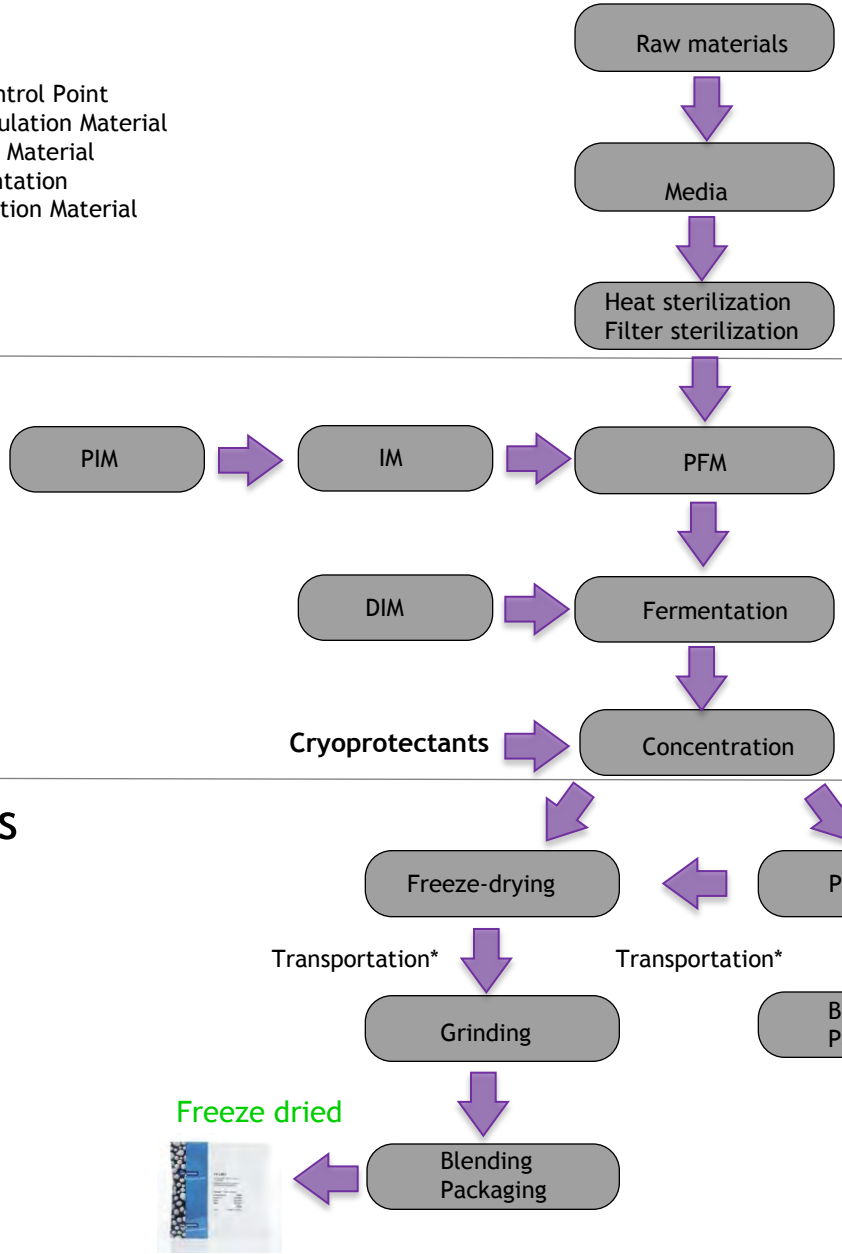
FSSC 22000

Production process flow – bacterial meat cultures

January 24, 2018
Valid two years from date of issue

Acronyms

CCP	Critical Control Point
DIM	Direct Inoculation Material
IM	Inoculation Material
PFM	Pre Fermentation
PIM	Pre Inoculation Material



CCPs/oPRPs

↓

Time & temperature/
Filter integrity

Heat treatment
of media for PIM, DIM,
IM and PFM

Integrity of glass
electrodes and filters

Heat treatment
of cryoprotectants

Integrity of
sieves

Integrity of sieves
Foreign body control (for
automatic filling line)

* Transportation may occur, internally or between plants

CHR. HANSEN

Production of bacterial meat cultures
Critical Control Points (CCP's)

January 24, 2018
Valid two years from date of issue

FSSC 22000 standard

- Each plant comply with a set of basic GMP-rules, also called Pre-Requisite Program (PRP) according to Chr. Hansen's Quality, GMPs and Food Safety Principles, which is available from our website: www.chr-hansen.com
- On top of that, each plant has an appointed local OPRP (Operational Pre-Requisite Program), that includes PRP issues, which need to be documented, and are classified as specifically critical for the food safety.
- The following CCP's (Critical Control Points) are global, and per site adopted to the local equipment and processes:
  - Glass electrodes in fermentors
  - Heat treatments (heat sterilization, heat-in-place)
  - Filters and sieves

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CHR. HANSEN

SafePro® B-LC-48

Product Specification

Form:

Material No: 690607

Culture Composition: Lactobacillus curvatus

Performance	Specification
Total cell count cfu/g	>3.2E+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

References and analytical methods are available upon request

Storage and shelf life: See labels and product packaging

Electronically generated - signature not required

BactoFlex® B-LC-48

Product Specification

Form:

Material No: 713185

Culture Composition: Lactobacillus curvatus

Performance	Specification
Total cell count cfu/g	>4.0E+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.

References and analytical methods are available upon request

Storage and shelf life: See labels and product packaging

Electronically generated - signature not required

Statement

January 24, 2018

Valid two years from date of issue

To whom it may concern

Analysis on Meat Cultures, Freeze-dried and Frozen.

We are pleased to inform you that our finished goods are subject to the following list of analysis:

Microbiology	Frequency	Method
Cell count of specified bacteria strain	every batch	§ 64 LFGB
Bacillus cereus	every batch	§ 64 LMBG
Enterobacteriaceae	every batch	AFNOR ISO 16140
Enterococci	every batch	NMKL No. 68/1992
Staphylococcus aureus	every batch	§ 64 LFGB, Feuersenger, D., Knauf, H.J., Baumgart, J. (1994)
Yeast and mould	every batch	ISO 7954/1987
Salmonella spp.	on regular basis	based on AOC2004.03
Listeria	on regular basis	based on AOC2004.06
Acidification	fast fermenting culture	Internal method

In addition to this all intermediate batches of product are analyzed for clostridia according to ISO 15213.

Yours sincerely

Chr. Hansen A/S
Viktor Mierau
QC/Process Manager
Production Pohlheim

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Statement

December 12, 2016

Valid two years from date of issue

General Handling of Chr. Hansen's Freeze-dried Meat Cultures

Chr. Hansen's freeze-dried cultures are free flowing powders ranging in color from off-white to medium tan. This range in color is typical and does not affect performance. Visually, cultures should be free-flowing when package is opened. Culture that is lumped in the pouch should not be used as this indicates temperature abuse.

The freeze-dried cultures are packed in white/blue alufoil pouches in an outer corrugated white cardboard box. Each pouch has printed label information which includes a "best before date". For consistent performance the cultures should be used before this date. After this date additional inoculation may be necessary to ensure optimal performance.

Chr. Hansen's freeze-dried cultures are stored in temperature-controlled warehouses consistent with the specified storage conditions. To maintain best performance over shelf-life the cultures should be tored at -18 ºC (0 ºF) or below. Chr. Hansen guarantees a shelf life of 18 months from the date of manufacture, for freeze-dried meat cultures stored at -18 ºC (0 ºF). Higher storage temperatures will reduce expected shelf-life. Cultures stored at +5 ºC (41 ºF), have a shelf life of 6 weeks.

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Statement

Freeze-dried cultures are shipped either under ambient conditions, or under refrigerated/frozen conditions.
Shipping under ambient temperatures will not reduce performance below the specified activity. However, the culture should not be subjected to temperatures aboce 30 °C (86 °F) for more than 10 days, as this may compromise product quality. Freeze-dried meat cultures are relatively stable at temperatures below 30 °C (86 °F) because of their low water activity.

If you have further questions, please contact us.

Yours sincerely
Chr. Hansen A/S – Food Cultures & Enzymes

Diana Mattern
Business Support Specialist

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BactoFlex® B-LC-48

Product Information

Version: 3 PI GLOB EN 10-05-2016

Range

The BactoFlex ® range includes both traditional and fast fermenting cultures in a frozen, pelletized, easy-to-use format for high throughput operations.

Description

BactoFlex® B-LC-48 is a single strain meat culture for cooked or cured meat products. The strain in the culture is able to suppress growth of spoilage and pathogenic bacteria such as indigenous lactic acid bacteria and *Listeria monocytogenes* . The strain in the culture grows within a wide temperature range down to 4°C (39°F) and survives freezing.

Culture composition:
Lactobacillus curvatus

Application

Usage
B-LC-48 is recommended for different types of Ready-To-Eat food products which are packed under vacuum or modified atmosphere and cold-stored.
The culture works particularly well in cooked and sliced meat products and Wiener sausages .

The culture does not ferment lactose and consequently, if lactose is used as filler, the acid formation will be limited. The producer obtains the advantages of maintaining both product safety and sensory quality during shelf life.

Dosage
for 10000kg

Physical Properties

Color:	Off-white to brownish	Form:	Frozen pellets
Solubility:	Water soluble suspension		

Packaging

Material No:	Size	Type
713185	10x100 U	Bag(s) in box

Storage and handling

Temperature:	< -45 °C / < -49 °F
Conditions:	Dry

Transport condition

Shipment with dry ice

Ingredients

Culture

Shelf life

For frozen pellets at least 12 months when stored according to recommendations.

BactoFlex® B-LC-48

Product Information
Version: 3 PI GLOB EN 10-05-2016

Technical Data

Physiological data

Culture composition	*Lactobacillus curvatus*
Growth temperature	
Opt/max/min	37°C/40°C/4°C (98°F/104°F/39°F)
Salt limit	10% salt-in-water
Characteristics	Facultative anaerobic L(+)-lactic acid
Fermentable sugars	
Glucose (dextrose)	+
Fructose	+
Maltose	+
Lactose	-
Saccharose (sucrose)	+
Starch	-

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

Analytical methods

References and analytical methods are available on request.

Legislation

Chr. Hansen's cultures comply with the general requirements on food safety laid down in Regulation 178/2002/EC. Lactic acid bacteria are generally recognized as safe and can be used in food, however, for specific applications we recommend to consult national legislation.

The product is intended for food use.

Food Safety

No guarantee of food safety is implied or inferred should this product be used in applications other than those stated above. Should you wish to use this product in another application, please contact your Chr. Hansen representative for assistance.

Labeling

Suggested labeling “starter culture” or "culture", however as legislation may vary, please consult local legislation.

Trademarks

Product names, names of concepts, logos, brands and other trademarks referred to in this document, whether or not appearing in large print, bold or with the ® or TM symbol are the property of Chr. Hansen A/S or used under license. Trademarks appearing in this document may not be registered in your country, even if they are marked with an ®.

Dietary information

Technical support

Chr. Hansen's Application and Product Development Laboratories and personnel are available if you need further information.

GMO Information

In accordance with the legislation in the European Union* BactoFlex® B-LC-48 does not contain GMOs and does not contain GM labeled raw materials**. In accordance with European legislation on labeling of final food products** we can inform that the use of BactoFlex® B-LC-48 does not trigger a GM labeling of the final food product. Chr. Hansen's position on GMO can be found on: www.chr-hansen.com/About us/Policies and positions/Quality and product safety.

** Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms with later amendments, and repealing Council Directive 90/220/EEC.*
*** Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed with later amendments.*
Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labeling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms amending Directive 2001/18/EC, and with later amendments.

Allergen Information

List of common allergens in accordance with the US Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) and EU Regulation 1169/2011/EC with later amendments	Present as an ingredient in the product
Cereals containing gluten* and products thereof	No
Crustaceans and products thereof	No
Eggs and products thereof	No
Fish and products thereof	No
Peanuts and products thereof	No
Soybeans and products thereof	No
Milk and products thereof (including lactose)	No
Nuts* and products thereof	No
List of allergens in accordance with EU Regulation 1169/2011/EC only	
Celery and products thereof	No
Mustard and products thereof	No
Sesame seeds and products thereof	No
Lupine and products thereof	No
Mollusks and products thereof	No
Sulphur dioxide and sulphites (added) at concentrations of more than 10 mg/kg or 10 mg/litre expressed as SO_2	No

** Please consult the EU Regulation 1169/2011 Annex II for a legal definition of common allergens, see European Union law at: www.eur-lex.europa.eu*

Challenge study: efficiency of a DSM 18775 – based culture to inhibit the growth of Listeria monocytogenes in emulsified sausages – IFIP, September 2017

I- Design of the challenge study

Product: Emulsified sausages: Pork meat 41%, water, chicken stock 11%, mechanically separated poultry meat, pork fat, pork rind, SOY proteins, glucose syrup, salt, stabilizers: di- and triphosphates (E450, E451), MILK proteins, flavor (contains CELERY), antioxidant: sodium erythorbate (E316), colors: paprika extract (E160), carmine (E120), smoke flavor, preservative: sodium nitrite (E250 – concentration in agreement with the French Code des Usages < 120ppm), natural flavor.

- Control batch
- Batch previously inoculated with the DSM 18775-based culture to a 6.3 Log_{10} CFU/g level

Targeted microorganisms: 2 *L. monocytogenes* strains, one isolated from a listeriosis episode and the other isolated from cooked ham (IFIP collection).

Inoculum preparation: For each strain, cultivation in Brain Heart Infusion (B.H.I.) broth at 37°C for 16 hours, transfer in a new B.H.I. broth at 37°C for 6 hours, pooling and incubated at 4°C ± 1.0°C for 24 hours ± 1 hour.

Inoculation: Samples are unpacked, inoculated on the surface with 0.1 mL of the bacterial suspension of *L. monocytogenes* diluted in tryptone salt to reach 2.1 Log_{10} CFU/g. Products are then vacuum-packed. Each sample are composed of the emulsified sausages and when present the remaining purge of the package.

Storage conditions: Storage at 4°C ± 1.0°C during 16 days followed by 34 days of storage at 8°C ± 1.0°C.

Sampling plan: Preparation and inoculation of test units: number of test units prepared per batch

Microbiology	D_0	D_{16}	D_{50}
Enumeration of *L. monocytogenes* in inoculated test units Control batch and batch inoculated with DSM 18775 (Method: BRD 07/17-01/09*)	3 samples	3 samples	3 samples
Detection of *L. monocytogenes* in non-inoculated test units Control batch (Method: BRD 07-16-01/09*)	1 sample	Not applicable	Not applicable
Enumeration of L.A.B. in inoculated test units Control batch (Method: NF EN ISO 15214)	1 sample	1 sample	1 sample
Enumeration of L.A.B. in inoculated test units Batch inoculated with DSM 18775 (Method: NF EN ISO 15214)	1 sample	1 sample	1 sample

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Measurement of physico-chemical characteristics	D_0	D_{16}	D_{50}
pH – value in non-inoculated test units Control batch and batch inoculated with DSM 18775 (Method: NF V04-408)	1 sample	1 sample	1 sample
a_W – value in non-inoculated test units Control batch (Method: NF EN ISO 21807)	1 sample	Not applicable	Not applicable

BRD 07/17-01/09*: alternative method to the ISO standard NF EN ISO 11290-2 – approved.
BRD 07-16-01/09*: alternative method to the ISO standard NF EN ISO 11290-1 – approved.

II- Results of the challenge study

Microbiology: enumeration of *L. monocytogenes* (in Log_{10} CFU/g)

	Control					Batch inoculated with DSM 18775				
Day	**1**	**2**	**3**	**Average**	**SD***	**1**	**2**	**3**	**Average**	**SD***
D_0	2.06	2.05	2.15	2.09	0.052	2.08	2.06	2.05	2.06	0.015
D_{16}	2.43	2.41	2.52	2.45	0.057	< 0.6	< 0.6	< 0.6	< 0.6	-
D_{50}	3.66	3.78	3.93	3.79	0.136	< 0.6	< 0.6	< 0.6	< 0.6	-

SD*: Standard deviation

Microbiology: detection of *L. monocytogenes*

Day	Control
D_0	Absence in 25 g
D_{16}	-
D_{50}	-

Microbiology: enumeration of L.A.B. (in Log_{10} CFU/g)

	Control	Batch inoculated with DSM 18775				
Day	**1**	**1**	**2**	**3**	**Average**	**SD***
D_0	1.30	6.28	6.08	6.40	6.25	0.161
D_{16}	3.66	7.65	7.72	7.92	7.77	0.141
D_{50}	5.63	7.80	8.08	8.20	8.03	0.207

SD*: Standard deviation

Physico-chemical properties: pH-value

Day	Control	Batch inoculated with DSM 18775
D_0	6.04	6.06
D_{16}	5.91	5.83
D_{50}	5.17	5.26

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Physico-chemical properties: a_w-value

Day	Control
D_0	0.987
D_{16}	-
D_{50}	-

III- Evaluation of the challenge study

Calculation of the growth potential of *L. monocytogenes* in emulsified sausages (δ)

The growth potential, δ, is the log increase above the initial inoculum level throughout the intended shelf life of the product. When δ is below 1, the product is generally considered as not able to support the growth of *L. monocytogenes*[1].

Batch	δ (in Log_{10}(CFU/g)
Control	**1.71**
Inoculated with DSM 18775	**< -1.46**

Conclusion

Emulsified sausages – as currently produced (control batch) – support the growth of *L. monocytogenes* during 16 days of storage at 4°C ± 1.0°C followed by 34 days of storage at 8°C ± 1.0°C. If inoculated on the surface with the tested DSM 18775 – based culture and stored in the same conditions, emulsified sausages do no longer support the growth of *L. monocytogenes*.

Maisons-Alfort, October the 24th, 2017,

(b) (6)

Sabine Jeuge

Projects manager in Microbiology

[1] National Advisory Committee on Microbiological Criteria for Foods, 2010, Parameters for determining inoculated pack / challenge study protocols, Journal of Food Protection, Vol. 73, No. 1, p 140 - 202

Challenge study: efficiency of a DSM 18775 – based culture to inhibit the growth of Listeria monocytogenes in cooked ham – IFIP, January 2012

I- Design of the challenge study

Product: Batch of cooked ham

- Control batch
- Batch previously inoculated with the DSM 18775-based culture to a 6.7 Log_{10}(CFU/g) level

The cooked ham contains sodium nitrite (E250) in a concentration in agreement with the French Code des Usages < 120ppm)

Targeted microorganisms: 1 *L. monocytogenes* strain, isolated from a listeriosis episode (IFIP collection).

Inoculum preparation: Cultivation in Brain Heart Infusion (B.H.I.) broth at 37°C, subculture in BHI at 37°C to reach end of the growth phase, followed by an exposition to a cold stress at 4°C ± 1.0°C.

Inoculation: Inoculation is performed on the surface with the bacterial suspension of *L. monocytogenes* diluted in tryptone salt to reach 1.7 Log_{10} CFU/g. Inoculation by 0.1ml of suspension throw a septum to respect original packaging of the products. Each sample are composed of the cooked ham and when present the remaining purge of the package.

Storage conditions: Storage at 7°C ± 1.0°C during 35 days.

Sampling plan: Preparation and inoculation of test units: number of test units prepared per batch

Microbiology	**D_0**	**$D_{2.\ 4.\ 8.\ 12.\ 17.\ 21.\ 28}$**	**D_{35}**
Enumeration of *L. monocytogenes* in inoculated test units Control batch and batch inoculated with DSM 18775 (Method: NF EN ISO 11290-2)	3 samples	3 samples	3 samples
Detection of *L. monocytogenes* in non-inoculated test units Batch inoculated with DSM 18775 (Method: NF EN ISO 11290-1)	1 sample	Not applicable	1 sample
Enumeration of L.A.B. in inoculated test units Control batch and batch inoculated with DSM 18775 (Method: NF EN ISO 15214)	3 samples	3 samples	3 samples
Measurement of physico-chemical characteristics	**D_0**	**D_{17}**	**D_{35}**
pH – value in non-inoculated test units Batch inoculated with DSM 18775 (Method: NF V04-408)	3 samples	3 samples	3 samples
a_W – value in non-inoculated test units Batch inoculated with DSM 18775 (Method: NF EN ISO 21807)	3 samples	Not applicable	Not applicable

II- Results of the challenge study

Microbiology: enumeration of *L. monocytogenes* (in Log_{10} CFU/g)

	Control					Batch inoculated with DSM 18775				
Day	**1**	**2**	**3**	**Average**	**SD***	**1**	**2**	**3**	**Average**	**SD***
D_0	1.84	1.77	1.68	1.76	0.07	1.76	1.63	1.73	1.71	0.06
D_2	2.00	2.08	1.89	1.99	0.08	1.86	2.41	1.92	2.07	0.25
D_4	2.53	2.11	2.51	2.38	0.19	2.04	2.45	1.86	2.12	0.25
D_8	3.36	4.11	3.71	3.73	0.31	2.23	2.45	2.61	2.43	0.16
D_{12}	1.91	1.43	0.95	1.43	0.39	2.32	2.34	2.45	2.37	0.06
D_{17}	4.15	3.90	3.40	3.81	0.31	2.68	2.34	2.41	2.48	0.15
D_{21}	3.87	4.63	3.89	4.13	0.35	2.41	2.43	2.69	2.51	0.13
D_{28}	4.83	5.48	3.54	4.62	0.81	2.76	2.26	2.43	2.48	0.21
D_{35}	3.46	3.40	4.30	3.72	0.41	3.11	2.53	2.40	2.68	0.31

SD*: Standard deviation

Microbiology: detection of *L. monocytogenes*

Day	Batch inoculated with DSM 18775
D_0	Absence in 25 g
$D_{2.\ 4.\ 8.\ 12.\ 17.\ 21.\ 28}$	-
D_{35}	Absence in 25 g

Microbiology: enumeration of L.A.B. (in Log_{10} CFU/g)

	Control					Batch inoculated with DSM 18775				
Day	**1**	**2**	**3**	**Average**	**SD***	**1**	**2**	**3**	**Average**	**SD***
D_0	2.90	2.15	2.75	2.60	0.32	6.40	7.04	6.53	6.66	0.28
D_2	3.83	4.34	4.90	4.36	0.44	7.45	7.40	7.56	7.47	0.07
D_4	5.57	5.48	5.79	5.61	0.13	8.64	8.74	8.85	8.74	0.09
D_8	8.51	8.00	8.04	8.18	0.23	9.04	9.11	9.11	9.09	0.03
D_{12}	8.98	9.00	8.98	8.99	0.01	8.93	8.93	9.00	8.95	0.03
D_{17}	9.00	8.81	9.26	9.02	0.18	9.36	6.96	9.32	8.55	1.12
D_{21}	8.96	9.04	8.99	9.00	0.03	9.08	9.18	9.15	9.13	0.04
D_{28}	8.53	8.49	9.08	8.70	0.27	9.41	9.30	9.32	9.35	0.05
D_{35}	8.20	8.69	8.54	8.48	0.20	8.60	8.72	8.94	8.76	0.14

SD*: Standard deviation

Physico-chemical properties: pH-value

	Batch inoculated with DSM 18775				
Day	**1**	**2**	**3**	**Average**	**SD***
D_0	6.0	6.0	6.0	6.0	0.0
D_{17}	5.3	5.3	5.3	5.3	0.0
D_{35}	5.3	5.3	5.3	5.3	0.0

SD*: Standard deviation

Physico-chemical properties: a_W-value

	Batch inoculated with DSM 18775				
Day	**1**	**2**	**3**	**Average**	**SD***
D_0	0.977	0.977	0.978	0.977	0.000
D_{17}	-				
D_{35}	-				

SD*: Standard deviation

III- Evaluation of the challenge study

Calculation of the growth potential of *L. monocytogenes* in emulsified sausages (δ)

The growth potential, δ, is the log increase above the initial inoculum level throughout the intended shelf life of the product. When δ is below 1, the product is generally considered as not able to support the growth of *L. monocytogenes*[1].

Batch	δ (in Log_{10}(CFU/g)
Control	**1.96**
Inoculated with DSM 18775	**0.97**

Conclusion

The growth of *L. monocytogenes* in cooked ham during its storage at 7°C ± 1.0°C for 35 days is lowered when using the tested DSM 18775 – based culture.

Maisons-Alfort, October the 24th, 2017,

(b) (6)

Sabine Jeuge

Projects manager in Microbiology

[1] National Advisory Committee on Microbiological Criteria for Foods, 2010, Parameters for determining inoculated pack / challenge study protocols, Journal of Food Protection, Vol. 73, No. 1, p 140 - 202

Challenge study: efficiency of a DSM 18775 – based culture to inhibit the growth of Listeria monocytogenes in cooked chicken – IFIP, January 2012

I- Design of the challenge study

Product: roasted cooked and sliced chicken stored under modified atmosphere (50% N_2, 50% CO_2).

- Control batch
- Batch previously inoculated with the DSM 18775-based culture to a 7.0 Log_{10}(CFU/g) level

The cooked chicken contains sodium nitrite (E250) in a concentration in agreement with the French Code des Usages < 120ppm).

Targeted microorganisms: 2 *L. monocytogenes* strains, one isolated from a listeriosis episode and the other isolated from chicken meat (IFIP collection).

Inoculum preparation: Cultivation in Brain Heart Infusion (BHI) at 30°C for 24 hours, pooling and exposition to a starvation stress at 3°C ± 1.0°C for 24 hours ± 1 hour.

Inoculation: Samples are unpacked, inoculation is performed on the surface, with the bacterial suspension of *L. monocytogenes* diluted in tryptone salt to reach a contamination level of 1.7 Log_{10} CFU/g on the products. Once inoculated by 0.1 ml of suspension, products are repackaged under modified atmosphere (50% N_2, 50% CO_2). Each sample are composed of the cooked chicken and when present the remaining purge of the package.

Storage conditions: Storage at 4°C ± 1.0°C during 12 days then at 8°C ± 1.0°C.

Sampling plan: Preparation and inoculation of test units: number of test units prepared per batch.

Microbiology	**D_0**	**D_{12}**	**D_{23}**	**D_{35}**
Enumeration of *L. monocytogenes* in inoculated test units Control batch and batch inoculated with DSM 18775 (Method : NF EN ISO 11290-2)	3 samples	3 samples	3 samples	3 samples
Detection of *L. monocytogenes* in non-inoculated test units Control batch and batch inoculated with DSM 18775 (Method: NF EN ISO 11290-1)	1 sample	Not applicable	Not applicable	1 sample
Enumeration of L.A.B. in inoculated test units Control batch and batch inoculated with DSM 18775 (Method: NF EN ISO 15214)	3 samples	3 samples	3 samples	3 samples
Measurement of physico-chemical characteristics	**D_0**	**D_{12}**	**D_{23}**	**D_{35}**
pH – value in non-inoculated test units Control batch and batch inoculated with DSM 18775 (Method: NF V04-408)	1 sample	1 sample	1 sample	1 sample
a_W – value in non-inoculated test units Control batch and batch inoculated with DSM 18775 (Method: NF EN ISO 21807)	1 sample	1 sample	1 sample	1 sample
gas atmosphere in non-inoculated test units Control batch and batch inoculated with DSM 18775	1 sample	1 sample	1 sample	1 sample

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II- Results of the challenge study

Microbiology: enumeration of *L. monocytogenes* (in Log_{10} CFU/g)

	Control					Batch inoculated with DSM 18775				
Day	**1**	**2**	**3**	**Average**	**SD***	**1**	**2**	**3**	**Average**	**SD***
D_0	1.71	2.04	1.63	1.79	0.18	1.76	1.79	1.63	1.73	0.07
D_{12}	2.46	2.41	2.18	2.35	0.13	0.60	1.20	1.08	0.96	0.26
D_{23}	4.95	4.60	4.85	4.80	0.15	1.60	1.90	1.51	1.67	0.17
D_{35}	7.04	6.53	6.04	6.54	0.41	1.08	1.75	1.20	1.34	0.29

SD*: Standard deviation

Microbiology: detection of natural *L. monocytogenes* contamination

Day	Control	Batch inoculated with DSM 18775
D_0	Absence in 25 g	Absence in 25 g
D_{12}	-	-
D_{23}	-	-
D_{35}	Absence in 25 g	Absence in 25 g

Microbiology: enumeration of L.A.B. (in Log_{10} CFU/g)

	Control					Batch inoculated with DSM 18775				
Day	**1**	**2**	**3**	**Average**	**SD***	**1**	**2**	**3**	**Average**	**SD***
D_0	1.80	< 1	< 1	1.80	-	7.08	6.88	7.04	7.00	0.09
D_{12}	5.45	5.26	5.20	5.30	0.10	8.56	8.74	8.60	8.63	0.08
D_{23}	9.11	8.34	8.92	8.79	0.33	9.30	9.30	9.20	9.27	0.05
D_{35}	8.46	7.91	8.40	8.26	0.24	9.15	9.08	9.04	9.09	0.05

SD*: Standard deviation

Physico-chemical properties: pH-value

Day	Control	Batch inoculated with DSM 18775
D_0	6.35	6.37
D_{12}	6.15	6.32
D_{23}	5.97	6.29
D_{35}	6.23	6.97

Physico-chemical properties: a_w-value

Day	Control	Batch inoculated with DSM 18775
D_0	0.973	0.981
D_{12}	0.975	0.974
D_{23}	0.976	0.980
D_{35}	0.975	0.976

Physico-chemical properties: gas atmosphere of the packaging

	Control		Batch inoculated with DSM 18775	
Day	% O_2	% CO_2	% O_2	% CO_2
D_0	0.024	48.60	0.021	48.80
D_{12}	0.286	42.00	0.296	43.00
D_{23}	0.455	37.50	0.014	38.40
D_{35}	0.038	32.10	0.022	31.90

III- Evaluation of the challenge study

Calculation of the growth potential of *L. monocytogenes* in cooked chicken (δ)

The growth potential, δ, is the log increase above the initial inoculum level throughout the intended shelf life of the product. When δ is below 1, the product is generally considered as not able to support the growth of *L. monocytogenes*[1].

Batch	δ (in Log_{10} CFU/g)
Control	**4.7**
Inoculated with DSM 18775	**- 0.4**

Conclusion

The growth of *L. monocytogenes* in sliced cooked chicken stored under modified atmosphere packaging (50% N_2 and 50% CO_2) at 4°C ± 1.0°C during 12 days then at 8°C ± 1.0°C during 23 days is stopped when using the tested DSM 18775 – based culture. This is not the case if the products are not previously inoculated with this culture.

Maisons-Alfort, October the 24th, 2017,

(b) (6)

Sabine Jeuge

Projects manager in Microbiology

[1] National Advisory Committee on Microbiological Criteria for Foods, 2010, Parameters for determining inoculated pack / challenge study protocols, Journal of Food Protection, Vol. 73, No. 1, p 140 - 202

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(54) **A NEW LACTIC ACID BACTERIA STRAIN AND ITS USE FOR THE PROTECTION OF FOOD PRODUCTS**

NEUER MILCHSÄUREBAKTERIENSTAMM UND DESSEN VERWENDUNG ZUM SCHUTZ VON LEBENSMITTELPRODUKTEN

NOUVELLE SOUCHE DE BACTÉRIES D'ACIDE LACTIQUE ET SON UTILISATION POUR LA PRÉSERVATION DE PRODUITS ALIMENTAIRES

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Description

[0001] The present invention relates to a new lactic acid bacterium that produces proteasesensitive antimicrobial agents (bacteriocins) at low storage temperatures. Particularly, the present invention refers to *Lactobacillus curvatus* DSM 18775, which has been found to be useful for bioprotection of refrigerated products such as Ready-To-Eat (RTE) meat and dairy products.

BACKGROUND ART

[0002] Bacterial contamination of food products is known to be responsible for spoilage and for the transmission of food borne illness. This problem is particularly important in RTE meats and dairy products which are not normally reheated by consumers prior to ingestion and which are stored for extended times in refrigerators at 2-10°C. An exemplary case is *Listeria monocytogenes* which is a pathogenic bacterium of particular concern in food products, such as vacuum- or modified atmosphere (MA)-packed RTE meat products, due to its tolerance to refrigeration temperatures, relatively high concentrations of NaCl and anaerobic conditions or in products, such as Fresh Cheese, due to the lack of a heat inactivation step. As a result, a great deal of effort has been expended in attempts to identify natural products that can be safely added to foods for the purpose of inhibiting bacterial growth.

[0003] It is well-known to use lactic acid bacteria as starter cultures to induce fermentation of meat products, typically raw salted meat products. The term "starter culture" refers to a preparation containing microbial cells that is intended for inoculating a food matrix to be subjected to fermentation. Starter cultures for meat fermentation are commonly comprised by one or more lactic acid bacteria. The starter culture is intended for providing the desired change in the characteristics of the food matrix during fermentation (e.g. a desired acidification, and certain other sensory and technological parameters). Typically, a starter culture will proliferate during the fermentation process. During the fermentation process the lactic acid bacteria primarily produce lactic acid whereby pH drops to the desired pH-value depending on the culture and the processing conditions (temperature, sugar type/content etc.), and importantly, the sensory properties of the product are distinctly changed.

[0004] Antagonistic cultures added to food to inhibit pathogens and/or extend shelf life without changing the sensory properties of the product are termed "protective cultures". In contrast to starter cultures, protective cultures are not intended to change the sensory properties of the product. Their use or that of their metabolic products (organic acids, hydrogen peroxide, enzymes and bacteriocins) is often referred to as "biopreservation" or "bioprotection" (Castellano, P. and Vignolo, G., "Inhibition of Listeria innocua and Brochothrix thermosphacta in vacuum-packaged meat by addition of bacteriocinogenic Lactobacillus curvatus CRL705 and its bacteriocins", 2006, Letters in Applied Microbiology. Vol. 43: 194-199). This study demonstrates a bacteriostatic effect on a non-patogenic Listeria species. No bacteriocidal effect to Listeria is reported. Furthermore the "sensoric" evaluation performed was limited to pH measurements.

[0005] Besides the establishment of biopreservation as a method to ensure microbiological safety without changing the sensoric characteristics of the product, bioprotective cultures have also been evaluated for their potential of preventing growth of spoilage bacteria (Vermieren, L. et al., "Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products", 2004, International Journal of Food Microbiology, 96: 149-164).

[0006] The sensory acceptability of cooked meat products treated with bioprotective cultures may limit the use of the preservation method, and the buffering capacity as well as the content of glucose have shown to be key elements to avoid sensory deviations when applying bioprotective cultures (Vermieren *et al.*, *supra*).

[0007] A re-growth of *Listeria monocytogenes* has often been observed with the use of bioprotective cultures after an initial phase of inhibition. Re-growth has been ascribed to the development of resistance of *L. monocytogenes* to the bacteriocins, degradation of bacteriocin molecules with endogenous proteases produced during the growth phase, adsorption of the bacteriocins to the surface of the producer strain, or specific interactions with the food matrix (Dicks, L.M.T. et al., "Use of bacteriocin-producing starter cultures of Lactobacillus plantarum and Lactobacillus curvatus in production of ostrich meat salami", 2004, Meat Science, 66: 703-708).

[0008] The European patent application EP 1.475.432 discloses two *Lactobacillus curvatus strains*, deposited as PTA-5150 and PTA-5159 and their use for reducing the growth of a microbe in a food or pharmaceutical composition.

[0009] The patent no. US 4.886.673 discloses three bacteria strains *Lactobacillus curvatus* DSM 4265, *Micrococcus varians* DSM4263, and *Debaromyces hansenii* DSM 4260 and their use for preserving meat products. Example 1 describes the use of *Lactobacillus curvatus* DSM 4265 in the production of cut raw sausage.

[0010] The European patent application EP 0.640.291 discloses the use of *Lactobacillus curvatus* DSM8430 as a starter culture in salami production. It is specifically mentioned that optimal bacteriocin production occurs at temperatures between 15 and 20°C and that the activity decreases at low temperatures (+4°C).

[0011] Vogel, R.F. et al., (1993, System. Appl. Microbiol., 16: 457-462) discloses the use of *Lactobacillus curvatus* strain LTH 1174 as a starter culture in salami production. It is specifically mentioned that optimal bacteriocin production occurs at temperatures between 15 and 20°C and that the activity decreases at low temperatures (+4°C).

[0012] Benkerroum, N. et al. (2005, J. Appl. Microbiol., 98: 56-63) discloses the use of *Lactobacillus curvatus* strain LBPE as a starter culture in the production of dry-fermented sausages. The fermentation is performed at 30°C, and the drying at 14-16°C. No bioprotective effect was demonstrated at low temperatures.

[0013] Mauriello, G. et al. (2004, J. Appl. Microbiol., 97: 314-322) discloses the use of polyethylene films for food packing that are treated with partially purified bacteriocin of *Lactobacillus curvatus* strain 32Y. In order to produce the bacteriocin this particular strain is grown at 30°C.

[0014] Mataragas et al. (2003, Food Microbiology, 20 :259-265) discloses a *Lactobacillus curvatus* L442 which has antimicrobial activity against *Listeria monocytogenes.* Changes of pH have been investigated and the results are provided in Fig. 3 showing a pH drop from 6.5 to about 5.5 due to acetate production (Fig. 4). Such a pH drop is known to reduce the amount of pathogenic microorganisms by itself. The amount of acetate produced is quite high and likely to produce sensory changes, i.e. have an impact on flavor.

SUMMARY OF THE INVENTION

[0015] The problem to be solved by the present invention is the provision of a bacterial strain which inhibits the growth of food-borne pathogenic and spoilage bacteria at low temperatures (2-10°C) without changing the sensory properties of the food product.

[0016] The solution is based on a *Lactobacillus curvatus* strain deposited under the accession number DSM 18775.

[0017] As it is illustrated below in a non-restricted way, it has been found that the *Lactobacillus curvatus* strain deposited under the accession number DSM 18775 is useful for the bioprotection of food products. The *Lactobacillus curvatus* strain deposited under the accession number DSM 18775 can be useful and has proven particularly useful for the inhibition of food-borne pathogenic bacteria and spoilage bacteria owing to the production of bacteriocins.

[0018] Food-borne pathogenic and spoilage bacteria can be aerobic, anaerobic or facultative anaerobic, and thus, the elimination of oxygen alone from a food package or from a food storage environment will not effectively eliminate all types of undesired bacteria. Moreover, control of the temperature in the storage of food is not totally effective to preclude the growth of such bacteria because several types of pathogenic and spoilage bacteria are able to grow at various temperatures. On the other hand, there are pathogenic bacteria, which due to their tolerance to refrigeration temperatures, relatively high concentrations of NaCl and anaerobic conditions, are of particular concern in RTE food products.

[0019] The inventors of the present invention have observed that under refrigeration conditions, the *Lactobacillus curvatus* strain deposited under the accession number DSM 18775 produces bacteriocins, providing considerable reductions in numbers of food-borne pathogenic bacteria without causing undesirable sensory changes, and also preventing growth of spoilage bacteria in the food product. The fact that the *Lactobacillus curvatus* deposited under the accession number DSM 18775 is able to produce bacteriocins at a refrigeration temperature implies that said strain can be used for the bioprotection of refrigerated products, and particularly of ready to eat refrigerated products packaged in vacuum or modified atmosphere.

[0020] Thus, in one aspect the present invention relates to a strain of *Lactobacillus curvatus* deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmBH (DSMZ) under the accession number DSM 18775 characterized in that it has the ability at a temperature ranging from 2 to 10ºC of inhibiting the growth of at least one food-borne pathogenic bacterium without causing sensory changes in food.

[0021] A culture sample of the microorganism was deposited on 09.11.2006 in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under the accession number DSM 18775.

[0022] An important aspect in the evaluation of the use of a strain as a bioprotective culture is the ability of the strain to work in the food product for which it is intended. In this respect it is not only important that the strain is able to inhibit any undesired food-borne pathogenic bacteria in the product under relevant storage conditions but also that it does not produce any undesired sensory effects (off-taste, off-odors or unwanted color changes). The *Lactobacillus curvatus* strain deposited under the accession number DSM 18775 inhibits the food-borne pathogenic bacteria considerably when applied on a wide range of real RTE meat products throughout storage at relevant storage conditions. The inventors have proven by sensory analyses that the culture of the *Lactobacillus curvatus* strain deposited under the accession number DSM 18775 does not negatively affect the sensory quality of the food products (such as various RTE meat products) under relevant storage conditions, as it is illustrated below.

[0023] Furthermore, in contrast to other strains used as bioprotective cultures, when the culture of the *Lactobacillus curvatus* strain deposited under the accession number DSM 18775 is used as a bioprotective culture, no re-growth of *L. monocytogenes* is observed.

[0024] Hence, when using a culture of a strain according to the present invention there are reduced health risks associated with the ingestion of refrigerated products, due to increased safety of the product during the shelf life. Consequently, the economic loss to the food industry can be considerably reduced.

[0025] In a third aspect the present invention relates to a process for preparing a composition capable of inhibiting the growth of at least one food-borne pathogenic bacterium, said process comprising: (a) culturing cells of a strain of

Lactobacillus curvatus according to the first aspect of the invention, which upon culturing in a culture medium produces a bacteriocin which has inhibitory activity against bacterial strains including *Listeria monocytogenes*, to obtain a supernatant comprising the bacteriocin; and (b) separating the supernatant from the cultured cells to obtain the supernatant, thus obtaining a supernatant composition comprising the bacteriocin.

[0026] In one embodiment of this aspect the bacteriocin comprising supernatant composition is further subjected to a drying step to obtain a dried culture eluate product. The drying step may conveniently be freeze drying or spray drying. As described in example 8 the process results in a dried culture eluate product which inhibits *Listeria monocytogenes* on meat products packed in a modified atmosphere or in vacuum.

[0027] The growth inhibiting composition has a bacteriocidal effect on at least one food-borne pathogenic bacterium when sufficient amounts are provided. An example is a growth inhibiting composition having a bacteriocidal effect on *Listeria monocytogenes* when provided in sufficient amounts. This is illustrated in example 8 fig. 7a.

[0028] A culture eluate composition may result from the above-mentioned processes, e.g. from step (a) of the process of the third aspect of the invention or from step (b) of the process of the third aspect of the invention.

[0029] In a further aspect, the present invention relates to compositions for preserving food products which comprise the *Lactobacillus curvatus* strain according to the first aspect of the invention.

[0030] In a yet further aspect, the present invention relates to the use of a strain of *Lactobacillus curvatus* according to the first aspect of the invention or the supernatant composition of step b) of the process according to the third aspect of the invention for preserving food products.

[0031] Also, the present invention relates to the use of a strain of *Lactobacillus curvatus* according to the first aspect of the invention or the supernatant composition of step b) of the process according to the third aspect of the invention for inhibiting the growth of food-borne pathogenic bacteria or spoilage bacteria in food products preserved in a refrigerated state at a temperature ranging from 2 to 10 ºC.

[0032] In a further aspect, the present invention relates to a method for controlling *Listeria* contamination in a food product, on food processing equipment, or on food storage containers, comprising applying the *Lactobacillus curvatus* strain with the accession number DSM 18775, characterized in that it has the ability at a temperature ranging from 2 to 10ºC of inhibiting the growth of at least one food-borne pathogenic bacterium without causing sensory changes in food, to a food product or food processing equipment in an amount sufficient to reduce the amount or prevent growth of *Listeria.*

[0033] Throughout the description and claims the word "comprise" and variations of the word, such as "comprising", is not intended to exclude other technical features, additives, components, or steps. Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following examples and drawings are provided by way of illustration, and are not intended to be limiting to the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034]

FIG. 1 represents the cell counts of *L. monocytogenes* in slices of Mortadella sausages inoculated with (■) or not inoculated with (♦) DSM 18775 (initial level of 10^7 CFU/g). The slices of Mortadella sausages were packed in modified atmosphere (30% CO_2 and 70% N_2) and stored at 7°C. Cell counts were based on determinations made on two different slices of meat, and the bars indicate the standard deviations between these duplicate determinations.

FIG. 2 represents the cell counts of *L. monocytogenes* in slices of Mortadella sausages made of finely chopped meat inoculated with (■) or not inoculated with (♦) DSM 18775 (initial level of 10^7 CFU/g). The slices of Mortadella sausages were packed in modified atmosphere (30% CO_2 and 70% N_2) and stored at 7°C. Cell counts were based on determinations made on two different slices of meat, and the bars indicate the standard deviations between these duplicate determinations.

FIG. 3 represents the cell counts of *L. monocytogenes* in slices of cooked, smoked ham, inoculated with (■) or not inoculated with (♦) DSM 18775 (initial level of 10^7 CFU/g). The ham slices were packed in modified atmosphere (30% CO_2 and 70% N_2) and stored at 7°C. Cell counts were based on determinations made on two different slices of meat, and the bars indicate the standard deviations between these duplicate determinations.

FIG. 4 represents the cell counts of *L. monocytogenes* in Wiener Sausage, inoculated with (■) or not inoculated with (♦) DSM 18775 (initial level of 10^7 CFU/g). The Wiener Sausages were vacuum-packed and stored at 7°C. Cell counts were based on determinations made on two different sausages, and the bars indicate the standard deviations between these duplicate determinations.

FIG. 5 represents the cell counts of *L. monocytogenes* in slices of cooked ham, inoculated with (■) or not inoculated with (♦) DSM 18775 (initial level of 10^7 CFU/g). The ham slices were packed in modified atmosphere (30% CO_2 and 70% N_2) and stored at 5°C. Cell counts were based on determinations made on two different slices of cooked ham, and the bars indicate the standard deviations between these duplicate determinations.

FIG. 6 represents the development in pH in cheeses inoculated with DSM 18775 (■) or not inoculated with DSM 18775 (♦) (initial level of 10^7 CFU/g). The cheeses were packed in vacuum and stored at 9°C. pH is measured in a suspension of cheese and water (1:1) stirred for 30 min before measurement of pH (using a PHM 92 pH-meter, Radiometer, Copenhagen, DK).

FIG. 7. Cell counts of L. monocytogenes exposed to culture eluate of DSM18775 or MicroGARD 730 in different concentrations, as explained in the figure legends to the right, on slices of emulsion sausage stored under Modified Atmosphere (MA, 30% CO_2 and 70% N_2) 7a, or vacuum (7b) at 7°C. Cell counts were based on determinations made on two different slices of meat, and the bars indicate the standard deviations between these duplicate determinations.

DETAILED DESCRIPTION OF PARTICULAR EMBODIMENTS

[0035] The *Lactobacillus curvatus* strain DSM 18775 of the present invention is a lactic acid bacterium. It was isolated from fermented food and was identified as *Lactobacillus curvatus* by protein gel electrophoresis followed by analyzing and clustering with the reference profiles of the LMG culture collection database. Furthermore, by using an API profile characterization, DSM 18775 was identified as *Lactobacillus curvatus* with 63.4% probability. The strain was characterized by full metabolization of: D-Ribose, D-Galactose, D-Glucose, D-Fructose, D-Mannose, N-Acetylglucosamine, Esculine, D-Maltose and D-Trehalose and partial metabolization of D-Saccharose after 48 h incubation at 30ºC. The strain was deposited on November 9, 2006 under the terms of the Budapest treaty at 'Deutsche Sammlung von Microorganismen und Zellkulturen' GmbH (DSMZ). It was assigned deposit number DSM 18775. The anti-listerial bacteriocin produced by DSM 18775 was determined in an agar well diffusion assay using *Lactobacillus sakei* NCFB 2714 as the indicator organism.

[0036] The scope of the present invention also encompasses a strain of *Lactobacillus curvatus* obtained by mutation of the strain of *Lactobacillus curvatus* DMS 18775, provided that the resulting strain has the ability at a temperature ranging from to 2 to 10ºC of inhibiting the growth of at least one food-borne pathogenic bacterium without causing sensory changes in food.

[0037] In the process for preparing the bacteriocin, the strain of the present invention which produces the bacteriocin, is cultured in a medium and under conditions which are favorable for growth, the supernatant is isolated from the resulting culture by separating the supernatant from the cultured cells to obtain a supernatant containing the bacteriocin, and to effect separation, the resulting culture is centrifuged and a supernatant extract containing the bacteriocin is obtained. The supernatant may be concentrated to obtain a concentrate comprising the bacteriocin, and an isolated and purified bacteriocin may be obtained from the supernatant and concentrate and may be dehydrated.

[0038] In a particular preferred embodiment of this process the supernatant composition is further subjected to a drying step to obtain a dried culture eluate. The drying step may conveniently be freeze drying or spray drying, but any drying process which is suitable for drying of bacteriocins, also including vacuum drying and air drying, are contemplated. Although the bacteriocin produced at low temperatures by *Lactobacillus curvatus* DSM 18775 is not yet characterized in details, it is known that certain *Lactobacillus curvatus* may produce class IIa bacteriocins including Sakacin. Class IIa bacteriocins are small heat-stable proteins, therefore we expect that even drying methods, which result in moderate heating of the culture eluate, will result in active compositions.

[0039] The bacteriocin according to the present invention is characterized in more detail below with the aid of various microbiological, biochemical and genetic findings which illustrate its properties. The percentages are given by weight. Unit of antibacterial activity is according to the "agar well test". Within the context of the present exposition, inhibitory activity is defined in terms of arbitrary units.

[0040] The agar well test is used to determine whether the culture supernatant containing the bacteriocin according to the present invention exhibits inhibitory activity against different strains of spores and bacteria. The inhibition spectrum of the supernatant is thus determined.

[0041] The term "food product" as used herein refers to any food that is susceptible to spoilage as a result of bacterial growth and proliferation. Such food products include, but are not limited to, meat, dairy products, vegetables, fruits and grains.

[0042] The terms "refrigerated product" or "preserved in a refrigerated state" are equally used and refer to food products which are stored at temperatures ranging from to 2 to 10ºC. The food product can be either packaged under vacuum or at modified atmosphere.

[0043] As used herein, the term "meat" refers to any meat product or meat by-product (including those processed) from an animal which is consumed by humans or animals, including, without limitation, meat from bovine, ovine, and porcine species, poultry, fish and crustaceous seafood. As used in the present application, the term "ready to eat meat product", also referred to as RTE meat product, is intended to include any meat product which does not require cooking prior to consumption.

[0044] The term "dairy product" is intended to include any food product made using milk or milk products, including, but not limited to, milk, yogurt, ice cream, cheese, butter, and cream.

[0045] As used herein the term "shelf life" means the period of time that a food product remains saleable to retail customers. In traditional meat processing, the shelf life of meat and meat by-products is about 30 to 40 days after an animal has been slaughtered. Refrigeration of meat during this period of time is expected to largely arrest and/or retard the growth of pathogenic bacteria, and to a lesser extent, spoilage bacteria. After about 30 to 40 days, however, refrigeration is no longer able to effectively control the proliferation of spoilage bacteria below acceptable levels.

[0046] The term "bacteriocidal effect" as used herein refers to any type of treatment which effect the killing of bacteria (i.e. which reduce their numbers). This is in contrast to a "bacteriostatic effect" which refers to the situation where the treatment only inhibits the growth or reproduction of the bacteria. An agent is said to be a bactericide or a bacteriocide if the agent is able to kill one or more type of bacteria. A bacteriocide is said to possess bacteriocidal or bactericidal activity.

[0047] By "bacteriocins" we refer to peptides or protein molecules released extracellularly that are able to kill certain other closely related bacteria by a mechanism by which the producer cell exhibits a degree of specific immunity.

[0048] The term "spoilage bacteria" as used herein refers to any type of bacteria that act to spoil food. Spoilage bacteria may grow and proliferate to such a degree that a food product is made unsuitable or undesirable for human or animal consumption. Bacteria are able to proliferate on food surfaces, such as meat surfaces, by assimilating sugars and proteins on such surfaces. By metabolizing these components, spoilage bacteria create by-products including carbon dioxide, methane, nitrogenous compounds, butyric acid, propionic acid, lactic acid, formic acid, sulfur compounds, and other undesired gases and acids. The production of such by-products alter the color of meat surfaces, often turning meat from a red color to a brown, grey or green color. Gaseous by-products generated by spoilage bacteria also give spoiled meat an undesirable odor. The color and odor alterations of meat due to the growth of spoilage bacteria on the surface of a meat product often make such food product unsaleable to consumers.

[0049] In addition to the control of spoilage bacteria, another significant concern in the food processing industry is controlling the growth of food-borne pathogenic bacteria. As used herein, the term "food-borne pathogenic bacteria" refers to any food poisoning organism that is capable of causing disease or illness in animals or humans. The term "food-borne pathogenic bacteria" will be understood to include bacteria that infect the food product (for instance meat) and thereby cause disease or illness, as well as bacteria that produce toxins that cause disease or illness. Preferably, the food-borne pathogenic bacteria is selected from the group: *Aeromonas caviae; Aeromonas hydrophila; Aeromonas sobria; Bacillus cereus; Campylobacter jejuni; Citrobacter* ssp.; *Clostridium botulinum; Clostridium perfringens; Enterobacter* ssp.; *Enterococcus* ssp.; *Escherichia coli* enteroinvasive strains; *Escherichia coli* enteropathogenic strains; *Escherichia coli* enterotoxigenic strains; *Escherichia coli* O157:H7; *Klebsiella* ssp.; *Listeria monocytogenes; Plesiomonas shigelloides; Salmonella* ssp.; *Shigella* ssp.; *Staphylococcus aureus; Streptococcus* ssp.; *Vibrio cholerae; Yersinia enterocolitica.* More preferably, the pathogenic-bacteria are *Listeria monocytogenes.*

[0050] As used herein, the expression "effective amount" refers to the amount of *Lactobacillus curvatus* bacteria according to the first aspect of the invention which gives rise to an inhibition of the bacterial growth or a reduction of the number of other bacteria from the food product.

[0051] Preferred aspects and embodiments of the invention are given below.

[0052] A strain of *Lactobacillus curvatus* deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmBH (DSMZ) under the accession number DSM 18775 characterized in that it has the ability at a temperature ranging from 2 to 10ºC of inhibiting the growth of at least one food-borne pathogenic bacteria without causing sensory changes in food.

[0053] A composition for preserving a food product characterized in that it comprises a *Lactobacillus curvatus* strain according to the invention.

[0054] The composition according to the invention, wherein the composition delays the development of food-borne pathogenic bacteria or spoilage bacteria.

[0055] The composition according to the invention, wherein the food product is Ready-To-Eat (RTE) meat or a dairy product.

[0056] A process for preparing a composition capable of inhibiting the growth of at least one food-borne pathogenic bacterium said process comprising:

(a) culturing cells of a strain of *Lactobacillus curvatus* of the invention, which upon culturing in a culture medium produces a bacteriocin which has inhibitory activity against bacterial strains including *Listeria monocytogenes* to obtain a supernatant comprising the bacteriocin; and

(b) separating the supernatant from the cultured cells to obtain the supernatant, thus obtaining a supernatant composition comprising the bacteriocin.

[0057] The process may further comprise isolating the bacteriocin from the supernatant composition to obtain a purified bacteriocin product.

[0058] The process may further comprise concentrating the supernatant composition to obtain a concentrate comprising the bacteriocin.

[0059] Use of a strain of *Lactobacillus curvatus* according to the invention or the supernatant composition of step (b) of the process according to the invention for preserving food products.

[0060] Use of a strain of *Lactobacillus curvatus* according to the invention or the supernatant composition of step (b) of the process according to the invention for inhibiting the growth of food-borne pathogenic bacteria or spoilage bacteria on food products preserved in the refrigerated state at a temperature ranging from 2 to 10ºC.

[0061] Strains and cultures of *Lactobacillus curvatus* or the supernatant may be useful for delaying the appearance of unwanted sensory effects (off-taste, off-odors or unwanted color changes) in food products preserved in the refrigerated state.

[0062] The food product may be packaged under vacuum or at modified atmosphere. The food product may be a RTE meat product or a dairy product.

[0063] The food-borne pathogenic bacterium may be *Listeria monocytogenes.*

[0064] A process of preserving food products may be characterized in that *Lactobacillus curvatus* according to the invention or the supernatant composition of step (b) of the process according to the invention is added in an effective amount to said food products.

[0065] The *Lactobacillus curvatus* strain or the supernatant composition may be added during the manufacture process of the food product or to the food product so as to form a barrier on the surface of said product.

[0066] The food product may be a RTE meat or a dairy product.

[0067] A method for controlling *Listeria* contamination in a food product, on food processing equipment, or on food storage containers, comprising applying the Lactobacillus strain with the accession number DSM 18775 characterized in that it has the ability at a temperature ranging from 2 to 10ºC of inhibiting the growth of at least one food-borne pathogenic bacterium without causing sensory changes in food, to a food product or food processing equipment in an amount sufficient to reduce the amount of *Listeria.*

EXAMPLES

Example 1. Strain identification and bacteriocin production

[0068] After growth of DSM 18775 in MRS (De Man, Rogosa and Sharpe, Difco, VWR, Herlev, Denmark) broth for 22 h at 25ºC, the cell suspension was centrifuged at 4,000 x g for 15 min. The supernatant was adjusted to pH 6.0 $\pm$ 0.1 with 1 N NaOH and filter sterilized (0.45 μm). Two-fold dilutions of the supernatant were made in sterile ion-exchanged water and 50 μl of each bacteriocin dilution were added to wells in MRS agar containing the indicator organism, *Lactobacillus sakei* NCFB 2714, in a concentration of 10^6 CFU/ml. To verify the proteinaceus nature of the inhibitory substances, a solution of the proteolytic enzyme, Proteinase K, was applied next to one well of the agar well diffusion assay. The bacteriocin activity of the cell supernatant was defined as the reciprocal of the highest dilution causing an inhibition zone in the agar assay. Inhibition zones caused by a proteinaceus compound, bacteriocin, were observed with an activity of 500 units/ml cell supernatant, clearly indicating the ability of DSM 18775 to produce considerable amounts of anti-listerial bacteriocin.

Example 2. Application trial with *Lactobacillus curvatus* DSM 18775 on sliced Mortadellatype sausage (I)

[0069] The anti-listerial effect of DSM 18775 and the sensory impact of the culture were evaluated on sliced Mortadella sausage. A 5 strain *L. monocytogenes* cocktail was added to the surface of the RTE meat product (10^3 CFU/g) followed by inoculation of the bioprotective culture (10^7 CFU/g). The product was packed in a modified atmosphere (30% CO_2 and 70% N_2) and stored at 7°C for 27 days.

[0070] Cell counts of the bioprotective culture were determined by plating appropriate 10-fold dilutions made in peptone saline onto MRS-agar plates and incubating anaerobically for 3 days at 30°C. DSM 18775 proliferated on the product and reached approx. 10^8 CFU/g after 1 week of storage and approx. 10^9 CFU/g by the end of storage. *Listeria* cell counts were determined by plating appropriate 10-fold dilutions made in peptone saline onto listeria selective PALCAM agar plates (Oxoid A/S, Glostrup, Denmark) and incubating microaerophilic for 48 h at 37°C. These cell counts can be seen in Fig. 1, showing a bacteriocidal effect of DSM 18775 on *L. monocytogenes.*

[0071] Sensory descriptive triangle tests carried out by a panel of 10 judges did not show any significant effect of

adding the bioprotective culture upon 11 days of storage. After 21 days of storage (end of shelf life), the products with added bioprotective culture were perceived as fresher in taste and odor compared to the products without DSM 18775 added, which were characterized as more insipid.

Example 3. Application trial with *Lactobacillus curvatus* DSM 18775 on sliced Mortadellatype sausage (II), very finely chopped

[0072] The anti-listerial effect of DSM 18775 and the sensory impact of the culture was evaluated on sliced Mortadella sausage made of very finely chopped meat. A 5 strain *L. monocytogenes* cocktail was added to the surface of the RTE meat product (10^3 CFU/g) followed by inoculation of the bioprotective culture (10^7 CFU/g). The product was packed in a modified atmosphere (30% CO_2 and 70% N_2) and stored at 7°C for 27 days.

[0073] Cell counts of the bioprotective culture were determined by plating appropriate 10-fold dilutions made in peptone saline onto MRS-agar plates and incubating anaerobically for 3 days at 30°C. DSM 18775 proliferated on the product and reached approx. 10^8 CFU/g after 1 week of storage and approx. 10^9 CFU/g by the end of storage.

[0074] *Listeria* cell counts were determined by plating appropriate 10-fold dilutions made in peptone saline onto listeria selective PALCAM agar plates (Oxoid A/S, Greve, Denmark) followed by microaerophilic incubation for 48 h at 37°C. *Listeria* cell counts can be seen in FIG. 2, clearly illustrating a bacteriocidal effect of DSM 18775 on *L. monocytogenes* throughout 27 days of storage.

[0075] Sensory descriptive triangle tests carried out by a panel of 10 judges did not show any significant sensory effect of adding the bioprotective culture upon 11 days of storage or at the end of shelf life, i.e. after 21 days of storage.

Example 4. Application trial with *Lactobacillus curvatus* DSM 18775 on cooked, smoked and sliced ham

[0076] The anti-listerial effect of DSM 18775 and the sensory impact of the culture was evaluated on cooked, smoked and sliced ham. A 5 strain *L. monocytogenes* cocktail was added to the surface of the RTE meat product (10^3 CFU/g) followed by inoculation of the bioprotective culture (10^7 CFU/g). The product was packed in a modified atmosphere (30% CO_2 and 70% N_2) and stored at 7°C for 27 days.

[0077] Cell counts of the bioprotective culture were determined by plating appropriate 10-fold dilutions made in peptone saline onto MRS-agar plates and incubating anaerobically for 3 days at 30°C. DSM 18775 proliferated on the product and reached approx. 10^8 CFU/g after 1 week of storage and approx. $5x10^8$ CFU/g by the end of storage.

[0078] *Listeria* cell counts were determined by plating appropriate 10-fold dilutions made in peptone saline onto listeria selective PALCAM agar plates followed by microaerophilic incubation for 48 h at 37°C. This product did not support growth of *L. monocytogenes* (probably due to the smoking processing step), but larger reductions in cell counts of *L. monocytogenes* throughout 27 days of storage were observed in the presence compared to the absence of DSM 18775 (FIG. 3).

[0079] Sensory descriptive triangle test carried out by a panel of 10 judges did not show any significant sensory effect of adding the bioprotective culture upon 11 days of storage or at the end of shelf life, i.e. after 21 days of storage.

Example 5. Application trial with *Lactobacillus curvatus* DSM 18775 on Wiener Sausages

[0080] The anti-listerial effect of DSM 18775 and the sensory impact of the culture were evaluated on Wiener Sausages. A 5 strain *L. monocytogenes* cocktail was added to the surface of the RTE meat product (10^3 CFU/g) followed by inoculation of the bioprotective culture (10^7 CFU/g). The sausages were vacuum packed and stored at 7°C for 27 days.

[0081] Cell counts of the bioprotective culture were determined by plating appropriate 10-fold dilutions made in peptone saline onto MRS-agar plates and incubating anaerobically for 3 days at 30°C. DSM 18775 proliferated on the product and reached approx. 10^8 CFU/g after 1 week of storage and approx. $5x10^8$ CFU/g by the end of storage.

[0082] *Listeria* cell counts were determined by plating appropriate 10-fold dilutions made in peptone saline onto listeria selective PALCAM agar plates followed by microaerophilic incubation for 48 h at 37°C. An instant bacteriocidal effect of DSM 18775 was found on *L. monocytogenes* and the reduced cell count was constant throughout storage in contrast to the pronounced growth of *L. monocytogenes* found in the absence of bioprotective culture in this RTE meat product (FIG. 4).

[0083] An agar well diffusion assay was used to detect bacteriocins produced by DSM 18775 in the Wiener Sausages using *Lactobacillus sakei as* indicator organism. Bacteriocin was extracted from the sausages by homogenizing with 0.02M HCl (1:2, w/v), centrifuging at 16,000 x g for 5 min at 5°C, adjusting the supernatant to pH 6.0 $\pm$ 0.1 with 1 N NaOH and filter sterilizing (0.45 μm). To verify the proteinaceus nature of the inhibitory substances, a solution of the proteolytic enzyme, Proteinase K, was applied next to the sausage extract in the agar well diffusion assay. Inhibition zones caused by a proteinaceus compound, presumably bacteriocin, were observed from extracts derived from Wiener Sausage after 11, 21 and 28 days of storage.

In sensory descriptive triangle tests carried out by a panel of 10 judges Wiener Sausages with added DSM 18775 were evaluated as slightly different, and fresher, than sausages without bioprotective culture after 11 days of storage, whereas no significant effect of DSM 18775 was found on the sensory quality after 21 days of storage, i.e. at the end of shelf life.

Example 6. Application trial with *Lactobacillus curvatus* DSM 18775 on cooked sliced ham

[0084] The anti-listerial effect of DSM 18775 and the sensory impact of the culture were evaluated on cooked sliced ham. A 5 strain *L. monocytogenes* cocktail was added to the surface of the RTE meat product (10^3 CFU/g) with or without the concomitant inoculation of the bioprotective culture (10^7 CFU/g). The product was packed in a modified atmosphere (30% CO_2 and 70% N_2) and stored at 5°C for 4 weeks.

[0085] Cell counts of the bioprotective culture were determined by plating appropriate 10-fold dilutions made in peptone saline onto MRS-agar plates and incubating anaerobically for 3 days at 30°C. DSM 18775 proliferated on the product and reached approx. 10^8 CFU/g by the end of storage.

[0086] *Listeria* cell counts were determined by plating appropriate 10-fold dilutions made in peptone saline onto listeria selective PALCAM agar plates followed by microaerophilic incubation for 48 h at 37°C. A clear bacteriocidal effect of DSM 18775 was found on *L. monocytogenes* as seen in Figure 6 with 1-2 $\log_{10}$ unit reductions observed throughout storage (FIG. 5).

[0087] In sensory descriptive triangle tests carried out by a panel of 8 judges, no significant differences were found in the sensory quality of the products in the presence compared to the absence of bioprotective culture throughout the storage period.

Example 7. Application trial with *Lactobacillus curvatus* DSM 18775 in Fresh Cheese

[0088] 20 kg whole milk (pasteurized at 72-73°C, homogenized and standardized to 3.0% of butter fat) were added 3.5×10^{10}CFU of the bioprotective culture DSM 18775, corresponding to approx. 10^7 CFU/g of cheese. At the time of inoculation the temperature of the milk was 35°C. DSM 18775 was pre-ripened in the milk for 30 min at 35 °C before addition of the rennet. The manufacture of the fresh cheese followed a standard protocol for fresh cheese: cutting the curd, heating the curd at 43°C for 30 min, drainage of 50% whey, dry salting to obtain a final salt content of 2% in the cheese. After manufacture, the cheese was divided into smaller pieces, vacuum packed separately and stored for 4 weeks at 9°C.

[0089] Cell counts of the bioprotective culture were determined by plating appropriate 10-fold dilutions made in peptone saline onto MRS-agar plates and incubating anaerobically for 3 days at 30°C. The initial cell count of DSM 18775 was 10-times lower than expected (approx. 10^6 CFU/g of cheese), but during storage, DSM 18775 proliferated in the cheese and reached approx. 10^8 CFU/g after 4 weeks.

[0090] Throughout storage, a faster reduction of pH was observed in the cheese with the added bioprotective culture, but by the end of storage no significant difference was observed in the cheese with or without the bioprotective culture as seen in FIG. 6.

[0091] A trained panel of three assessors evaluated the sensory impact of adding the bioprotective culture to fresh cheese. The cheeses were mainly characterized on taste and texture. The assessment was done at 10°C. The assessors described the cheese with the added DSM 18775 as fresher, more intense, with improved texture, compared to the cheese without the DSM 18775, which was characterized as neutral, tasteless and crumbling.

[0092] Bacteriocins produced by DSM 18775 in the Fresh Cheese during storage were determined in an agar well diffusion assay using *Lactobacillus sakei* NCFB 2714 as the indicator organism. Bacteriocin was extracted from the cheese by homogenizing with 0.02M HCL (1:5, w/v), centrifuging at 16,000 x g for 5 min at 5°C, adjusting the supernatant to pH 4.5 $\pm$ 0.1 with 1 N NaOH and filter sterilizing (0.45 μm). To verify the proteinaceus nature of the inhibitory substances, a solution of the proteolytic enzyme, Proteinase K, was applied next to the cheese extract in the agar well diffusion assay.

[0093] Bacteriocin was produced and detected in the fresh cheese during storage in increasing amounts during the first 3 weeks (Table 1) and in a concentration range expected to inhibit growth of *L. monocytogenes* in the cheese.

Table 1.

Day of analysis during storage	Bacteriocin activity in cheese with added DSM 18775 (unit/mg)	Bacteriocin activity in cheese without added DSM 18775 (unit/mg)
0	-	-
7	8	-
14	16	-

(continued)

Day of analysis during storage	Bacteriocin activity in cheese with added DSM 18775 (unit/mg)	Bacteriocin activity in cheese without added DSM 18775 (unit/mg)
21	32	-
28	32	-

Example 8. Comparison of *Lactobacillus curvatus* DSM18775 culture eluate with a commercially available culture eluate

[0094] The antilisterial effect of freeze dried eluate from DSM18775 was compared to the effect of a similar type of product from Danisco, MicroGARD 730, on sliced emulsion sausages.

[0095] A 5-strain cocktail of *Listeria monocytogenes* in final concentrations of approx. 5e03 CFU/g was added to slices of emulsion sausages. Solutions of culture eluates of DSM18775 and MicroGARD 730 dissolved in Milli-Q water and filter sterilized (0.2 mm) were added in final concentrations of 0.01 and 0.1% (w/w). As a control, the same volume of saline peptone instead of culture eluate was added to slices of emulsion sausage. The inoculated slices of emulsion sausages were packed in Modified Atmosphere (MA 30% CO2 + 70% N2) or vacuum packed and stored at 7°C.

[0096] After 1 and 7 days of storage, the MA-packed and vacuum-packed meat were examined for the content of listeria by making appropriate 10-fold dilutions in saline peptone and spread-plating on PALCAM agar plates followed by 2 days of microaerophilic incubation at 30°C. Slices without added listeria were sensory evaluated after 1 and 7 days of storage.

[0097] The results of the cell counts are presented in Figure 7 showing a superior ability of DSM18775 eluate to inhibit growth of *Listeria monocytogenes* under both packaging conditions. MicroGARD 730 only had a slight growth inhibitory effect at the highest concentration (0.1%) and under MA-conditions. Culture eluate of DSM18775, on the contrary, was able to prevent growth of *Listeria monocytogenes* at the high concentration of 0.1% under MA-packaging and almost prevented growth under vacuum.

[0098] Sensory evaluations showed no effect of either DSM18775 or MicroGARD on the color or the taste of emulsion sausage when MA- or vacuum packed. However, the sensory panel noted a slightly acidic odor of the vacuum-packed emulsion sausage slices with added MicroGARD 730.

Regarding Deposited Microbial Organisms [EXPERT SOLUTION, Rule 13 bis.6 (PCT)]

[0099] For all deposited microbial organisms mentioned in the present patent application the following applies.

[0100] As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms stated above only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

[0101] In particular it is requested, that regarding:

EUROPE

[0102] In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample, and approved either i) by the Applicant and/or ii) by the European Patent Office, whichever applies (Rule 32 EPC).

CANADA

[0103] The applicant requests that, until either a Canadian patent has been issued on the basis of an application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the Commissioner of Patents only authorizes the furnishing of a sample of the deposited biological material referred to in the application to an independent expert nominated by the Commissioner, the applicant must, by a written statement, inform the International Bureau accordingly before completion of technical preparations for publication of the international application.

AUSTRALIA

[0104] The applicant hereby gives notice that the furnishing of a sample of a microorganism shall only be affected prior to the grant of a patent, or prior to the lapsing, refusal or withdrawal of the application, to a person who is a skilled addressee without an interest in the invention.

SINGAPORE

[0105] The applicant hereby requests that the furnishing of a sample of a microorganism shall only be made available to an expert.

Claims

1. A strain of *Lactobacillus curvatus* deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under the accession number DSM 18775 **characterized in that** it has the ability at a temperature ranging from 2 to 10ºC of inhibiting the growth of at least one food-borne pathogenic bacterium without causing sensory changes in food.

2. The strain of *Lactobacillus curvatus* according to claim 1 wherein the food-borne pathogenic bacterium is *Listeria monocytogenes.*

3. The strain of *Lactobacillus curvatus* according to claim 1 or 2 which in addition to having the ability of inhibiting the growth of at least one food-borne pathogenic bacterium also has a bacteriocidal effect on at least one food-borne pathogenic bacterium at a temperature ranging from 2 to 10°C.

4. A composition for preserving a food product **characterized in that** it comprises the *Lactobacillus curvatus* strain according to any of the claims 1 to 3.

5. The composition according to claim 4, wherein the composition delays the development of food-borne pathogenic bacteria or spoilage bacteria.

6. The composition according to claim 4 or 5, wherein the food product is Ready-To-Eat (RTE) meat or a dairy product.

7. A process for preparing a composition capable of inhibiting the growth of at least one food-borne pathogenic bacterium said process comprising:

   (a) culturing cells of a strain of *Lactobacillus curvatus* as claimed in any of claims 1 or 3, which upon culturing in a culture medium produces a bacteriocin which has inhibitory activity against bacterial strains including *Listeria monocytogenes* to obtain a supernatant comprising the bacteriocin; and
   (b) separating the supernatant from the cultured cells to obtain the supernatant, thus obtaining a supernatant composition comprising the bacteriocin.

8. The process according to claim 7 which further comprises a drying step to obtain a dried culture eluate.

9. The process according to claim 8 wherein the drying step is freeze drying or spray drying.

10. The process according to any of claims 7 to 9, wherein an effective amount of the growth inhibiting composition has a bacteriocidal effect on the at least one food-borne pathogenic bacterium.

11. The process according to any of claims 7 to 10, wherein the at least one food-borne pathogenic bacterium is *Listeria monocytogenes.*

12. Use of a strain of *Lactobacillus curvatus* according to any of the claims 1 to 3 or the supernatant composition of step (b) of the process claim 7 for preserving food products.

13. Use of a strain of *Lactobacillus curvatus* according to any of the claims 1 to 3 or the supernatant composition of step (b) of the process claim 7 for inhibiting the growth of food-borne pathogenic bacteria or spoilage bacteria on

food products preserved in the refrigerated state at a temperature ranging from 2 to 10 ºC.

14. A method for controlling *Listeria* contamination in a food product, on food processing equipment, or on food storage containers, comprising applying the *Lactobacillus curvatus* strain with the accession number DSM 18775 **characterized in that** it has the ability at a temperature ranging from 2 to 10ºC of inhibiting the growth of at least one foodborne pathogenic bacterium without causing sensory changes in food, to a food product or food processing equipment or food storage containers in an amount sufficient to reduce the amount of *Listeria.*

Patentansprüche

1. Stamm von *Lactobacillus curvatus,* in der Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) unter der Zugangsnummer DSM 18775 hinterlegt, **dadurch gekennzeichnet, dass** er bei einer Temperatur von 2 bis 10°C fähig ist, das Wachstum mindestens eines durch Lebensmittel übertragbaren krankheitserregenden Bakteriums zu hemmen, ohne sensorische Veränderungen in Lebensmitteln zu verursachen.

2. Stamm von *Lactobacillus curvatus* nach Anspruch 1, wobei das durch Lebensmittel übertragbare krankheitserregende Bakterium *Listeria monocytogenes* ist.

3. Stamm von *Lactobacillus curvatus* nach Anspruch 1 oder 2, der zusätzlich zur Fähigkeit des Hemmens des Wachstums mindestens eines durch Lebensmittel übertragbaren krankheitserregenden Bakteriums auch bei einer Temperatur von 2 bis 10°C eine bakterizide Auswirkung auf mindestens ein durch Lebensmittel übertragbares krankheitserregendes Bakterium hat.

4. Zusammensetzung zum Konservieren eines Lebensmittels, **dadurch gekennzeichnet, dass** es den *Lactobacillus-curvatus*-Stamm nach einem beliebigen der Ansprüche 1 bis 3 umfasst.

5. Zusammensetzung nach Anspruch 4, wobei die Zusammensetzung die Entwicklung von durch Lebensmittel übertragbaren krankheitserregenden Bakterien oder Verderbnisbakterien verzögert.

6. Zusammensetzung nach Anspruch 4 oder 5, wobei das Lebensmittel essfertiges (RTE) Fleisch ist oder ein Milchprodukt.

7. Verfahren zur Herstellung einer Zusammensetzung, die fähig ist, das Wachstum mindestens eines durch Lebensmittel übertragbaren krankheitserregenden Bakteriums zu hemmen, wobei das Verfahren folgende Phasen umfasst:

(a) Kultivierung von Zellen eines Stammes von *Lactobacillus curvatus* nach einem beliebigen der Ansprüche 1 oder 3, der bei Kultivierung in einem Kulturmedium ein Bakteriozin produziert, das hemmende Wirkung gegen Bakterienstämme einschließlich *Listeria monocytogenes* hat, um einen Überstand mit dem Bakteriozin zu erhalten; und
(b) Trennung des Überstandes von den kultivierten Zellen, um den Überstand zu erhalten; man erhält so eine Überstandszusammensetzung mit dem Bakteriozin.

8. Verfahren nach Anspruch 7, das des Weiteren eine Trocknungsphase umfasst, um ein getrocknetes Kultureluat zu erhalten.

9. Verfahren nach Anspruch 8, wobei die Trocknungsphase Gefriertrocknung oder Sprühtrocknung ist.

10. Verfahren nach einem beliebigen der Ansprüche 7 bis 9, wobei eine wirksame Menge der wachstumshemmenden Zusammensetzung eine bakterizide Auswirkung auf das mindestens eine durch Lebensmittel übertragbare krankheitserregende Bakterium hat.

11. Verfahren nach einem beliebigen der Ansprüche 7 bis 10, wobei das mindestens eine durch Lebensmittel übertragbare krankheitserregende Bakterium *Listeria monocytogenes* ist.

12. Verwendung eines Stammes von *Lactobacillus curvatus* nach einem beliebigen der Ansprüche 1 bis 3 oder der Überstandszusammensetzung von Phase (b) des Verfahrensanspruchs 7 zum Konservieren von Lebensmitteln.

13. Verwendung eines Stammes von *Lactobacillus curvatus* nach einem beliebigen der Ansprüche 1 bis 3 oder der Überstandszusammensetzung von Phase (b) des Verfahrensanspruchs 7 zur Hemmung des Wachstums von durch Lebensmittel übertragbaren krankheitserregenden Bakterien oder Verderbnisbakterien auf Lebensmitteln, die im gekühlten Zustand bei einer Temperatur von 2 bis 10°C aufbewahrt werden.

14. Verfahren zur Steuerung der Listeria-Kontamination in einem Lebensmittel, auf Ausrüstungen für die Nahrungsmittelverarbeitung oder auf Lebensmittelvorratsbehältern, unter Anwendung des *Lactobacillus-curvatus*-Stamms mit der Zugangsnummer DSM 18775, **dadurch gekennzeichnet dass** er bei einer Temperatur von 2 bis 10°C fähig ist, das Wachstum mindestens eines durch Lebensmittel übertragbaren krankheitserregenden Bakteriums zu hemmen, ohne sensorische Veränderungen in Lebensmitteln zu verursachen, in einem Lebensmittel oder auf Ausrüstungen für die Nahrungsmittelverarbeitung oder Lebensmittelvorratsbehältern in ausreichender Menge, um die Menge an *Listeria* zu reduzieren.

Revendications

1. Souche de *Lactobacillus curvatus* déposée à la Collection Allemande de Microorganismes et de Cultures Cellulaires (DSMZ) sous le numéro d'entrée DSM 18775 **caractérisée en ce qu'**elle a la capacité à une température allant de 2 à 10°C d'inhiber la croissance d'au moins une bactérie pathogène d'origine alimentaire sans provoquer des changements sensoriels dans les aliments.

2. Souche de *Lactobacillus curvatus* selon la revendication 1 où la bactérie pathogène d'origine alimentaire est *Listeria monocytogenes.*

3. Souche de *Lactobacillus curvatus* selon la revendication 1 ou 2 qui en plus d'avoir la capacité d'inhiber la croissance d'au moins une bactérie pathogène d'origine alimentaire a aussi un effet bactéricide sur au moins une bactérie pathogène d'origine alimentaire à une température allant de 2 à 10°C.

4. Composition pour la conservation d'un produit alimentaire **caractérisée en ce qu'**elle comprend la souche de *Lactobacillus curvatus* selon l'une quelconque des revendications 1 à 3.

5. Composition selon la revendication 4, où la composition retarde le développement de bactéries pathogènes d'origine alimentaire ou bactéries d'altération.

6. Composition selon la revendication 4 ou 5, où le produit alimentaire est de la viande prête à manger (RTE) ou un produit laitier.

7. Procédé pour préparer une composition capable d'inhiber la croissance d'au moins une bactérie pathogène d'origine alimentaire, ledit procédé comprenant:

   (a) cultiver des cellules d'une souche de *Lactobacillus curvatus* selon l'une quelconque des revendications 1 ou 3, qui lors de la culture dans un milieu de culture produit une bactériocine qui a de l'activité inhibitoire contre des souches bactériennes comprenant *Listeria monocytogenes* pour obtenir un surnageant comprenant la bactériocine; et
   (b) séparer le surnageant des cellules cultivées pour obtenir le surnageant, obtenant ainsi une composition surnageante comprenant la bactériocine.

8. Procédé selon la revendication 7 qui comprend en outre une étape de séchage pour obtenir un éluat de culture séchée.

9. Procédé selon la revendication 8 où l'étape de séchage est la lyophilisation ou le séchage par atomisation.

10. Procédé selon l'une quelconque des revendications 7 à 9, où une quantité efficace de la composition inhibitrice de la croissance a un effet bactéricide sur l'au moins une bactérie pathogène d'origine alimentaire.

11. Procédé selon l'une quelconque des revendications 7 à 10, où l'au moins une bactérie pathogène d'origine alimentaire est *Listeria monocytogenes.*

12. Utilisation d'une souche de *Lactobacillus curvatus* selon l'une quelconque des revendications 1 à 3 ou de la composition surnageante de l'étape (b) de la revendication de procédé 7 pour la conservation de produits alimentaires.

13. Utilisation d'une souche de *Lactobacillus curvatus* selon l'une quelconque des revendications 1 à 3 ou de la composition surnageante de l'étape (b) de la revendication de procédé 7 pour inhiber la croissance de bactéries pathogènes d'origine alimentaire ou de bactéries d'altération sur des produits alimentaires conservés dans l'état réfrigéré à une température allant de 2 à 10°C.

14. Méthode pour contrôler la contamination par *Listeria* dans un produit alimentaire, sur un équipement de traitement d'aliments, ou sur des récipients de stockage d'aliments, comprenant l'application de la souche de *Lactobacillus curvatus* avec le numéro d'entrée DSM 18775 **caractérisée en ce qu'**elle a la capacité à une température allant de 2 à 10°C d'inhiber la croissance d'au moins une bactérie pathogène d'origine alimentaire sans provoquer des changements sensoriels des aliments, à un produit alimentaire ou un équipement de traitement d'aliments ou des récipients de stockage d'aliments en une quantité suffisante pour réduire la quantité de *Listeria.*

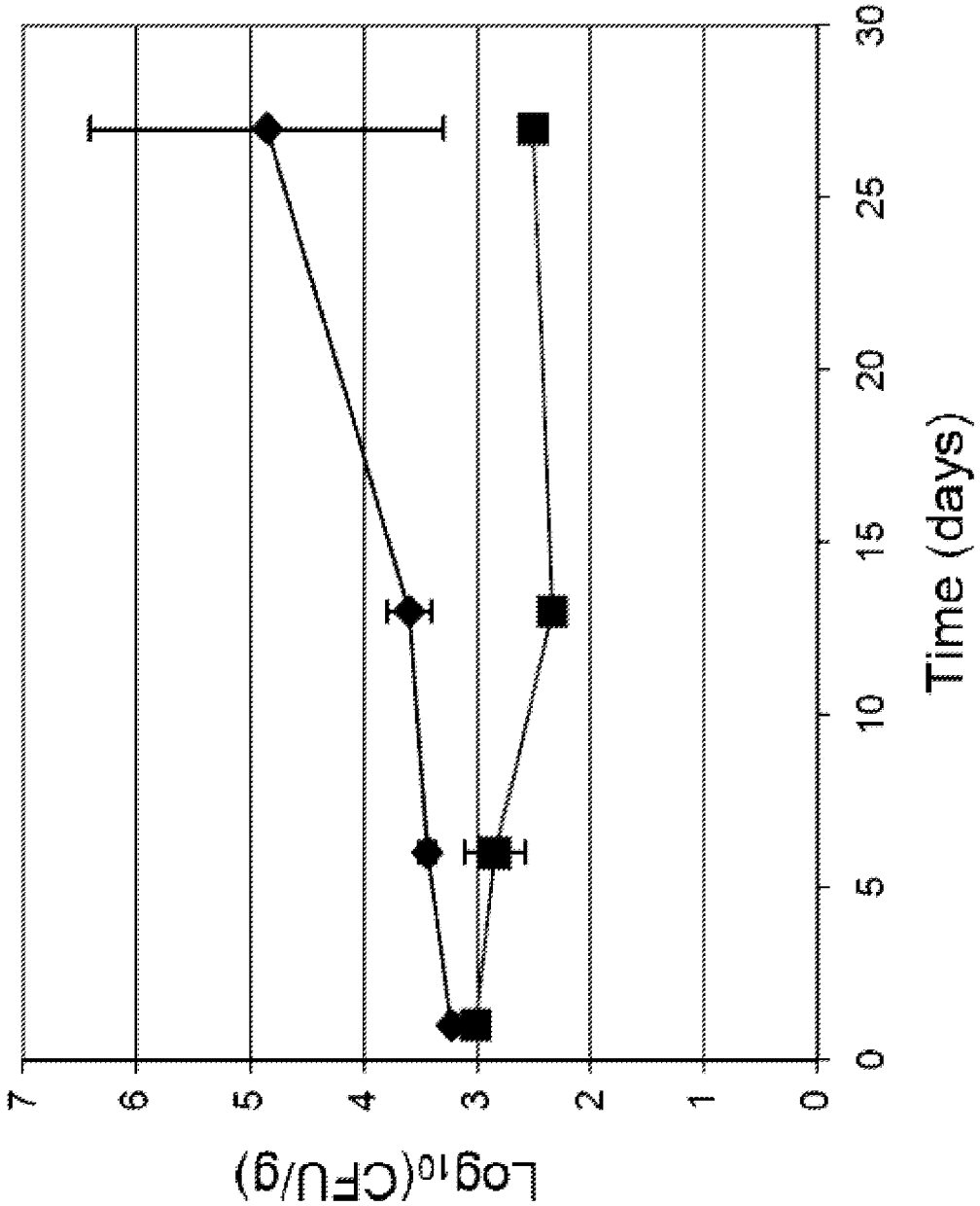

Fig. 1

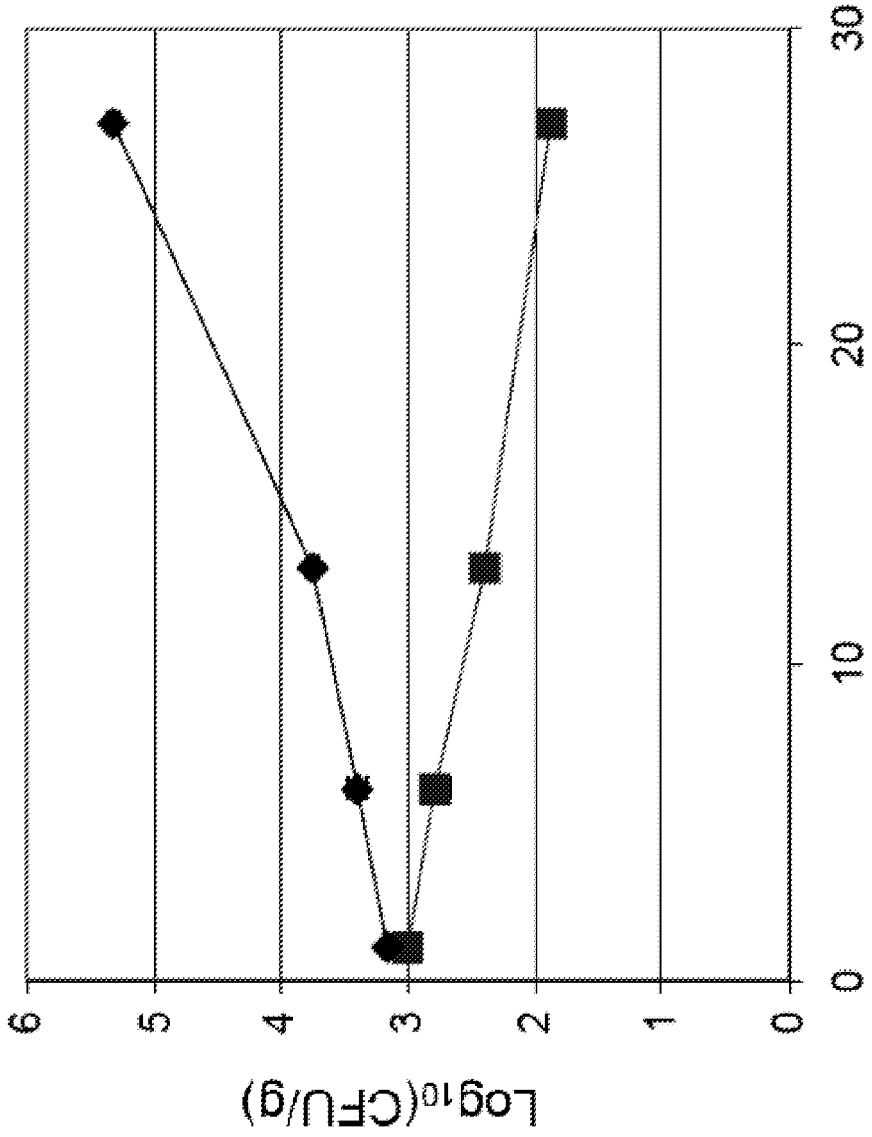

Fig. 2

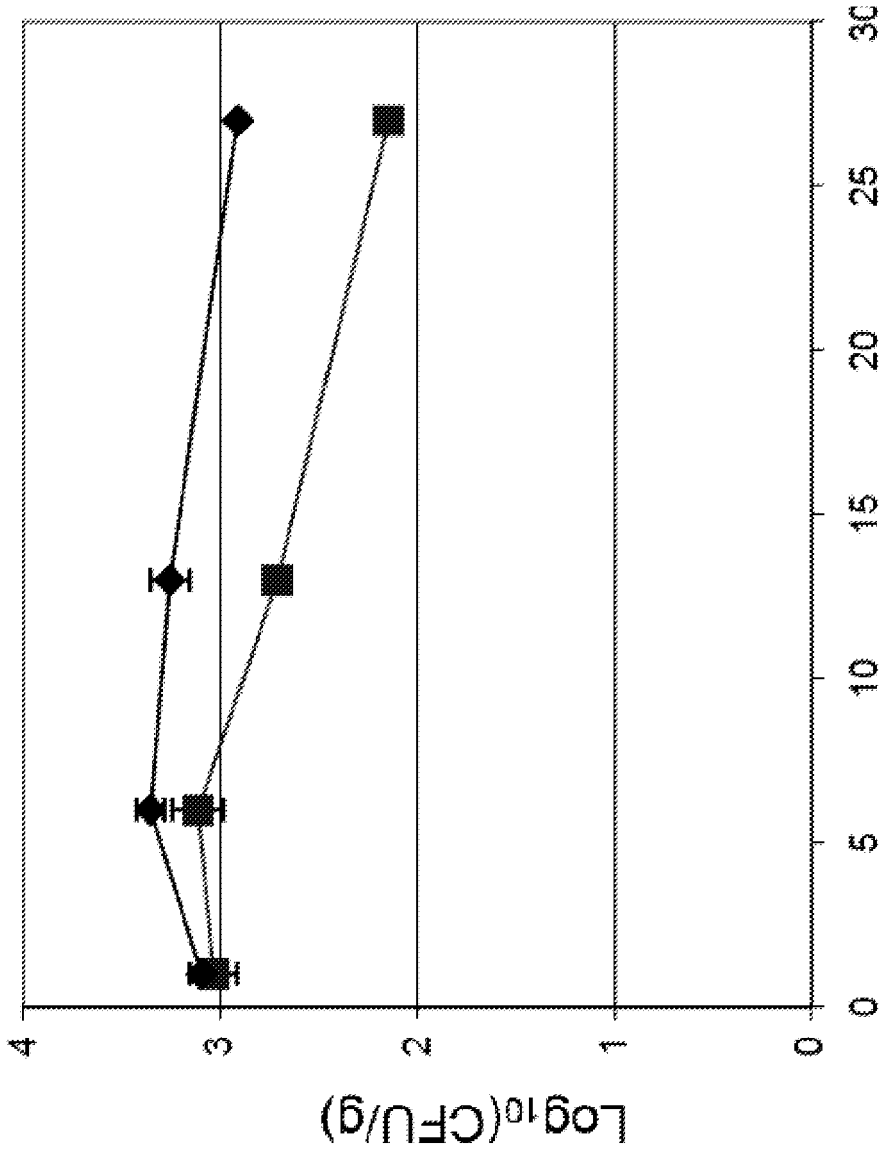

Fig. 3

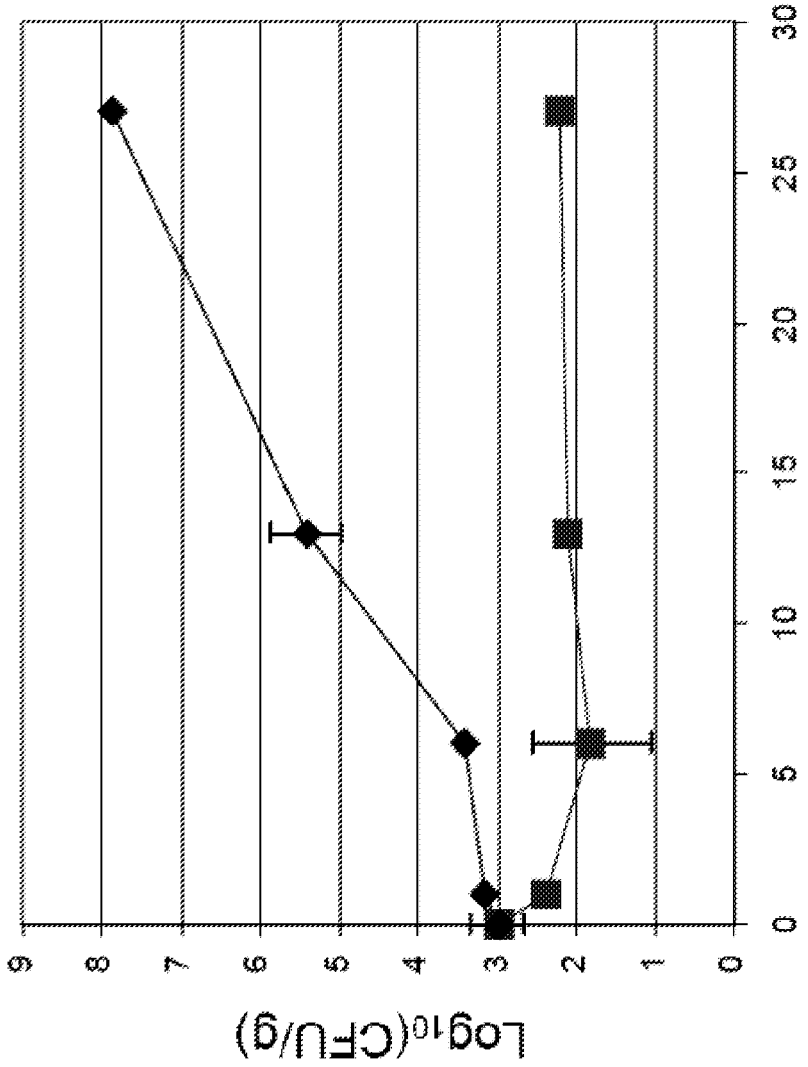

Fig. 4

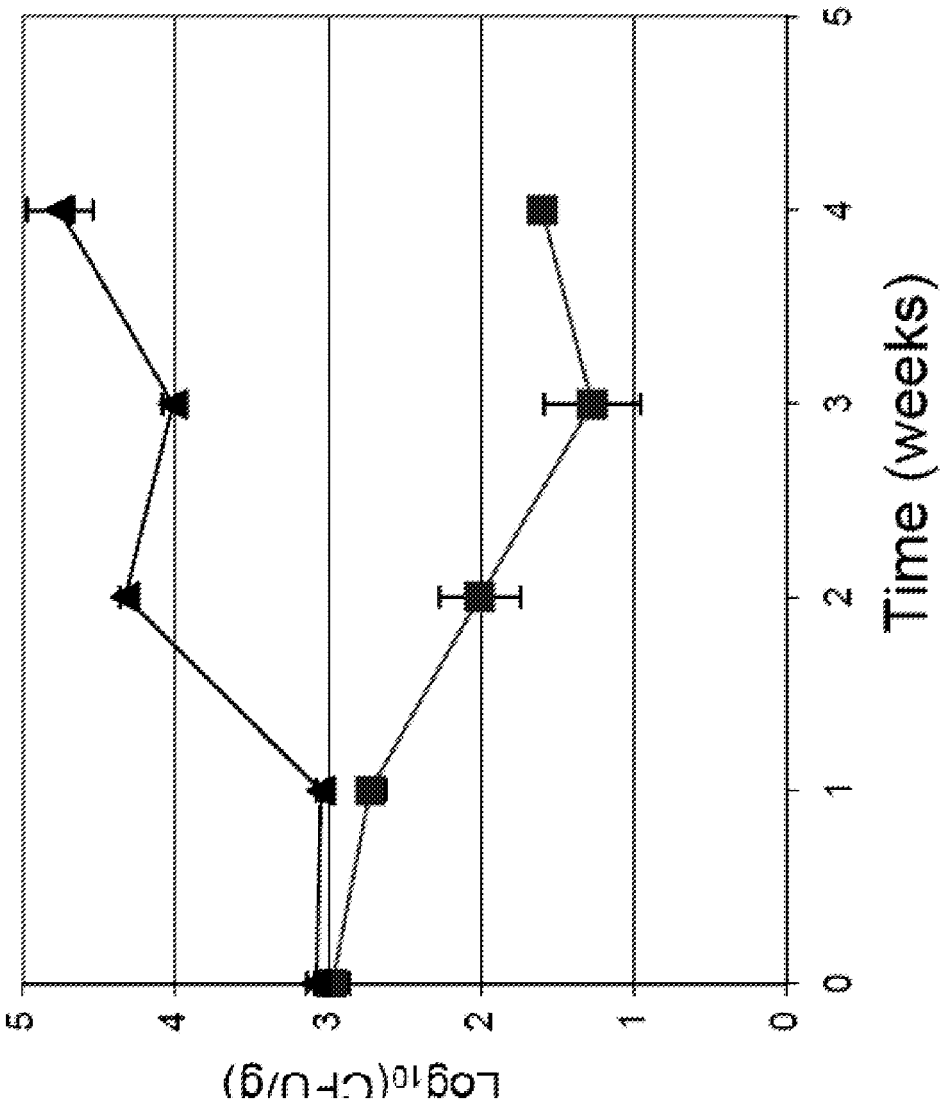

Fig. 5

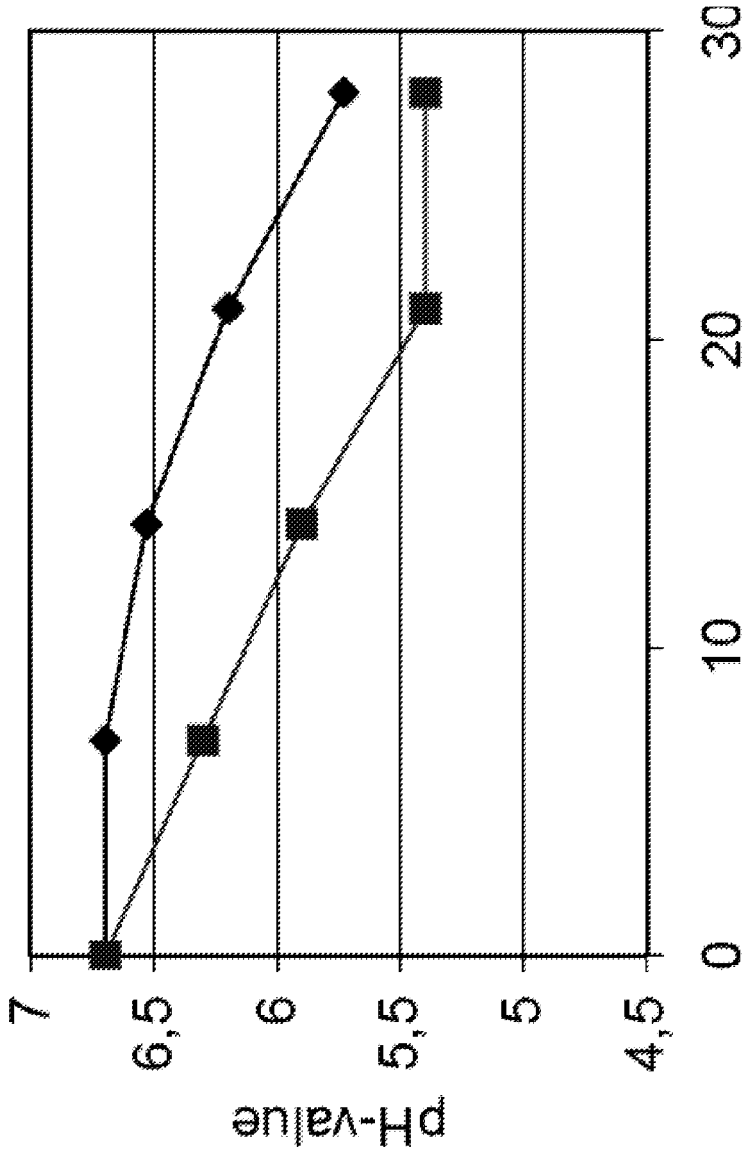

Fig. 6

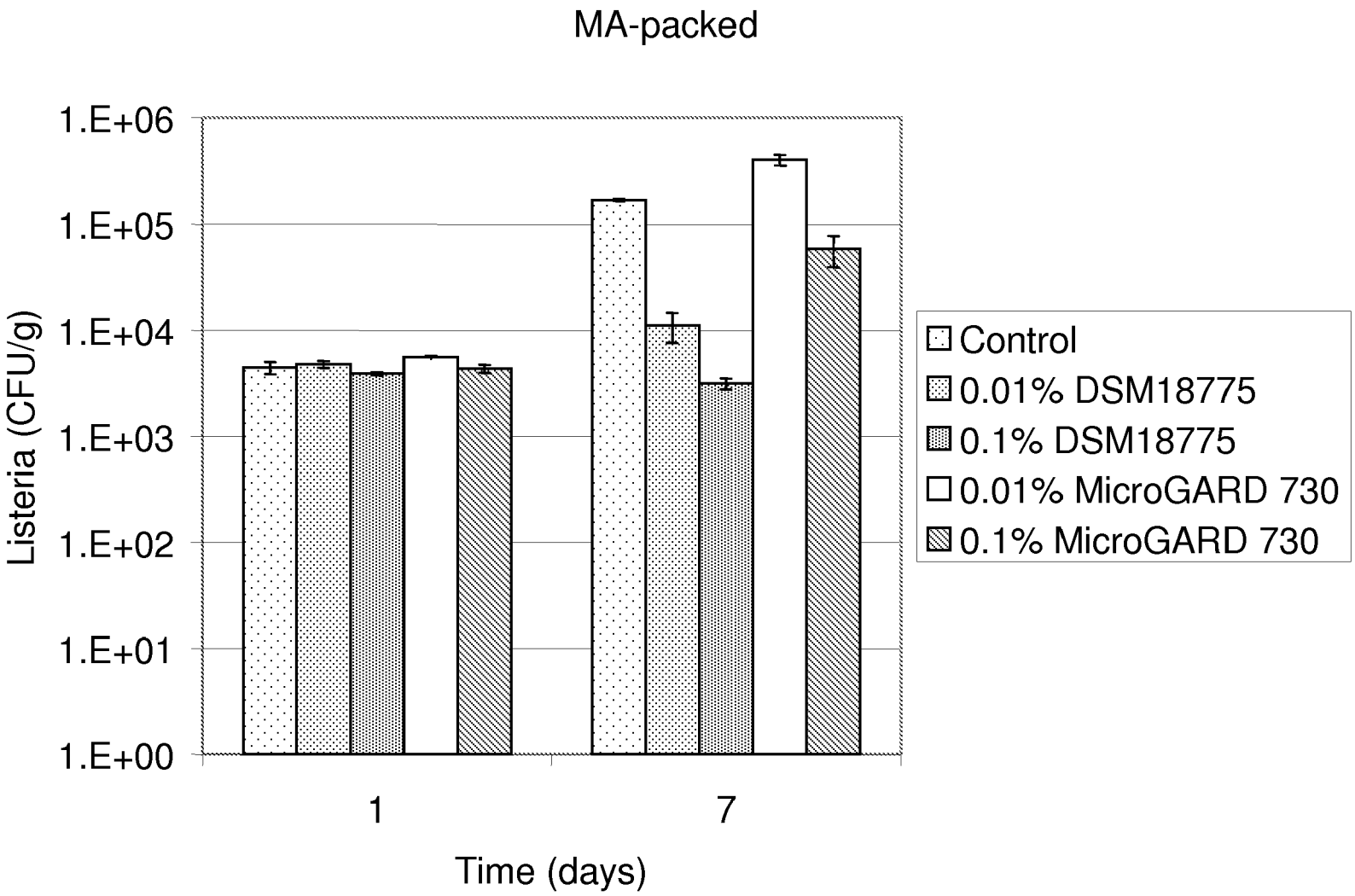
MA-packed
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Listeria (CFU/g)
1
7
Time (days)
Control
0.01% DSM18775
0.1% DSM18775
0.01% MicroGARD 730
0.1% MicroGARD 730

Fig. 7a

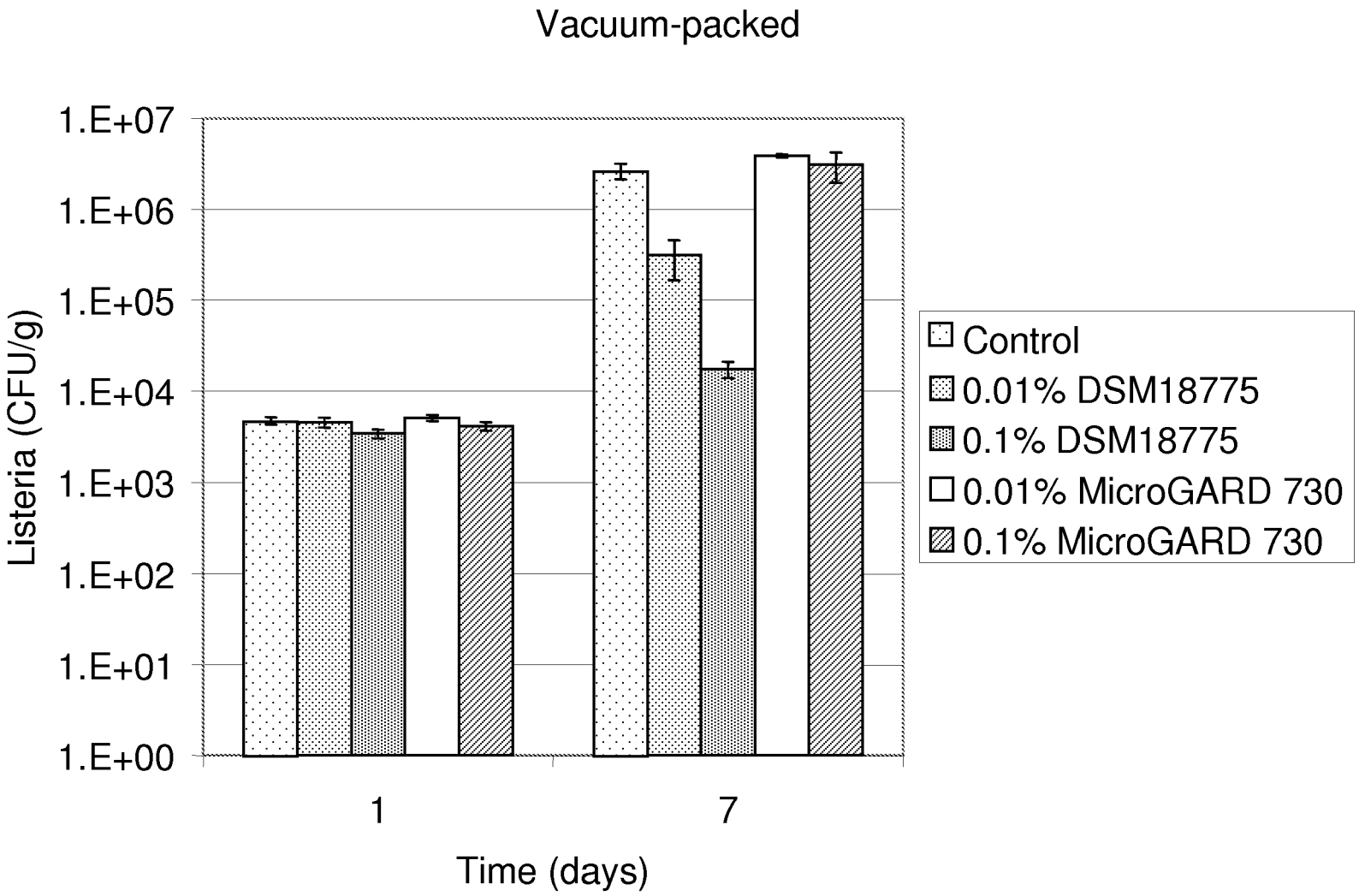
Vacuum-packed
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Listeria (CFU/g)
1
7
Time (days)
Control
0.01% DSM18775
0.1% DSM18775
0.01% MicroGARD 730
0.1% MicroGARD 730

Fig. 7b

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- EP 1475432 A **[0008]**
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- EP 0640291 A **[0010]**

Non-patent literature cited in the description

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- **VERMIEREN, L. et al.** Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products. *International Journal of Food Microbiology,* 2004, vol. 96, 149-164 **[0005]**
- **DICKS, L.M.T. et al.** Use of bacteriocin-producing starter cultures of Lactobacillus plantarum and Lactobacillus curvatus in production of ostrich meat salami. *Meat Science,* 2004, vol. 66, 703-708 **[0007]**
- **VOGEL, R.F. et al.** *System. Appl. Microbiol.,* 1993, vol. 16, 457-462 **[0011]**
- **BENKERROUM, N. et al.** *J. Appl. Microbiol.,* 2005, vol. 98, 56-63 **[0012]**
- **MAURIELLO, G. et al.** *J. Appl. Microbiol.,* 2004, vol. 97, 314-322 **[0013]**
- **MATARAGAS et al.** *Food Microbiology,* 2003, vol. 20, 259-265 **[0014]**

Safety Data Sheet

SafePro® B-LC-48

Version: 5 GHS / EN
Revision Date: 03-30-2016

1. IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND OF THE COMPANY/UNDERTAKING

1.1 Product identifier

Product name: SafePro® B-LC-48
Material No: 706142

1.2 Relevant identified uses of the substance or mixture and uses advised against

Application: Bacteria for production of meat products.
Spray application only in closed systems or in clean rooms (ISO-14644-(1 - 8)).

1.3 Details of the supplier of the safety data sheet

Supplier:
Chr. Hansen Inc.
9015 West Maple Street
53214-4298 Milwaukee - WI
Phone: +1 414 607-5700

Headquarters:
Chr. Hansen A/S
Boge Allé 10-12
DK-2970 Horsholm
Tel. +45 45 74 74 74

1.4 Emergency telephone number

Emergency telephone: +45 45 74 74 74

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2. HAZARDS IDENTIFICATION

2.1 Classification of the substance or mixture

The product is not classified.

2.2 Label elements

Signal Word None.

Hazard statements None

Precautionary statements None

2.3 Other hazards

Physical and Chemical Hazards:
Small quantities: The hazardous properties of the product are considered to be limited. Large quantities: In high concentrations, fine particles may form explosive dust/air mixtures.

Human health:
Dust may irritate the eyes and the respiratory system. Inhalation of high concentrations of dust or aerosols may cause toxic alveolitis. Frequent inhalation of dust over a long period of time increases the risk of developing lung diseases.

The product does not contain any carcinogenic substances in amounts to be declared.

Environment:
The harmful effects of the product in the environment are considered to be limited.

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3. COMPOSITION/INFORMATION ON INGREDIENTS

3.2 Mixtures
The product contains: bacteria.

Does not contain substances that must be indicated according to current regulations.

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4. FIRST-AID MEASURES

4.1 Description of first aid measures

Inhalation: Dust inhalation: Move into fresh air and keep at rest.
In case of inhalation of spray mist: Move person into fresh air and keep at rest.
In case of persistent throat irritation or coughing: Seek medical attention and take along these instructions.

Skin contact: Remove contaminated clothing immediately and wash skin with soap and water.

Eye contact: Do not rub eye. Immediately flush with plenty of water for up to 15 minutes. Remove any contact lenses and open eyelids widely. If irritation persists: Seek medical attention and bring these instructions.

Ingestion: Immediately rinse mouth and drink plenty of water.

4.2 Most important symptoms and effects, both acute and delayed
Dust may irritate the eyes and the respiratory system. Inhalation of high concentrations of dust or aerosols may cause toxic alveolitis. Frequent inhalation of dust over a long period of time increases the risk of developing lung diseases.

4.3 Indication of any immediate medical attention and special treatment needed
Symptomatic treatment.

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5. FIRE-FIGHTING MEASURES

5.1 Extinguishing media
Use fire-extinguishing media appropriate for surrounding materials.

5.2 Special hazards arising from the substance or mixture
Potential dust explosion hazard. Fine dust dispersed in air in sufficient concentrations and in the presence of an ignition source is a potential dust explosion hazard.
The explosion limits and the flash point are stated in section 9.

5.3 Advice for firefighters
Selection of respiratory protection for fire fighting: follow the general fire precautions indicated in the workplace.

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6. ACCIDENTAL RELEASE MEASURES

6.1 Personal precautions, protective equipment and emergency procedures
Avoid generation and spreading of dust. Avoid inhalation of dust and aerosols. Avoid contact with skin and eyes. Follow precautions for safe handling described in this safety data sheet.

6.2 Environmental precautions
Avoid discharge into drains, water courses or onto the ground.

6.3 Methods and material for containment and cleaning up
Powder: Use a vacuum cleaner. If not possible, moisten dust with water before it is collected with shovel, broom or the like. Dust Deposits should not be allowed to accumulate on surfaces, as these may form an explosive mixture if they are released into the atmosphere in sufficient concentration. Avoid dispersal of dust in the air.
Liquid: Flush contaminated area with plenty of water.

6.4 Reference to other sections
For personal protection, see section 8.
For waste disposal, see section 13.

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7. HANDLING AND STORAGE

7.1 Precautions for safe handling
Safe handling advice: Avoid inhalation of dust and aerosols. Avoid contact with skin and eyes. Observe good industrial hygiene practices.

Technical measures: Keep the workplace clean. Avoid generation, spreading and accumulation of dust.
Large quantities: Ensure appropriate exhaust and ventilation at machinery and at places where dust can be generated. Take precautionary measures against static discharges when there is a risk of dust explosion.

Technical precautions: Mechanical ventilation may be required.
Spray application: Mechanical ventilation or local exhaust ventilation is required.

7.2 Conditions for safe storage, including any incompatibilities
Store in tightly closed original container at a temperature: Store at deep frozen temperature conditions. For detailed information consult PI sheet.

Technical measures for safe storage: Risk of dust explosion: Comply with the regulations on protection against dust explosions.

---

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

8.1 Control parameters
Exposure limits are listed below. No data - no exposure limits noted for ingredient(s).
Occupational exposure limits:
Total dust: 15 mg/m^3. Respirable dust: 5 mg/m^3.
Chr. Hansen company limit, microorganisms: 10,000 CFU/m^3.

8.2 Exposure controls

Engineering measures: Provide adequate ventilation. Use work methods which minimize dust production.
Spray application: Observe the Chr. Hansen company limit and minimize the risk of inhalation of aerosols.

Respiratory equipment: In case of inadequate ventilation or risk of inhalation of aerosols: Use respiratory equipment with particle filter:
EU: FFP3 filter [e.g. 3M 8835 mask]
US: P100 filter [e.g. 3M 8293 mask]
For daily use of more than 3 hours a respirator with a powered air blower should be used.

Hand protection: Gloves are recommended for prolonged use.

Eye protection: Use tight fitting goggles if dust is generated.

Skin protection: No special precautions.

Hygiene measures: Wash hands after contact.

Environmental Exposure Controls: None.

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9. PHYSICAL AND CHEMICAL PROPERTIES

9.1 Information on basic physical and chemical properties

Appearance:	Powder, ground
Color:	Off-white to brownish
Odor:	Slightly meaty
pH:	5,00 - 7,00
Melting point:	Not relevant
Boiling point:	Not relevant
Decomposition temperature:	Not relevant
Flash point:	Not relevant
Relative density:	No data available
Solubility:	Water soluble suspension

9.2 Other information
No information available.

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10. STABILITY AND REACTIVITY

10.1 Reactivity
None known.

10.2 Chemical stability
Stable under normal temperature conditions and recommended use.

10.3 Possibility of hazardous reactions
None known.

10.4 Conditions to avoid
Increased temperatures and humidity.

10.5 Incompatible materials
None known.

10.6 Hazardous decomposition products
None known.

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11. TOXICOLOGICAL INFORMATION

11.1 Information on toxicological effects

Inhalation: Dust may irritate respiratory system. Inhalation of high concentrations of dust or aerosols may cause toxic alveolitis. Symptoms like fever, coldshivering, coughing, difficulties in breathing, headache, muscle and joint pains etc. may appear 6 to 8 hours after exposure. The symptoms normally disappear completely over night without any treatment.

Skin contact: Dust has an irritating effect on moist skin.

Eye contact: Dust in the eyes will cause irritation.
Ingestion: May irritate and cause malaise.

Specific effects: Frequent inhalation of dust over a long period of time increases the risk of developing lung diseases.
The product does not contain any carcinogenic substances in amounts to be declared.

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12. ECOLOGICAL INFORMATION

12.1 Ecotoxicity
The harmful effects of the product in the environment are considered to be limited.

12.2 Persistence and degradability
The product is expected to be biodegradable.

12.3 Bioaccumulative potential
Bioaccumulation: Is not expected to be bio-accumulable.

12.4 Mobility in soil
The product is water soluble and may spread in water systems.

12.6 Other adverse effects
None known.

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13. DISPOSAL CONSIDERATIONS

13.1 Waste treatment methods
Dispose of waste and residues in accordance with local authority requirements.

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14. TRANSPORT INFORMATION

The product is not covered by international regulations on the transport of dangerous goods (IMDG, IATA, DOT).

14.1 UN number -

Air (ICAO/IATA):
14.3 Transport hazard class(es) -
14.4 Packing group -

Sea (IMDG):
14.3 Transport hazard class(es) -

14.4 Packing group -
EmS -
MFAG -

Land (DOT):
14.3 Transport hazard class(es) -
14.4 Packing group -

14.5 Environmental hazards
Marine pollutant (IMDG): -

14.6 Special precautions for user
None known.

14.7 Transport in bulk according to Annex II of MARPOL 73/78 and the IBC Code
Not relevant.

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15. REGULATORY INFORMATION

15.1 Safety, health and environmental regulations/legislation specific for the substance or mixture

NFPA: Health: 1 Fire: 3 Reactivity: 0 Other: -

GHS regulation
Globally Harmonized System of Classification and Labelling of Chemicals (GHS)

---

16. OTHER INFORMATION

The user must be instructed in the proper work procedure and be familiar with the contents of these instructions.

The following sections contain revisions or new statements : 1, 2, 4, 6, 7, 8.

Wording of Hazard Statements
-

BactoFlex® B-LC-48

Certificate of Analysis

Form:	
Material No:	713185
Batch no:	3377673
Date of Manufacture:	11.2017
Best Before Date:	11.2018

Performance	**Result**	**Specification**
Total cell count cfu/g	4.6E+11	>4.0E+10

Purity	**Result**	**Specification**
Bacillus cereus cfu/g	<100	<100
Enterobacteriaceae cfu/g	<10	<10
Enterococci cfu/g	<100	<1000
Staphylococcus aureus cfu/g	<1	<50
Yeasts and moulds cfu/g	<100	<100
Listeria monocytogenes *	* See note below	Absent in 25 g
Salmonella spp. *	* See note below	Absent in 25 g

* Production is systematically tested on an ongoing basis - details can be supplied on request

Electronically generated - signature not required

22 pages removed in accordance with the Privacy Act of 1974.