Carnobacterium divergens M35 Culture for Use as a bio-preservative in fish product

CARNOBACTERIUM DIVERGENS M35 CULTURE FOR USE AS A BIO-PRESERVATIVE IN FISH PRODUCTS
Submitted by Fumoir Grizzly Inc.

To: Office of Food Safety Additive (HFS-200), Center for Food Safety and Applied Nutrition, Food and Drug Administration

March 2022

GRIZZLY

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Part 1: Signed Statement and Certification

1.1 Statement of Intent

We want to submit a GRAS notice concerning the microorganism Carnobacterium divergens M35 used as a protective culture in ready-to-eat fish product (excluding catfish in this document) in accordance with the regulation 21 CFR §170, subpart E of the US Food and Drug Administration.

1.2 Name and Address of the Organization

Fumoir Grizzly Inc.
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Québec, Canada, G3A 2V5
Contact name:
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+1 (418) 878-8941 extension 26
mt@grizzly.qc.ca

1.3 Name of the Notified Substance

Carnobacterium divergens M35 is also known as BacM35, which is the commercial name of the bio-ingredient. It can be included in the ingredient list as "Bacterial culture".

1.4 Intended Conditions of Use

The bio-preservative preparation containing *Carnobacterium divergens* M35 cells and its metabolites, including the bacteriocin *divergicin* M35, is intended for the inhibition of *Listeria monocytogenes* in fresh, frozen or smoked fish (excluding catfish) See part 7.2. Its efficiency was scientifically demonstrated in cold-smoked Coho salmon, as well as in frozen tuna. The microorganism can be applied to fresh or frozen fish (catfish specifically excluded in this submission). The lyophilized microorganism shall be re-suspended in water of suitable microbiological quality (drinking water or higher grade of water) and applied by spraying or mixing to obtain a final concentration of 10^6 CFU (colony forming unit) per gram of product. The treated product can be stored at 4 °C or frozen during its respective conservation time.

There is no specific subpopulation expected to consume the microorganism, the targeted consumer is the general population.

1.5 Statutory basis for Conclusion of GRAS Status

The strain *Carnobacterium divergens* M35 GRAS status is based on scientific procedures §170.30(a) and (b) and literature information regarding the safety of the species *divergens* and the scientific data generated by experts from a research group at Université Laval, Québec, Canada and Innodal through a collaborative research project with Fumoir Grizzly. Based on all this information, there is a certitude that viable *Carnobacterium divergens* M35 is GRAS under the intended condition of use. Also, the bio-preservative preparation has already been accepted by Health Canada as a New Food Additive, as an

Antimicrobial Preservative, in Sliced Ready-to-Eat Cold-Smoked Salmon and Sliced Ready-to-Eat Cold-Smoked Trout (Reference Number: NOP/AVP-0018: https://www.canada.ca/en/health-canada/services/food-nutrition/public-involvement-partnerships/proposal-use-new-food-additive-carnobacterium-divergens-antimicrobial-preservative-sliced-ready-cold-smoked-salmon-sliced.html, consulted December 1st, 2021).

1.6 Premarket Approval Requirements

The bio-preservative is not subject to the premarket approval requirements of Federal Food drugs and Cosmetic act, because it is thought to be Generally Recognized as Safe (GRAS) under the conditions of use and it does not technically affect food from a nutritional and toxicological point of view. It does not impart color to the food or changes it's chemical and physical properties.

1.7 Availability of Information and Data

We agree to make the data and information available for the evaluation committee. When requested, available data will be sent (hard or electronic copy) to the committee and we allow the evaluation committee to review and copy the data and information. Fumoir Grizzly may be contacted at the address indicated in point #2, during business hours.

1.8 Disclosure under the Freedom of Information Act

Any data and information in Parts 2 through 7 of this GRAS notice are not exempt from disclosure under the Freedom of Information Act,5 U.S.C.552.

1.9 Certification

Based on the information and the best of our knowledge, we certify that our GRAS notice is complete, representative and balanced. It includes favorable and unfavorable information known to us and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

1.10 Signature

Signature of responsible official: 

Printed name and title: LAURA BOIVIN

Date (mm/dd/yyyy): 04/07/2022

If necessary, we authorize to send any trade secret to the Food Safety and Inspection Service of the U.S Department of Agriculture.

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

2.1 Scientific Data and Information that Identifies the Notified Substance

2.1.1 Identification of the strain, its metabolites and information substantiating the identification

The strain concerning this New Substances Notification form is *Carnobacterium divergens* M35. Isolate M35 was identified as *Carnobacterium divergens* by phenotypic characteristics, carbohydrate fermentation profile (API Gallery) and genetic identification (1). Numerous strains of lactic acid bacteria, isolated from ready-to-eat fish and seafood product, have demonstrated an inhibitory effect against the pathogen *Listeria monocytogenes*. *Carnobacterium divergens* M35 has shown higher potential and was therefore selected to make a protective culture for use in ready-to-eat food. Using standard methods of characterization, the biochemical and physiological profiles (e.g. production of D or L lactic acid, ability to grow at low temperatures) has been examined in order to establish its presumptive taxonomical classification. Isolate M35 was also selected for its significant inhibitory activity against *L. monocytogenes*.

Furthermore, using targeted primers (Cb1/Cb2) to amplify a specific ribosomal RNA sequence 340 base pairs in length, this isolate has been found to belong to the genus *Carnobacterium* (see figure 1). The full genome is not currently available.

Other primers were then used to amplify sequences peculiar to known species of *Carnobacterium* which allowed us to identify a 199-bp amplicon characteristic of the species *divergens*, obtained using the primer pair 27F/cdi (see figure 2) (2). Isolate M35 was thus assigned to the species *Carnobacterium divergens*.

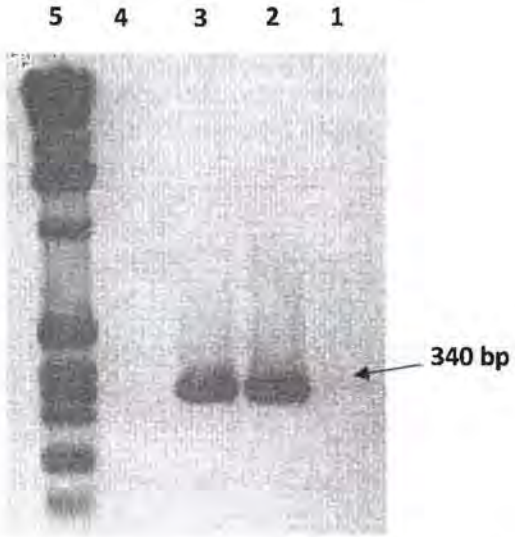


Figure 1. Genotyping of isolate M35 using primer pair Cb1/Cb2r to amplify a sequence of *Carnobacterium* 16S rRNA by PCR. Lane 1 – *Lactobacillus farciminis*; lane 2 – M35; lane 3 – *Carnobacterium piscicola* CS74; lane 4 – negative control; lane 5 – molecular mass mark

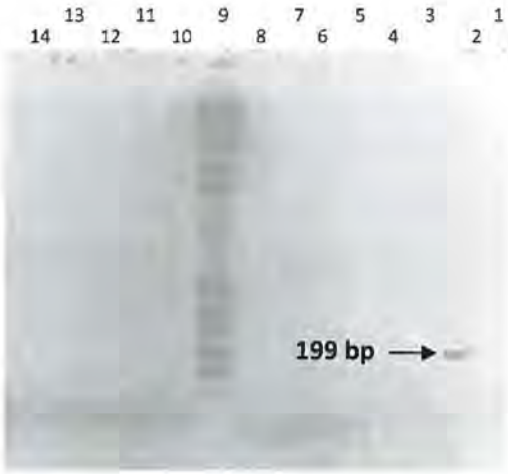


Figure 2. Genotyping of isolate M35 using PCR to amplify 16S rRNA with universal primer 27f and primers Cdi, Cmo, Cpg and Cga specific for *Carnobacterium* species *divergens*, *mobile*, *piscicola/gallinarum* and *gallinarum* respectively.

Isolate *C. divergens* M35 was deposited at the International Depository Authority of Canada in Winnipeg, Manitoba, under the number ADI 050404-01 (https://www.canada.ca/en/public-health/programs/international-depository-authority-canada.html, consulted December 1st, 2021).

Carnobacterium divergens M35 is known to produce the bacteriocin divergicin M35 (1). Bacteriocins are small peptides produced by bacteria that possess targeted antimicrobial activity against other genus and/or species of bacteria (3). This bacteriocin is 43 amino-acid long, as a molecular weight of 4525 Da and is classified as a class IIa bacteriocins, which are known for their consensus motif YGNGV (4). More precisely, the sequence of divergicin M35 is TKYYGNGVYC NSKKCWVDWG TAQGCIDVVI GQLGGGIPGK GKC and its Uniprot access number is P84962 (https://www.uniprot.org/uniprot/P84962, consulted January 10th, 2022).

2.1.2 The strain history

No genetic modifications have been done to the strain. It is used as it was isolated from frozen smoked mussels naturally found in the waters of St-Lawrence River in Gaspé, Québec, Canada. Bivalve shellfish are known to accumulate some marine toxins through their feeding process. Those bio toxins can cause different types of illness such as paralytic shellfish poisoning (PSP), amnesic shellfish poisoning caused by domoic acid (ASP) and diarrhetic shellfish poisoning (DSP) (5).

2.1.3 Methods that can be used to distinguish and detect the micro-organism

C. divergens M35 is a non-motile gram-positive rod lactic acid bacterium, non-spore forming, growing in short chains, catalase and oxidase negative. It produces only L-lactic acid and very little gas. Growth happens at 5° C but not at 45°C with an optimal growth temperature of 30°C, it can tolerate high amounts of salts and metabolize arginine at a glucose concentration of 0.5%. It appears that the growth of the strain is self-limited at 10^9 CFU (colony forming unit) in MRS broth at 30°C for 24h. On TSA media supplemented with 0.6% of Yeast Extract or MRS Agar, after 48h of incubation at 30°C, the colonies have a round shape, white opaque color and small size (1-2 mm). If put in broth (TSBYE) or STYA (fermentation media: 5g/L Sodium acetate, 5g/L dextrose, 43 g/L Yeast extract and 1g/L polysorbate 80), the supernatant

is loaded with bacteriocin and it can inhibit the growth of *Listeria monocytogenes* in microplate or soft agar assay.

To assess that the inhibitory activity of the strain was due only to the presence of the bacteriocin divergicin M35 and not other toxicants or metabolites, the antimicrobial activity of strain M35 was compared to another strain of *Carnobacterium divergens* (ATCC 35677) that does not produce bacteriocin (6). The results showed that the strain ATCC 35677, which does not produce any bacteriocin, has no effect on *Listeria monocytogenes* in a challenge test experiment or in an agar-well diffusion assay (see figure 3 and 4). So, the antimicrobial properties of strain M35 is attributable to its bacteriocin production.

Figure 3. Agar-well diffusion assay showing zones of inhibition of L. innocua HPB13 by (A) C. divergens M35 MRS culture supernatant concentrated 10-fold, (B) divergicin M35 bio-ingredient obtained from C. divergens M35 SCH culture supernatant concentrated 10-fold, (C) purified divergicin M35, (D) C. divergens M35 un-concentrated MRS culture supernatant and (E) C. divergens ATCC 35677 MRS culture supernatant. From Tahiri et *al.* 2009 (6).

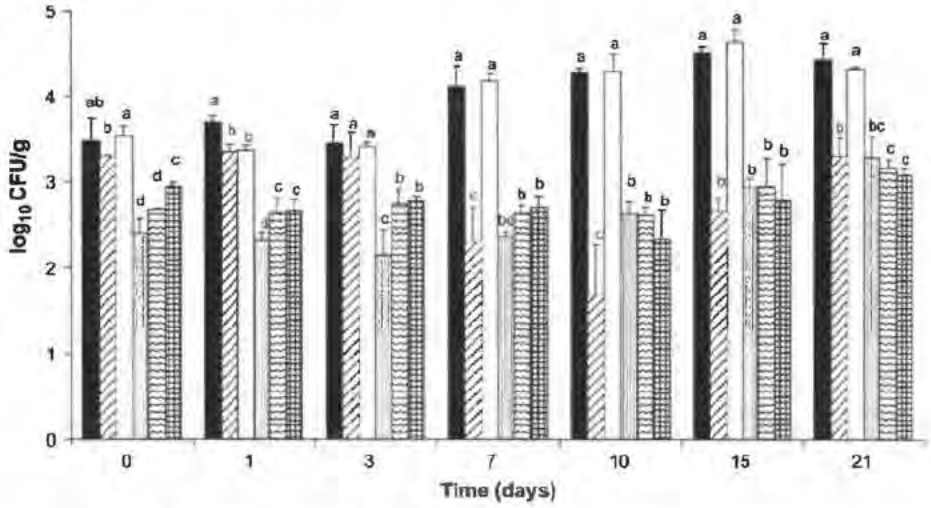


Figure 4. Evolution of viable *Listeria monocytogenes* LSD532 count in cold-smoked salmon inoculated with L. monocytogenes and stored at 4 °C for 21 days.☒Control (treatment A, always at zero here)■L. monocytogenes alone (treatment B),▨L. monocytogenes as a co-culture with C. divergens M35 (treatment C),□L. monocytogenes as a co-culture with C. divergens ATCC 35677 (treatment D),▥L. monocytogenes with purified divergicin M35; 50 mg/g (treatment E),▩L. monocytogenes with divergicin M35 bio-ingredient (treatment F) and ▦L. monocytogenes with MRS culture supernatant concentrated 10-fold

(treatment G). Error bars indicate standard deviations of treatments and duplicate enumerations per treatment. Different letters at the same time point indicate significant difference ($P < 0.05$). From Tahiri et *al.* 2009 (6).

Finally, two methods may be used for the specific detection and monitoring of *C. divergens* in foods. The first is based on a selective medium developed by Wasney et al. (7). CTSI agar contains sucrose, manganese sulfate, thallium acetate, inulin, thiamine hydrochloride, vancomycin, and nisin. On this medium, *C. divergens* produces highly characteristic pink colonies. Using a top layer of tryptic soy soft agar inoculated with *Listeria ivanovii* (a non-pathogenic organism), M35 colonies that produce divergicin can be counted specifically. The second method is based on the amplification of a specific DNA sequence of 199 base pairs in length using the primer pair 27F/cdi. The details of this molecular method are described in scientific articles published by Barakat et al., Tahiri et al. 2004 and 2009 (1, 2, 6). These two methods have been successfully used for detection and monitoring *C. divergens* M35 in cold and frozen smoked salmon. Finally, its ability to grow at pH 9 and low tolerance to acidic media can also be used to distinguish *Carnobacterium* from other lactic acid bacteria (8).

2.2 Method of manufacture of the notified substance

2.2.1 Culture media ingredients

An optimized culture medium (STYA) is used for the manufacture of the *C. divergens* M35-based bio-preservative composed of:

- Sodium acetate - **(5g/L)** (Cas No 127-09-3)
- Dextrose - **(5g/L)** (Cas No 50-99-7)
- Yeast extract - **(43g/L)** (Cas No 8013-01-2)
- Polysorbate 80 - **(1g/L)** (Cas No 9005-65-6)
- Maltodextrin - **(150g/L)** (Cas No 9050-36-6)

Each ingredient is certified food grade and allergen free by the manufacturer, following good manufacturing practices (GMP).

2.2.2 Fermentation conditions

- **Inoculation strain : *Carnobacterium divergens* M35**
- Inoculation volume ratio: 0.5 %
- Incubation temperature: 30-37 °C
- Agitation: 100 rpm
- pH controlled at 7.0 using NH_4OH solution (10M)
- Fermentation time: about 18h

Seed culture is kept at -80°C in fresh culture media supplemented with 20% glycerol to protect the cells. Each culture is started from the seed culture and propagated only three times to ensure genomic stability.

Under these culture conditions, an optical density of 1.04, equivalent to about 3×10^9 CFU/mL, is reached within 18 h. Based on agar diffusion and critical dilution methods, the inhibitory activity of the resulting culture broth is about 2.1×10^6 AU/ml, which corresponds to an inhibition zone diameter of 23 mm.

2.2.3 Stabilizing the *C. divergens* M35-based bio-preservative for storage

Composition of the lyophilization or spray-dried medium:

- Culture broth obtained from the preceding production step
- sucrose: 5-10 % w/v

The mixture is frozen at –25 °C then lyophilized or directly spray-dried under the following conditions:

Lyophilization:
- Lyophilization time: 48h
- Vacuum conditions: < 30 mTorr

Spray drying:
- Temperature during spray drying: between 180 to 110°C entrance

The product should be stored at 4 °C in the dark.

2.2.4 Labelling

Labelling shall be done according the figure 5.

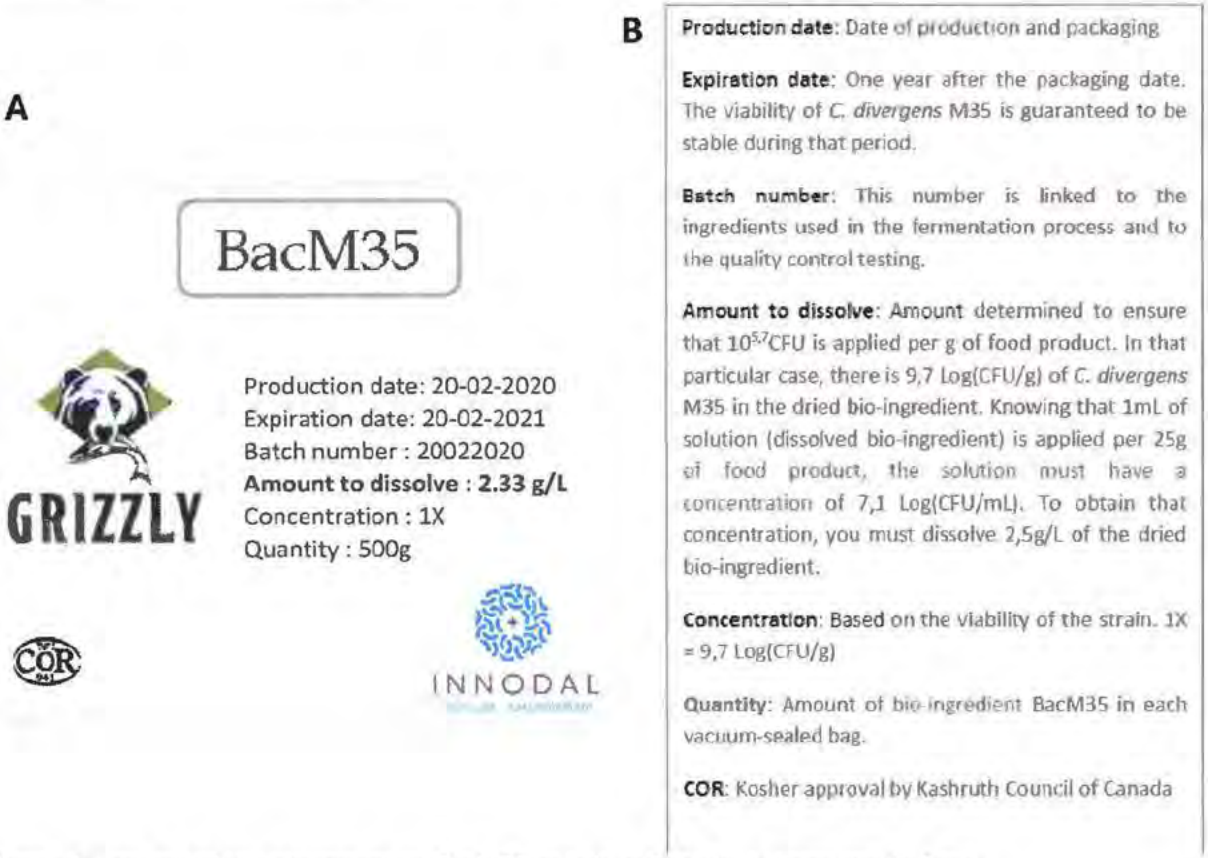
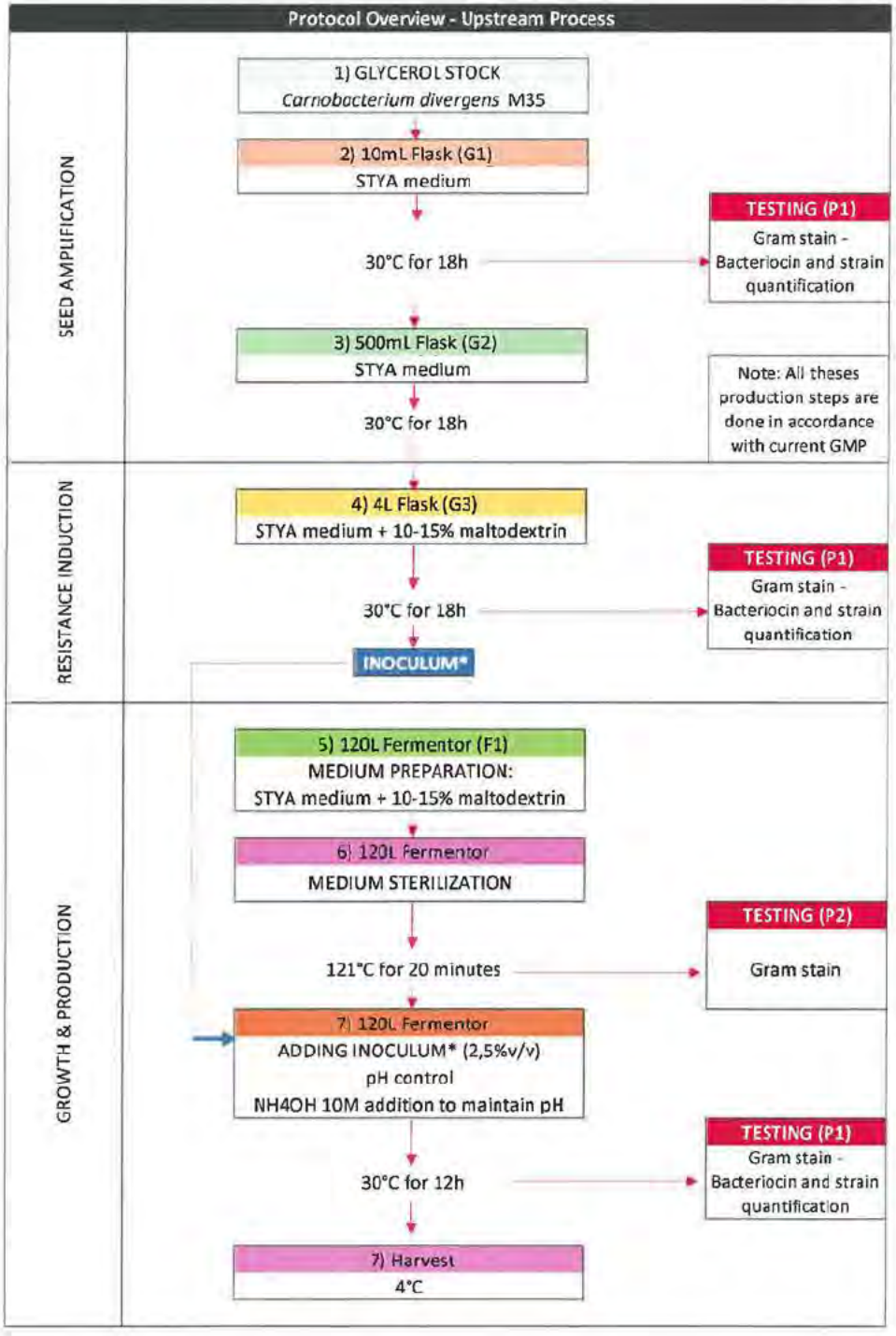
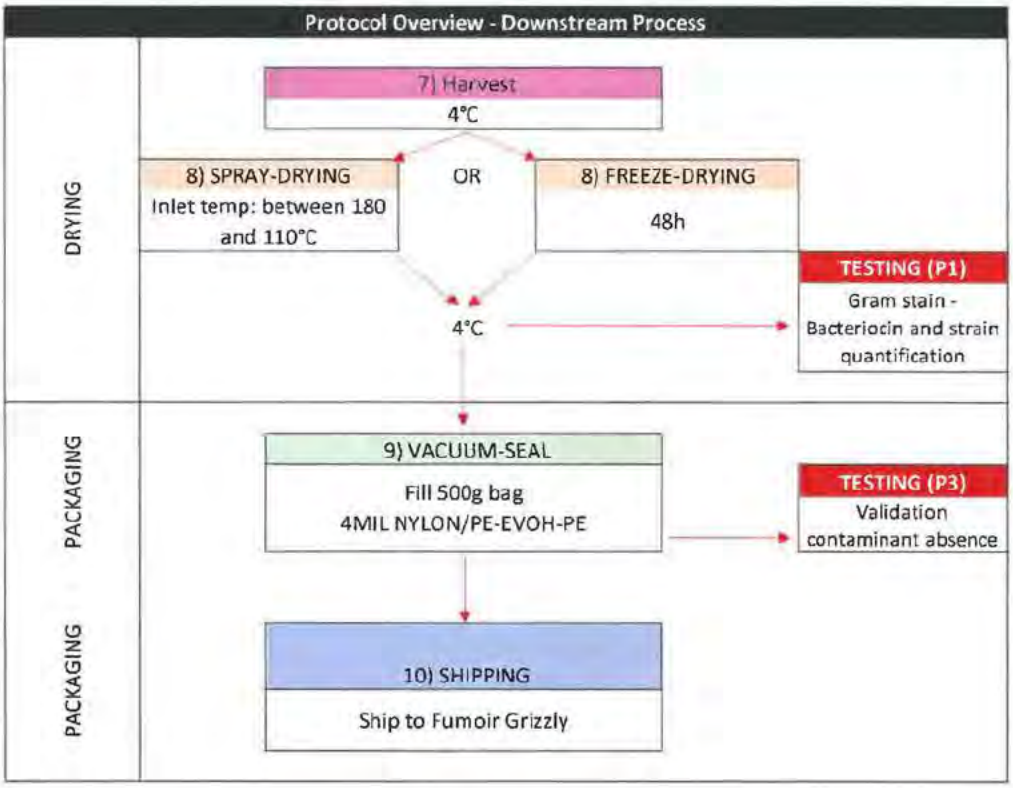


Figure 5. Labeling of the bio-ingredient BacM35 as sold in Canada. A: Front and B: Back.

The manufacturing process for the organism is described below. The purity of the culture is ensured by working in aseptic conditions and thorough microbiological testing (see below) between key steps of the process. Once lyophilized, the strain inoculum is stable for one years à 4 °C. All the ingredients are food grade and certified allergen free and thus, the bio-ingredient is produced in accordance with current good manufacturing practices (cGMP).

2.2.5 Manufacturing process flow



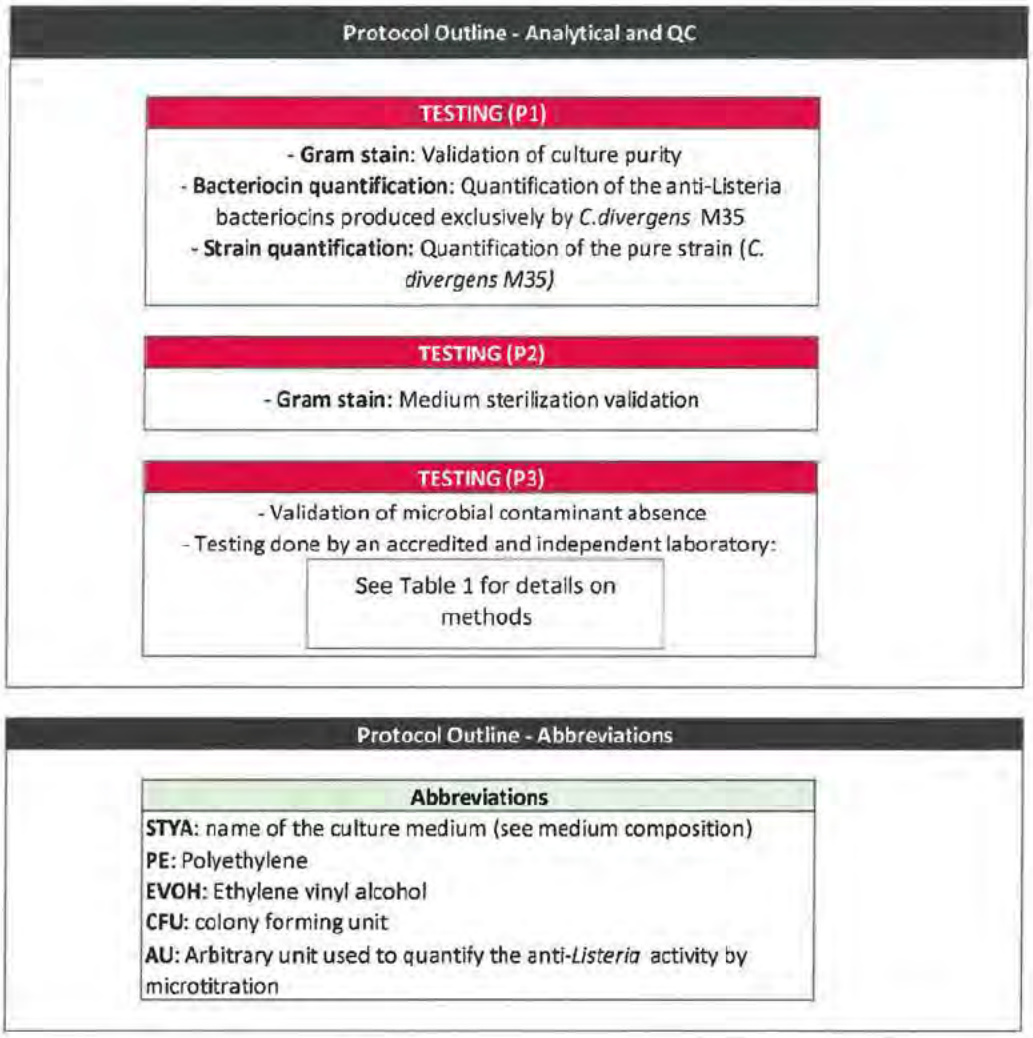


Protocol Outline - Medium recipes
Bio-ingredient composition

STYA - All fermentation steps
Yeast extract
Table sugar
Sodium acetate
Polysorbate 80
Every preparations (G1-G2-G3-F1) are sterilized at 121 °C during 20min

Note: All ingredients are food grade and their quality certificates are kept for each lot to maintain the traceability of these ingredients

Bio-ingredient final composition
Fermented STYA
10-15% maltodextrin
Carnobacterium divergens M35 9,7log(CFU/g)
Divergicin M35 Bacteriocin produced by *C. divergens* M35 $1,6x10^6$ AU/g
Trace amount of others metabolites and fermentative acids



2.2.6 The physical state of the microorganism

The microorganism is sold as a fine orange/yellow powder of lyophilized (or spray-dried) fermentation culture.

2.2.7 Concentration and viability of the microorganism in the formulation

The microorganism is produced to a concentration of 8Log to 9Log viable cells per gram of powder.

2.2.8 Identification and concentration of other ingredients and of any contaminants in the formulation

The formulation contains only culture media ingredients (Sodium acetate - (5g/L), Dextrose - (5g/L), Yeast extract - (43g/L), Polysorbate 80 - (1g/L), Maltodextrin - (150g/L), cells from the fermentation and the bacteriocin produced from this fermentation. The product is regularly analyzed for pathogens and heavy metals (see Table 1).

2.2.9 Description of any recommended storage and disposal procedures

The product should be stored at 4°C or -20°C for up to one years. The product can be disposed of in waste waters.

2.3 Specifications of food grade material

Every material and ingredient used for the manufacture of BaM35 is certified food grade. Certificates of the ingredients are present in Part 7. Table 1 resumes the testing done to each batch produced to monitor the microbiological contaminants and the presence of heavy metals.

- Seed and resistance induction steps: All these steps are carried out in glass falcons (Borosilicate) which are washed beforehand before adding the culture medium and sterilized in an autoclave (121°C for 15min) before inoculation of the strain. All the fermentation residues or remainders are then sterilized in a decontamination cycle (121°C for 45min) before being cleaned / reused.

- Growth and production steps: These two stages are carried out in one and the same fermenter which has been previously washed (CIP) and sterilized (SIP) before filling it with culture medium. This fermenter is of pharmaceutical grade (SS316) and allows the control of temperature, agitation, pH, as well as the monitoring of these data. The system also allows aseptic transfer to polypropylene transfer containers.

- Drying step: The drying step can be done by freeze-drying or by spray-drying depending on the availability of the equipment. In the case of lyophilization, the culture medium is placed on SS316 drying trays and dried for 48 hours in order to obtain dry crystals of product in a food grade freeze dryer. In the case of atomization, the liquid product is poured into a transfer tank in SS316. The spray dryer pumps the medium and atomizes it into a drying tank. The product dries instantly in a powder form and is recovered by a cyclone in a holding tank.

- Packaging step: The dried product is then packed in plastic bags under vacuum impermeable to oxygen. Generally, the product is portioned per 500g packet and vacuum packed in a vacuum bell.

Table 1. Methods for microbiological testing and monitoring the presence of heavy metals and contaminants.

Method	Specification	Limits
MFHPB-18	Aerobic Bacteria Count	10Log
MFHB-22	Yeast and Moulds	<20 cfu/g
MFHPB-21	*Staphylococcus aureus*	<25 cfu/g
MFLP-09	Enterobacteria	<10 cfu/g
MFLP-06	*Salmonella* spp.	Absent in 25g
MFHPB-72	*Listeria monocytogenes*	Absent in 25g

MFHPB-73	*Escherichia coli* O157:H7	Absent in 25g
SOP MET-101-6102F	Mercury Analysis MA. 200 Hg 1.1	<0.2
SOP FC-102-1501F	Arsenic (Food) AOAC MET-101-6107F	<0.1
SOP FC-102-1512F	Cadmium (Food) AOAC MET-101-6107F	<0.1
SOP FC-102-1512F	Lead (Food) AOAC MET-101-6107F	<0.1

2.3.1 Description of the location of manufacturing facilities in Canada

The first stages of preculture takes place at the Innodal microbiological laboratory (201 Mgr-Bourget, Lévis (QC) G6V6Z3). Innodal also has a license (L-R2-80312-18-KC-00) for its biosafety level 2 laboratory. This laboratory possesses all the equipment required for these steps, but also to perform all types of manipulations typical of classical microbiology. Product efficacy tests are carried out in this laboratory. The team there is composed of microbiologists and laboratory technicians. All access is controlled with key-cards, registry of entry, security cameras and security guards.

Innodal performs the final fermentation step, drying and packaging of the product at Saint-Hyacinthe Research and Development Center (AAC). This is a pilot-sized plant allowing fermentation productions of 100-300L and it has all the equipment required to dry the product, as well as its packaging. These are food grade installations and equipment that are frequently inspected by the MAPAQ. The equipment is therefore rented and operated by the Innodal team. The maintenance and cleaning of the equipment are carried out by the AAFC technical team according to standardized protocols. https://profils-profiles.science.gc.ca/en/research-centre/saint-hyacinthe-research-and-development-centre, consulted January 10th, 2022)

2.4 Safety, relevant data and information bearing on the physical or technical effect

2.4.1 Safety of the strain and relevant data

2.4.1.1 Life cycle

As mentioned previously, *Carnobacterium divergens* M35 is non-motile gram-positive rod, non-spore forming, growing in short chains, catalase and oxidase negative. It produces only L-lactic acid and very little gas. Growth happens at 5°C but not at 45°C with an optimal growth temperature of 30°C, it is facultatively anaerobic and it can tolerate high amounts of salts. It can metabolize arginine at a glucose concentration of 0.5% (8). On TSA media supplemented with 0.6% of Yeast Extract or MRS agar, after 48h of incubation at 30°C, the colonies have a round shape, white opaque color, shiny texture and small size (1-2 mm).

The genus *Carnobacterium* is composed of 9 species (*divergens, alterfunditum, funditum, gallinarum, inhibens, maltoramaticum, mobile, pleistocenium, viridans)* of which the species *divergens* is the type species of the genus. The genus is classified within the Lactic acid bacteria group and is generally found on food of animal origin and living fish, but also in Antarctic ice lakes, permafrost ice, Canadian winter soil and compost (9, 10). It seems to be ubiquitous to cold/temperate soil and water. *Carnobacterium divergens* has also been isolated from dairy products (10).

Bacteria from the *Carnobacterium* genus are psychrotolerant and can survive freeze-thaw cycles (8). They are indeed well adapted to cold temperatures due to cold adapted enzymes such as the β-galactosidase from *C. maltoramaticum* and alanine dehydrogenase in *Canobacterium* sp. (11, 12). They are able to survive in a wide range of environment because they can metabolise many different carbohydrate sources (8, 10) and can survive high pressure treatment and a wide pH variation (10). Not many studies have been done to understand the underlying mechanisms of its survival adaptation, but the sequencing of the genomes of some isolate showed a bigger genome size compared to other LABs. This suggests the presence of unknown genes that could contribute to its tolerance to harsh environments (10).

As the species *divergens* has been isolated from the gastrointestinal track of live fish, it can also adapt to seafood since *C. divergens* M35 has been isolated on commercial frozen smoked mussels in Gaspé, Qc, Canada (1). Also, *C. divergens,* has been isolated very often and is found is many ecological niches (vaccumed-paked meat, intestine of live fish, sphagnum pond and alpine permafrost) (10). *Carnobacterium divergens* M35 needs a rich culture media to grow, the high protein content requirement suggests that this strain is more adapted to a food ecological niche (meat product or intestine) than ice lakes and permafrost (10, 13). As seen by the growth curve (figure 6), *C. divergens* M35 has a lag phase of 5 hours and an exponential phase of 5 hours in MRS broth when cultured at 30°C with an inoculum of 0.1% (13).

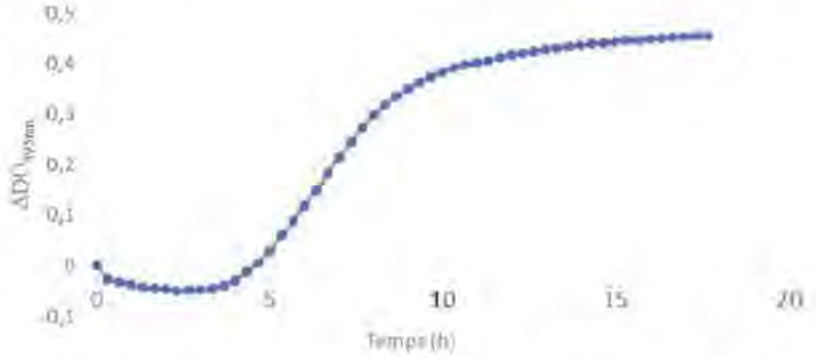


Figure 6. Growth curve of *Carnobacterium divergens* M35 in MRS broth at 30°C with an inoculum of 0.1% (13).

2.4.1.2 Infectivity, pathogenicity to non-human species and toxicity

A Published cases of bacteremia

Only two cases of bacteremia from *Carnobacterium divergens* has been reported in a woman and a male (14, 15). It is important to notice that the woman affected by that bacterium was previously very ill

from diabetes and exterior factors, such as severe undernutrition and chronic alcoholism that are known to weaken the body. It is also suspected that the bacteremia was caused by a contamination of the feeding tube or the solution given to the woman by enteral feeding and not by direct consumption. During enteral feeding, the stomach is usually bypassed, and the bacteria is not exposed to digestive fluids. In this case, the strain was directly in contact, in a very high concentration with the weaken intestine of a very ill woman, so we believe the infection was a very rare case of opportunistic infection, as it is the case for the male patient.

In this case, a 65 years-old male previously operated for a lymphoma removal surgery was admitted to the hospital due to a fever. Later, a blood culture uncovered the presence of *Carnobacterium divergens* and the presumed cause of the infection was thought to be the operation (15). So far other infections of other members of the *Carnobacterium* gender are very rare are always thought to be opportunistic from a cut or other contact with water of food (see table 2). People with systemic infections are always associated with risk factors that can make them susceptible to opportunistic infections and it is very important to note that the infection can be cured with a course of antibiotics without any known resistance. Finally, *Carnobacterium divergens* is naturally found in dairy products, meat and fish and no other case has been reported in the literature. Also, its approval and consumption in Canada since June 7th,2016 has not caused an increase of infection or any new reported case (notice number NOP/AVP-0018).

Table 2. Literature review of human infections with *Carnobacterium* spp. isolated in cultures.

Case	Age/ Sex	Country	Possible risk factors	Type of culture/ body Site	Isolate	Presentation	Treatment	Outcome
1	35/ M	Czech Republic	None	Wound swab of abscess	Mixed flora with *C. piscicola*	Traumatic hand from water sawmill	Amputation, debridement; Abx	Cured
2	1.5/F	China	None	Wound swab of gangrene	Mixed flora with *Carnobacterium* spp.	Traumatic hand with pool water exposure	Amputation, debridement; IPM	Cured
3	43/ M	Austria	Extensive hx of handling and consuming fish	½ blood culture sets	*C. mobile* or *C. funditum*	Sepsis suspected from GI source	CRO/AMP → MXF	Cured
4	57/F	France	DM; EtOH; TPN; post cardiac arrest	4 blood culture sets	*C. divergens*	Septic shock post cardiac arrest; esophagectomy for necrotizing esophagitis on TPN/EN	Broad spectrum Abx → AMX	Cured
5	65/ M	South Korea	Cancer; neutropenia on etoposide	½ blood culture sets	*C. divergens*	Febrile neutropenia with oral mucositis on TPN	TZP/VAN	Cured
6	81/ M	Canada	Cancer; chronic steroid use	½ blood culture sets	*C. inhibens*	Sepsis with multifocal pna	CRO/VAN → AMC	Cured

Table from Prameet *et al.* (16)

B. Virulence factors

There is no thorough research on possible virulence factors of *C. divergens* but assessment of the hemolytic activity was done for isolate M35 (Table 3). The method described by Giraffa et al. was used to evaluate the hemolytic activity of *C. divergens* M35 compared to *P. acidilactici* PAC1.0 commercially used in the food industry (Quest) (17). *C. divergens* (30°C) and *P. acidilactici* (35°C) were incubated simultaneously under aerobic or anaerobic conditions for 24 hours in different media containing defibrinated sheep blood. Zones of alpha or beta hemolysis were noted. Based on the growth obtained, the plates were incubated again under the same conditions. Neither *C. divergens* M35 nor *P. acidilactici* showed hemolytic activity, under aerobic or anaerobic conditions on TSA or MRS7 agar.

Table 3. Hemolytic capability of the tested strains.

Strain	°C	Medium	Hemolysis
C. divergens	30	TSA + defibrinated sheep blood	None
		Idem, anaerobic conditions	None
		MRS7 + defibrinated sheep blood	*
		Idem, anaerobic conditions	*
P. acidilactici	35	TSA + defibrinated sheep blood	None
		Idem, anaerobic conditions	None
		MRS7 + defibrinated sheep blood	*
		Idem, anaerobic conditions	*

*This medium was greenish colored at the start and likely of sub-standard quality

The genus is mostly associated to food spoilage bacteria, but it is mentioned that *Carnobacterium maltaromaticum* can be a fish pathogen in some cases (10, 18). In opportunistic cases of infection in stressed fish, only the species *maltaromaticum* has been reported (10, 19, 20). *C. divergens* has not been reported as a fish pathogen nor has been reported as pathogenic to any other organism. It has been isolated often from a variety of healthy fishes like salmon, Artic charr, wolffish, Atlantic cod and rainbow trout (10, 21). In some cases, *C. divergens* has even been studied as a fish probiotic bacteria for the control of infections from other bacteria like *Vibria* spp., *Aeromonas salmonicida* and *Pseudomonas fluorescens* (22–24).

C. Biogenic amine production (toxicity)

The analysis of biogenic amines in food (fishes, cheeses, and vegetables) is a current method to determine matrix degradation in the food industry and possible metabolism that could be toxic for humans. The decomposition process produces characteristic compounds frequently associated with bacterial microflora. This explains why the production of biogenic amines should be quantified after the application of *Carnobacterium divergens* M35 on food. This ensures that the bacteria do not accelerate the decomposition process or produce any harmful component. This was always performed by the external technology transfer center TransBIOTech. The method was developed by Maxim Maheux (Head of Chemistry and Biochemistry Sector) and always analyzed by a member of the chemistry department registered in the Ordre des Chimistes du Québec (Chemistry Order of Quebec).

Table 4: Level of toxicity of some biogenic amines in food.

Molecule	Maximale concentration (µg/g or ppm)
Histamine	100
Putrescine	2000
Tyramine	2000
Cadaverine	2000
Spermine	600
Spermidine	600

First, the production of biogenic amines in smoked salmon and in fresh tuna to which *C. divergens* M35 was added, was examined weekly. The quantification of eight amines, methylamine, tryptamine, putrescine, spermidine, spermine, tyramine, histamine, and cadaverine, was carried out using the method of Vallé et al. (1997) (25).

Table 5. Biogenic amine production (µg/g) in slices of cold-smoked Coho salmon (5 g) with and without the presence of *C. divergens* M35.

Strain	Day	Amine							
		MET	TRP	PUT	SPM	SPD	TYR	HIS	CAD
Control	1	5.94	59.41	1.98	3.96	3.96	ND	ND	ND
	8	7.94	59.52	1.98	3.97	ND	ND	ND	ND
	16	9.77	ND	1.95	3.91	1.95	ND	ND	ND
	22	5.94	ND	1.98	5.94	1.98	ND	ND	ND
	28	5.98	ND	1.99	3.98	ND	ND	ND	ND
M35	1	5.93	27.67	1.98	3.95	1.98	ND	ND	ND
	8	7.98	31.94	2.00	3.99	ND	ND	ND	ND
	16	7.84	21.57	ND	3.92	ND	62.75	ND	ND
	22	7.84	15.69	1.96	3.92	1.96	74.51	ND	ND
	28	15.24	13.33	3.81	3.81	1.90	80.00	ND	ND

Methylamine (MET), tryptamine (TRP), putrescine (PUT), spermine (SPM), spermidine (SPD), tyramine (TYR), histamine (HIS), cadaverine (CAD)
ND = not detect

The analysis for smoked salmon revealed that *C. divergens* M35 did not produce significant quantities of any of these substances compared to non-inoculated salmon, except for tyramine and tryptamine after 16 and 28 days of storage respectively, which is passed its consumption date of 14 days (Table 5). The tryptamine concentrations are also very low compared to those produced by other species of *Carnobacterium* in salmon (up to 4000 µg/g). Furthermore, this concentration is considered below the current toxic limit of 2000 ppm. It should be noted that *C. divergens* M35 does not produce the toxic amine histamine.

For fresh tuna, the results of the biogenic amines analysis are present in table 6. After 14 days of exposure to the bio-ingredient, the concentration of biogenic amines is very low or below the limit of detection. The only amines detected were cadaverine and tyramine between 327 µg/g to 378 µg/g for cadaverine

and 69 µg/g to 83 µg/g for tyramine, but as for smoked salmon this is passed the recommended consumption date for tuna, that is 48h after thawing. The maximal amount considered toxic in seafood and fish for those biogenic amines is 2000 µg/g for cadaverine and tyramine (26, 27). Therefore, the amount present on fresh tuna is not considered harmful or toxic for health. It is also noticeable that the addition of BacM35 did not increase the number of biogenic amines produces compared to the non-inoculated sample (batch 1) for any concentration of use tested (batch 2 to 4) and thus, the process of biodegradation is not accelerated by the ingredient.

Table 6. Biogenic amine production (ug/g) in fresh tuna (5g) with and without the presence of *C. divergens* M35 at three different concentration of use (10^4, 10^5 and 10^6 CFU of BacM35/g of fresh tuna) after 14 days.

Amine	Methylamine (µg/g)	Tryptamine (µg/g)	Putrescine (µg/g)	Cadaverine (nl/g)	Histamine (µg/g)	Tyramine (µg/g)	Spermidine (µg/g)	Spermine (µg/g)
Batch 1 (Control)	< 5 ug/g	< 20 ug/g	< 20 ug/g	357 ± 26.3	< 10 ug/g	79 ± 7.0	< 30 ug/g	< 100 ug/g
Batch 2 (10^4 CFU/g)	< 5 ug/g	< 20 ug/g	< 20 ug/g	327 ± 11.4	< 10 ug/g	69 ± 2.2	< 30 ug/g	< 100 ug/g
Batch 3 (10^5 CFU/g)	< 5 ug/g	< 20 ug/g	< 20 ug/g	378 ± 13.8	< 10 ug/g	83 ± 3.2	< 30 ug/g	< 100 ug/g
Batch 4 (10^6 CFU/g)	< 5 ug/g	< 20 ug/g	< 20 ug/g	334 ± 21.0	< 10 ug/g	76 ± 4.1	< 30 ug/g	< 100 ug/g

Biogenic amine was measured because there are some concerns about the use of *Carnobacterium divergens* as a bio-preservative in food. First, *C. divergens* and *C. maltaromaticum* can produce tyramine which is known to affect blood pressure and therefore causes migraines in sensitive individuals with decreased monoamine oxidase activity (10, 28). In this case, the concentration of tyramine obtained at day 0 for cold-smoked salmon inoculated with *Carnobacterium divergens* M35 was non detected, like the control. For day 16, cold-smoked salmon yielded a concentration of 63 µg/g. According to the Dietary Guideline for Americans (2020-2025) chapter 4, page 96, adults should consume a weekly amount of 8 to 10 ounces of fish/seafood, which is equivalent to about 225 to 285 grams of salmon (https://www.dietaryguidelines.gov/, consulted January 10th, 2022). So, the maximum amount of tyramine consumed with 285g of salmon at day 16 would be of 17.9 mg total, which is equivalent to 63 mg/kg of salmon. For tuna at day 14, it is 21.6 mg total, for an amount of 76 mg/kg of tuna.

Even with the addition of the bio-ingredient, the concentration of tyramine detected is still very low in contrast to some species of *Carnobacterium* and below the threshold value to trigger a vasoactive effect (100-800 mg/kg of food) (29). Those results are in accordance with another strain of *Carnobacterium divergens*, strain V41, who possesses similar characteristics (30). This strain produces about 34 µg/ml of tyramine and 43 µg/ml of cadaverine after 27 days (results obtained from different growth conditions) which is similar to the strain M35 at day 0 (31). This proves that even though some *Carnobacterium* have

a high tyrosine decarboxylase activity (32–34) and can produce high amounts of tyramine, some strains have a low activity and are perfect candidates for bio-preservatives.

Second, the conditions in which *Carnobacterium divergens* grows influences the biogenic amine production, especially tyramine (32). Masson et *al.* (1997) showed that tyramine production was greatly decreased (up to a 100-fold) at 5°C, initial pH of 6, no glucose and 10% NaCl or more (32). Considering the nutrition facts of cold smoked salmon of Fumoir Grizzly (for the absence of glucose (sugar) and an amount of 23% of salts (NaCl), the initial pH from 5.1 to 6 (Fumoir Grizzly, personal communication) and that the product should be kept frozen or at 4°C, *Carnobacterium divergens* M35 is in the ideal environment to have a very low tyramine production activity. Finally, no other biogenic amine, including histidine, which can cause allergy-like symptoms, was detected above the limit of the method and therefore we can assess that the strain M35 used as a bio preservative does not have any toxicity or allergenic potential. The total volatile base nitrogen (TVBN) production was also quantified and the difference between the control and the addition of *C. divergens* M35 was not significant ($p<0,05$) (6).

Finally, there is no known toxicant or harmful metabolite known in the literature that is or could be produced by *Carnobacterium divergens* strain and target humans.

Coho 85g

Valeur nutritive
Nutrition Facts

pour 1 emballage (85 g)
For 1 packaging (85 g)

Teneur / Amount	% valeur quotidienne / % Daily Value
Calories / Calories 150	
Lipides / Fat 9 g	14 %
saturés / Saturated 1,5 g + trans / Trans 0,1 g	8 %
Polyinsaturés / Polyunsaturated 3,5 g	
oméga-6 / Omega-6 1,5 g	
oméga-3 / Omega-3 2 g	
monoinsaturés / Monounsaturated 3 g	
Cholestérol / Cholesterol 40 mg	
Sodium / Sodium 540 mg	23 %
Glucides / Carbohydrate 1 g	1 %
Fibres / Fibre 0 g	0 %
Sucres / Sugars 0 g	
Protéines / Protein 17 g	
Vitamine A / Vitamin A	2 %
Vitamine C / Vitamin C	0 %
Calcium / Calcium	2 %
Fer / Iron	0 %

Figure 7. Nutrition facts for 85g of cold-smoked coho salmon f Fumoir Grizzly sold in grocery stores (provided by Fumoir Grizzly).

3.4.2.4 Resistance to antibiotics and tolerance to metals and pesticides

The antibiotic resistance of *C. divergens* M35 was evaluated simultaneously with a well-known strain of lactic acid bacteria used routinely in the food industry, *Pediococcus acidilactici* PAC 1.0 (commercial strain, Quest). An agar diffusion method based on the inhibition zone diameter around paper discs impregnated with antibiotic was used with a variety of culture media, including Mueller-Hinton medium enriched with 2–5 % lysed horse blood. Plates were incubated aerobically according to CLSI M-45A specifications (2006 and 2009) as is carried out routinely for testing candidates belonging to other genera of lactic acid bacteria

such as *Lactobacillus* and *Pediococcus*. The tested strains were first grown by surface inoculation. A suspension of bacterial cells was prepared in 5 mL of physiological saline solution (0.85 %) to obtain a McFarland density of 0.5 (0.08 – 0.13 DO625 nm). The suspension was inoculated on the surface of the Petri dish by streaking with a sterile cotton swab. The suspension was allowed to adsorb to the agar for 3-5 minutes and antibiotic discs (five per plate) were placed on the agar surface. The plates were incubated in the inverted position at 30 °C for *Carnobacterium divergens* M35 and 35 °C for *Pediococcus acidilactici* PA 1.0 during 20–24 hours.

Based on the presence of inhibition zones around the discs, *C. divergens* M35 appears to be sensitive to 11 of the 12 antibiotics tested, under almost all culture conditions tested (Table 7 and 8). Under some conditions, streptomycin did not produce a zone of inhibition, although it did produce a zone 13 mm in diameter under test conditions recommended by the CLSI.

Charteris et al. (2001) have proposed inhibition zone criteria that might be applicable to interpret the response to some of the antibiotics tested here (35). Based on these criteria, *C. divergens* M35 appears to be sensitive to all tested antibiotics except streptomycin. We note with interest that *P. acidilactici* appeared to be resistant to methicillin and vancomycin and somewhat resistant to gentamycin (Table 7).

Table 7. Inhibition zones (in mm) of *C. divergens* M35 on various agar media in the presence of different antibiotics.

Antibiotic	**µg per disc**	**Medium, hours of incubation**							
		MH, 24	MRS7, 24	MRS7, 48	MRS7, blood, 24	MRS7, blood, 24, *an*	MH, blood, 96*	MH, blood, 96*, *an*	TSAYE, 24
Ampicillin	10	25.5	28.3	27.9	25.2	27.4	31.0	36.3	26.5
Chloramphenicol	30	28.0	28.7	26.3	28.6	28.0	24.0	23.4	29.0
Ciprofloxacin	5	27.0	23.5	24.6	23.8	23.2	29.8.	31.5	28.0
Erythromycin	15	30.3	30.4	31.0	33.5	32.0	32.8	34.0	31.7
Gentamycin	10	18.1	9.7	8.9	20.3	19.7	25.0	29.0	13.4
Methicillin	5	0	10.4	9.1	12.3	13.5	19.2	21.0	11.2
Nitrofurantoin	300	19.0	25.0	23.5	25.8	26.0	24.2	–	21.0
Penicillin G	10 IU	20.0	24.1	21.1	24.1	26.6	30.5	38.7	24.8
Rifampicin	30	34.7	25.9	31.0	37.1	39.6	36.5	–	38.2
Streptomycin	10	0	0	0	9.1	8.7	11.0	11.0	0
Tetracycline	30	33.0	33.9	34.0	33.7	36.0	30.5	29.4	33.3
Vancomycin	5	21.3	18.4	19.5	17.2	17.4	21.8	23.0	20.1
Vancomycin	30	24.9	22.4	24.9	21.4	23.0	25.9	28.8	26.3

an – incubated anaerobically; *Poor growth, not visible until 96 h
MH – Mueller-Hinton agar; MRS7 – Mann-Rogosa-Sharpe agar with pH 7; TSAYE – Tryptic soy agar plus yeast extract

Table 8. Zones of inhibition (in mm) of *P. acidilactici* on various agar media in the presence of different antibiotics.

Antibiotic	µg per disc	MH, 24	MRS7, 24	MRS7, blood, 24	MRS7, blood, 24,*an*	MH cation adj. + blood, 48*	MH, cation adj. + blood 48*,*an*	TSAYE, 24*
Ampicillin	10	27.0	20.8	20.5	20.4	27.0	28.4	23.4
Chloramphenicol	30	35.0	27.0	31.2	32.8	38.0	33.0	31.4
Ciprofloxacin	5	10.0	0	0	Halo	9.0	9.0	10.6
Erythromycin	15	44.7	33.7	33.2	34.1	45.0	42.0	34.6
Gentamycin	10	25.0	11.9	12.6	12.3	34.0	24.0	14.3
Methicillin	5	0	0	0	0	0	0	0
Nitrofurantoin	300	20.0	25.0*	29.1**	27.4**	25.0	20.0	16.0**
Penicillin G	10 IU	33.0	29.3	28.3	28.0	40.0	33.0	27.8
Rifampicin	30	40.0	29.0	33.2	34.2	40.5	39.0	33.7
Streptomycin	10	12.0	0	0	0	13.0	9.0	0
Tetracycline	30	25.0	23.5	21.0	22.0	21.0	22.0	19.6
Vancomycin	5	0	0	0	0	0	0	0
Vancomycin	30	0	0	0	0	0	0	0

an – incubated anaerobically; *Poor growth, not visible until 96 h
MH – Mueller-Hinton agar; MRS7 – Mann-Rogosa-Sharpe agar with pH 7; TSAYE – Tryptic soy agar plus yeast extract

The results are consistent with those obtained by Brillet for the sensitivity of *C. divergens* strain V41, which was found sensitive to ampicillin, chloramphenicol, tetracycline, erythromycin, spiramycin, vancomycin, trimethoprim and rifampicin, but sensitive to cefalotin and norfloxacin, and intrinsically resistant to cefotaxime, streptomycin, gentamycin, kanamycin, colistin and nalidixic acid. Based on these results, Brillet concluded that strain V41 does not represent any risk in foods (36).

Table 9. Antibiotic resistance of *C. divergens* M35 – Interpretation of the results

Antibiotic (µg in the disc)	Suggested criteria (zone in mm)			Inhibition zone diameter (mm)	Interpretation of result
	R	I	S		
Penicillin G (10)	<19	20-27	>28	31	S
Ampicillin (10)	<12	13-15	>16	31	S
Vancomycin (30)	<14	15-16	>17	36	S
Rifampicin (5)	<14	15-17	>18	37	S
Metronidazole (5)	<14	15-17	>18	Not determined	–
Gentamycin (10)	<12	–	>13	25	S
Streptomycin (10)	<11	12-14	>15	11	R
Tetracycline (30)	<14	15-18	>19	31	S

Chloramphenicol (30)	<13	14-17	>18	24	S
Erythromycin (15)	<13	14-17	>18	33	S

The sensitivity of four strains of *Carnobacterium* (*C. divergens* V41, *C. piscicola* V1, *C. piscicola* SF668, and *C. piscicola* NCDO2762) to antibiotics was also established (37). Resistance to vefalotin, cefoxitin, moxalactam, cefuroxime, aztreonam, cefotaxime, ceftazidime, cefepime, cepirom, mecillinam, clindamycin, nalidixic acid, colistin, fosfomycin was observed, as was weak resistance to kanamycin, gentamycin, netilmicin, streptomycin, tobramycin, and amikacin. However, these strains were sensitive to amoxicillin, ticarcillin, piperacillin, imipenem, erythromycin, pristinamycin, ciprofloxacin, chloramphenicol, tetracycline, minocycline, vancomycin, teicoplanin, and rifampicin. These authors mentioned that resistance to cephalosporin, clindamycin, nalidixic acid, fosfomycin, and aminoglycosides is intrinsic (non-acquired) and non-transmissible via plasmids. Ringo et al. (2002) found *C. divergens* strain CCUG 30094 to be sensitive to each of 20 antibiotics tested, with the exception of deferoxamine (38). We note with interest that the preferred treatment against listeriosis uses a combination of gentamycin and ampicillin, two antibiotics to which *C. divergens* is sensitive, making it unable to transfer the resistance to cells of the genus *Listeria* (39).

There is little to no data as to weather or not *Carnobacterium divergens* is sensitive to pesticides or not. One article detected *Carnobacterium* spp. using sequencing from deltamethrin-resistance, deltamethrin-sensitive and field-caught diamondback moth, but no data has been performed to measure the sensitivity of different *Carnobacterium* species to various pesticides (40). Since *Carnobacteria* are mainly found in water and on food, we hypothesize that the strain would not be tolerant to pesticides are it would not normally be exposed to those substances.

As for heavy metals, it is known that some LABs are able to bioaccumulate some of the metals found in the environment and they can tolerate the presence of those metals (41). It was shown that *C. piscicola* was resistant to nickel and cobalt, but sensitive to cadmium in a bivariate culture model (42). It was also demonstrated that a strain of *C. divergens* has high removal capabilities of lead (Pb) and cadmium in culture conditions, thus showing a great bioremediation purpose for the species (43). There is no study specific to the strain M35, but it is hypothesized from the literature that it would tolerate and could even remove some heavy metals in the environment.

2.4.2.5 Genetic basis for pathogenicity to non-human species, toxigenicity and resistance to antibiotics

The species *divergens* is not a pathogen for human or non-human species. The genome of *C. divergens* strains is not very big (~2.7 Mpb). Some genome of the genus *Carnobactrium* (including the species *divergens*, but not strain M35) have been annotated using the MicroScope platform to automatically annotate the genes and perform a comparative analysis and a secretome prediction of the strains (44). This secretome showed that species *maltaromaticum* and *divergens* contained more genes encoding for carbohydrate transport, metabolism and peptidoglycan associated proteins, which could explain their implantation in animal based ecological niches compared to other strains of the same genus.

Those extract genes confer them an evolutive advantage, but no clear pathogenicity, toxigenicity or supplementary resistance to antibiotics.

2.4.2.6 Capability to transfer genes

It has been found that some strains of *C. divergens* have CRISPR systems (45). Those systems can act as a database of past infection from exogenous DNA and that the genome can limit its expansion thought the destruction of this DNA (46). The presence of those systems in *C. divergens* shows that invading DNA can be destroyed and therefore the genome won't be likely to acquire new genes by horizontal gene transfer. There is no data on the literature concerning the ability of *Carnobacterium divergens* to transfer genes horizontally, by putative prophages or any other mechanisms. This species is not known to be naturally competent either. So, there is no risk that the wild-type strain would mutate and carry or express harmful or toxin genes.

2.4.2.7 Conditions that might select for dispersal of traits of pathogenicity to non-human species toxigenicity and resistance to antibiotics

There are no specific conditions that might select for dispersal or traits of pathogenicity, toxigenicity or resistance to antibiotics. The species is not known or studied for its competence to transfer genes or is known to have any virulent genes considered as a hazard to be transferred to other pathogens.

2.4.2.8 Involvement in biogeochemical cycling

Carnobacterium divergens is found vey often in food of animal origin (meat, fish and dairy products), in water and soil (9, 10, 45). It is not a key member in any major biogeochemical cycle (carbon, nitrogen or sulpur). It main purpose is a food spoiler (for some strains) or a member of fish microbiota and even human gut microbiota (45). As a potential food spoiler, *C. divergens* or any other potential lactic acid bacteria on food could produce biogenic amines, thus playing a small roll in the degradation of amine and the avaibility of nitrogen. It was shown experimentally that on salmon, the addition of strain M35 did not increase the amount of biogenic amines already produced by the natural microflora. So the strain M35 has no big proteolytic activity and could not play a major role in biogeochemical cycles.

To a lesser extend, homologs of multidomain proteins were predicted as nucleotidases/metallophosphatases in *Carnobacterium divergens*. Those putative proteins catalyze dephosphorylation of exogenous adenine 5'-nucleotides to adenosine and phosphate and provide key function of phosphorous in aquatic ecosystems (45).

2.4.2.9 Geographic distribution of the microorganism

Carnobacterium sp. is ubiquitous to the environment, but mostly found in water and food. As for *C. divergens* itself, this strain is mostly found in food and on meat product, because it naturally colonizes various food (44, 47, 48). There is no global geographic distribution of the microorganism, but the following figure represents the relative abundance of *C. divergens* compared to other *Carnobacteria* in different ecological niches.

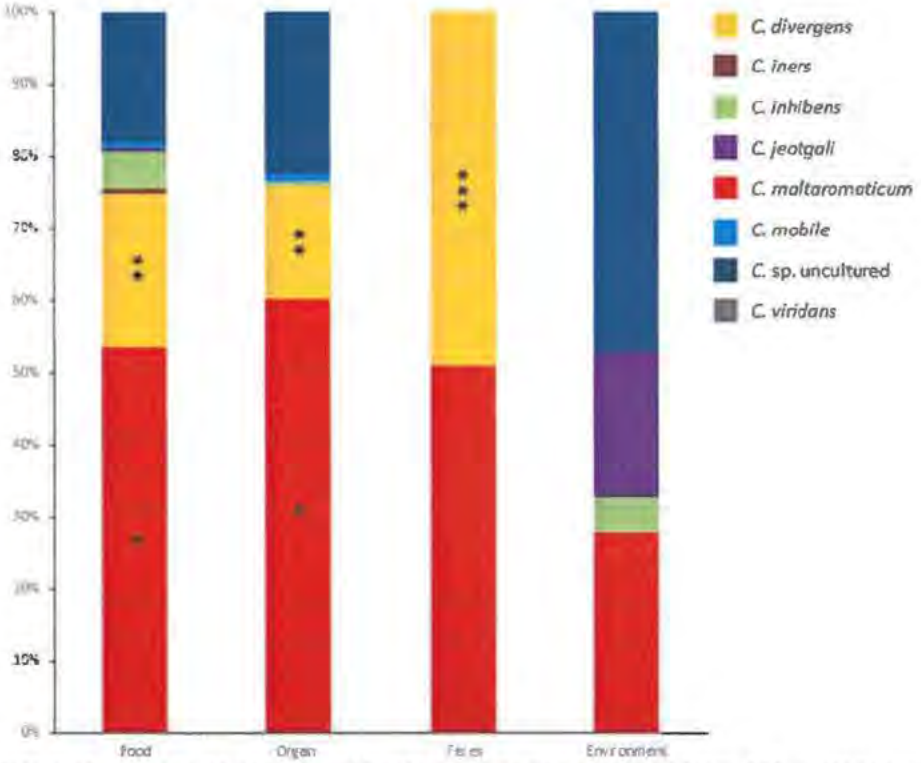


Figure 8. Relative abundance of each *Carnobacterium* species obtained from 16S metagenomic analyses of 681 samples originating from food, organs, feces, and environment. One asterisk indicates significant difference ($P < 0.0001$) compared to all other *Carnobacterium* sp, two asterisks indicate significant difference ($P < 0.01$) compared to *C. iners, C. inhibens, C. jeotgali, C. mobile*, and *C. viridans*. Three asterisks indicate significant difference ($P < 0.0001$) compared to all *Carnobacterium* sp. except *C. maltaromaticum* (44).

2.4.2.10 The potential for adverse immunologic reactions in persons exposed to the microorganism

Another *Carnobacterium* strain, *C. maltaromaticum* CB1, was also recognized as GRAS (GRN No. 305) by the U.S. Food and Drug Administration for the use as viable or heat-treated up to 1×10^9 CFU/day for preservation of ready-to-eat meat products, meat, poultry and fish products, frozen meals, processed fruit salads and vegetable salads, sauces, and soft cheese and cheese spread-type products. (http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=305, consulted January 7th, 2022)

Health Canada has approved the use of viable cells, pasteurized non-viable cells and the metabolites (bacteriocins) of *C. maltaromaticum* CB1 as a microbiological preparation in certain ready-to-eat (RTE) meat/poultry products as an intervention against human pathogenic microorganisms, such as *Listeria monocytogenes*. In table 7 are the biochemical and physiological characteristics of *Carnobacterium divergens* and *Carnobacterium maltaromaticum*. As seen in this table, *C. maltaromaticum* strains have the ability to produce acid from a wider variety of substrates compared to *C. divergens* strains. From a conservation point of view, we can assess that *C. divergens* is an even more suitable bio-preservative than *C. maltaromaticum*, because of its tendency to produce less acid and thus preserve the organoleptic proprieties of food. Other than this advantage, both species are described as gram-positive rods that occur singly or in short chain, non-motile, non-spore forming bacteria, catalase, and oxidase negative. The

colonies appear as round, white and convex, with smooth edges when grown on TSA at 25°C for 24h (Collins et al., 1987). It is also stated that phenotypic characteristics are not able to differentiate the species and that molecular methods are necessary (Birgit et al., 2004). Considering that one species is already labeled as GRAS, the differences present between *C. maltaromaticum* and *C. divergens* are not significant enough to doubt the safety of *Carnobacterium divergens* M35 in the indented conditions of use.

Table 10. Biochemical and physiological characteristics of *Carnobacterium divergens* and *Carnobacterium maltaromaticum*. From Collins *et al.* (49).

Characteristics	***C. divergens***	***C. maltaromaticum***
Acid produced from:		
Amidon	-	-
Amygdalin	+	+
Galactose	-	+
B-Gentiobiose	+	+
Gluconate	+	+
Inuline	-	+
2-Ketogluconate	-	-
Lactose	-	+
Mannitol	-	+
Melezitose	+	+
Melibiose	-	+
α-Methyl-D-glucoside	-	+
α-Methyl-D-mannoside	-	+
D-gatarose	-	-
D-Turanose	-	±
D-xylose	-	-
Voges-Proskauer	+	+
Motility	-	-
Gas production from glucose in arginine-MRS broth	+	+
Growth at temperature (°C):		
0	+	+
30	+	+
37	+	+
40	+	-
45	-	-

Finally, to assess the safety of the strain, a study of *Carnobacterium divergens* M35 was conducted in a simulated environment of the gastro-intestinal system (TIM-1 System, TNO Triskelion) (Fliss et *al.*, Unpublished). This study showed that the strain M35 is completely killed by gastric fluids and that no bacterial cells can survive the digestive process. This means that the strain is safe for consumption and that even in the case of consumption by people with weakened intestinal lining, no cells will be able to

cause an opportunistic infection as seen previously. Also, in 2016, a probiotic containing a strain of *Carnobacterium divergens,* Lavipan®, was approved for piglets, chickens and turkeys for fattening in Europe and an acute inhalation study was performed to determine the safety for the user (50). Clinical results by the inhalation of the product by rats, 4 hours a day for 14 consecutive days determined that the additive is not toxic for the user, but due to the proteinaceous nature of the probiotic, it should be kept in mind that it can be a potential respiratory sensitizer. An additional study on skin and eyes also concluded that the substance is not an irritant. So not only *Carnobacterium divergens* is safe for consumption because it cannot survive digestion, but the individuals handling the bio-ingredient on the production line can be considered safe from its exposure.

2.4.2 Safety of bacteriocins

In recent years, bacteriocins have been greatly studied as antimicrobial compounds due to their potential in food (preservatives and safety) and in pharmaceuticals (antibiotic alternative). Bacteriocins are small peptides produced by bacteria that target other bacteria to compete in nature, they often have a very narrow spectrum of activity. There are currently three classes of bacteriocins (51). Class I is called lantibiotics and is composed of post translationally modified peptides that can contain unusual amino acids. Class II is composed of unmodified peptides divided in three subclasses: IIa pediocin-like bacteriocins, IIb made of two polypeptide chains and IIc other single chain bacteriocins (52–54). Class III are composed of thermosensitive bacteriocins. Divergicin M35 is a bacteriocin from the class IIa pediocin-like that has the following sequence: TKYYGNGVYC NSKKCWVDWG TAQGCIDVVI GQLGGGIPGK GKC. It is made of all natural amino acids.

Humans are constantly exposed to bacteriocins because the majority of bacteriocins are produced by lactic acid bacteria and probiotics used in food (dairy fermentation, vegetable fermentation etc.) and normally found on food (51, 55–58). Nisin, a bacteriocin produced by many strains of lactic acid bacteria, has a GRAS status by the FDA (GRAS No 65, https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=65, consulted January 11th 2022) and is used as an antimicrobial agent. Also, in Canada, pediocin, also a class IIa bacteriocin, is the first bacteriocin approved as a processing aid since nisin (considered an additive) in food as an antimicrobial agent to control *Listeria monocytogenes* (File No ADDPS20013101). Moreover, you can often find starter cultures and antimicrobial strains commercially sold that produce bacteriocins such as ALTA™ 2341 from Quest International (59, 60) and the MicroGARD cultures from Dupont (IFF) active against a wide range of microorganisms (https://www.dupontnutritionandbiosciences.com/products/microgard.html, consulted January 12th, 2022). Thus, bacteriocins are already everywhere in our nutrition and have been used in food without any safety concerns for many decades.

The safety of bacteriocins reside mainly in the fact that they are small proteins, and for the case of divergicin M35 for example, they are made with the same amino acids normally found in our nutrition. Also, they are very sensitive to the proteolytic enzymes and low pH present in our digestive system, such as pepsin, trypsin and chymotrypsin (61–63). This means that our stomachs and small intestine are able to digest the bacteriocins like they would normally do with proteins sourced from our food (64). This is particularly true for class II bacteriocins, known to be extremely sensitive to potential gastro intestinal barriers (see figure 9). The fate of the bacteriocin is then the same fate as any other protein ingested.

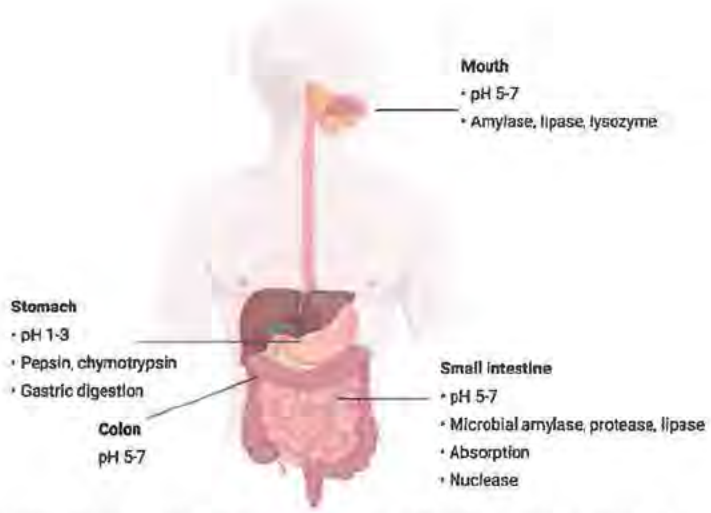


Figure 9. Physiological conditions that may influence the stability and biological activity of bacteriocins during the gastrointestinal transit. From Soltani *et al.* (65).

2.4.2.1 Absorption of bacteriocins

As for the absorption of bacteriocins, it varies due to their bioavailability following ingestion. For bacteriocins from the class II, very little is available once the stomach and the small intestine have digested the peptide. It has been shown that nisin can be absorbed thought the vaginal epithelium of rabbits and that other bacteriocins (enterocin E50-52, nisin and plantaricin 423 for example) could cross the gut-blood epithelium *in vitro* (62, 66, 67). But, Meade *et al.* have shown that bacteriocins can also be sensitive to proteases from homeostasis and fibrinolysis in the bloodstream (68, 69). This means that bacteriocins are subject to degradation but the digestive system and the bloodstream.

2.4.2.2 Toxicity and cytotoxicity of bacteriocins

Acute and subacute toxicity of bacteriocins can be done to determine the toxic characteristics of a studied compound and to assess the possible health hazards. Previous studies have shown that oral administration of nisin, bacteriocin TSU4, Lactocin 160 and pediocin N6 did not show signs of acute toxicity in *in vivo* animal models in amount normally found in food (70–73). With all that information, bacteriocins show a great potential to be used in the food industry, as well as in the pharmaceutical industry due to their low to absence of toxicity (65). Table 11 shows an overview of recent literature.

Table 11. Overview of recent literature and data on *in vivo* acute and subacute toxicity of bacteriocins. From Soltani *et al.* (65).

Acute and subacute toxicity			
Bacteriocin	Administration	Toxicity	References
Enterocin AS-48	1, 10, 20 μg/dose of 5 μL for 3 days	No skin sensitization	(Cebrián et al. 2019)
Lacticin 160	1.8 mL of 10 mg mL $^{-1}$ intravaginally in rat	No severe irritation	(Dover et al. 2007)
Nisin	1000 mg kg $^{-1}$ BW in rat 20 000 mg kg $^{-1}$ BW in mice	No sign of acute toxicity	(Frazer et al. 1962) (Marlida et al. 2016)
Pediocin N6	>20 000 mg kg $^{-1}$ in mice	LD50	(Marlida et al. 2016)
TSU4	Daily intake dose of 0.5 mg kg^{-1} for 21 days	No mortality and no changes in physical condition	(Sahoo et al. 2017)
	0.825 mg kg^{-1} daily intake for 21 days	Histological changes in spleen, liver and skin	(Vaucher et al. 2011)

As for cytotoxicity, many cell lines have been tested *in vitro* using different assays thought the years. Table 12 shows an overview of the recent literature. Some bacteriocins have shown levels of cytotoxicity like nisin at high concentration against different type of cells. But it is important to notice, because there is not a lot of data in the literature specifically for bacteriocins produced by *Carnobacterium*, that at concentration 100-fold higher than the MIC (Minimum Inhibitory Concentration) bacteriocins Cbn BM1 and Cbm B2 produced by *Carnobacterium maltoramaticum* CP5 were not cytotoxic to caco-2 cell line.

In brief, bacteriocins, more precisely divergicin, is composed of natural amino acids often found and currently in our nutrition. Some data suggests that certain bacteriocins could be absorbed through the gut, but class II bacteriocins are unlikely to survive the stomach and the small intestine. If a very small amount does make it to the gut epithelium and is absorbed in the blood stream, proteolytic activity from homeostasis and fibrinolysis will also digest the peptides. Overall data from toxicity and cytotoxicity shows that bacteriocin are safe for consumption and have a great potential to be used in the food industry.

Table 12. Overview of the recent literature on *in vitro* cytotoxicity of bacteriocins. From Soltani *et al.* (65).

		Cytotoxicity			
Bacteriocin	Producer organism	Cell line	Type of assay	Toxicity	References
Enterocin DD14	*Enterococcus faecalis* 14	IPEC-1	CCK-8 assay	No toxicity at MIC and 2× MIC concentrations	(Caly et al. 2017)
Enterocin S37	*Enterococcus faecalis* S37	Caco2-TS7	LDH release assay	No toxicity at 2 and 10 $\mu g\ mL^{-1}$	(Belguesmia et al. 2011)
Enterocin AS-48	*Enterococcus faecalis* UGRA 10	Melanoma cell line A2058	MTT assay	No toxicity at MIC and higher concentrations (up to 200 $\mu g\ mL^{-1}$)	(Abengózar et al. 2017)
Carnobacteriocin Cbn BM1					
Cbn B2	*Carnobacterium maltaromaticum* CP5	Caco-2	MTT assay	No toxicity at 100-fold MIC	(Jasniewski et al. 2009)
Plantaricin DM5	*Lactobacillus plantarum* DM5	Hek 293	MTT assay	No cytotoxic effect on mammalian cells	(Das and Goyal 2014)
Nisin (Nutrition 21/USA)		HeLa HT29	MTT assay	Cytotoxic at 4× MIC value in HT29	(Maher and McClean 2006)
		Caco-2	Neutral red	Cytotoxic at 2× MIC in Caco-2	
Nisin (Sigma-Aldrich)		SV40 HC	Trypan blue exclusion	Toxic to both cells at high concentration (<50% viability at >350 AU mL^{-1})	(Murinda et al. 2003)
		Vero cell			
Nisaplin® (Danisco)		Vero cell	MTT assay	EC50: 0.33 $\mu g\ mL^{-1}$ MTT	(Vaucher et al. 2011)
			LDH release assay	0.79 $\mu g\ mL^{-1}$ LDH	
			Neutral red assay	0.62 $\mu g\ mL^{-1}$ NUR	
Nisin Chrisin® (Chr. Hansen—Colors & Blends)		Vero cell MCF-7	MTT	IC50 13.48 μM Vero cell	(Paiva et al. 2012)
		HepG2		105.46 μM MCF-7 112.25 μM HepG2	
Pediocin PA-1	*Pediococcus acidilactis* PA-1/ACH	SV40 HC	Trypan blue exclusion	Toxic to both cells at high concentration (<50% viability at >170 AU mL^{-1})	(Murinda et al. 2003)
		Vero cell			
Col E1	*E. coli* 50164	SV40 HC	Trypan blue exclusion	Col E6 cytotoxic for both cells	(Murinda et al. 2003)
Col E3		Vero cell		Col E1, E3, E7 and Col K little toxic (350 and 700 AU mL^{-1})	
Col E6 Col E7 Col K					
Microcin E492	*K. pneumoniae* RYC492	HeLa	LDH release assay (at 14 $\mu g\ mL^{-1}$)	Toxic against Jurkat, HeLa, RJ2.2.5 (different degree)	(Hetz et al. 2002)
		Jurkat		AMG-3, KG-1 insensitive	
		RJ2.2.5		Ramos slightly sensitive	
		Ramos KG-1 AMG-3			
Bacteriocin-like P40	*Bacillus licheniformis* P40	Vero cell	MTT assay	EC50:	(Vaucher et al. 2011)
			LDH release assay	0.30 $\mu g\ mL^{-1}$ MTT	
			Neutral red assay	0.51 $\mu g\ mL^{-1}$ LDH 0.57 $\mu g\ mL^{-1}$ NUR	
Bovicin HC5	*Streptococcus bovis* HC5	Vero cell	MTT	IC50:	(Paiva et al. 2012)
		MCF-7 HepG2		65.42 μM in Vero 279.39 μM MCF-7 280.30 μM HPG2	

2.4.3 Technical effect of the notified substance

2.4.3.1 Description of the mode of action in relation to the intended use

Strain M35 is known for its bacteriocin production, divergicin. While using the strain and its supernatant on food, it was shown that this method of bio-preservation is able to inhibit the growth of *Listeria monocytogenes* on cold smoked salmon and cold smoked trout (6). Since its approval as an antimicrobial food additive (Reference number NOP/AVP-0018) in smoked salmon and trout, it has been used to protect this type of food from *L. monocytogenes* contaminations for food processors

Divergicin M35 is from the pediocin-like subclass of bacteriocin, known to often have an anti-listerial activity. Those bacteriocins can target the membrane of some *Listeria* species and cause non selective pores resulting in subsequent lysis and cell death (53, 74, 75). Divergicin M35 interacts with fatty acids at the surface of the membrane and causes depletion of intracellular ATP and also it might interact with Na+/K+ efflux in the cell (53). When isolate, the supernatant loaded with divergicin M35 showed powerful antimicrobial activity against *Listeria monocytogenes* and other species of *Listeria* and *Carnobacterium* but it was not active against strains of *Lactococcus, Pediococcus, Lactobacillus, Streptococcus, Propionibacterium, Bifidobacterium* and *Escherichia coli* in soft agar assay (see table 12) (1).

Table 13. Sensitivity to divergicin M35 of *Listeria* species and other LABs in soft agar assay. From Tahiri *et al.* (1)

Organism	Strain	Sensitivity to divergicin M35	Diameter of inhibition zone (mm)
Listeria monocytogenes	LSD[a] 15		NA
L. monocytogenes	LSD 333	–	18.5 ± 1
L. monocytogenes	LSD 336	+	16.5 ± 0.5
L. monocytogenes	LSD 338	+	18.0 ± 0.5
L. monocytogenes	LSD 339	+	19.0 ± 0.5
L. monocytogenes	LSD 340	+	17.0 ± 0.5
L. monocytogenes	LSD 341	+	13.0 ± 0.5
L. monocytogenes	LSD 346	+	17.5 ± 1
L. monocytogenes	LSD 348	+	17.5 ± 1
L. monocytogenes	LSD 523	+	10 ± 1[b]
L. monocytogenes	LSD 524	+	22.0 ± 1[b]
L. monocytogenes	LSD 525	–	NA
L. monocytogenes	LSD 526	+	21.0 ± 1
L. monocytogenes	LSD 529	+	14.5 ± 0.5
L. monocytogenes	LSD 530	+	19.0 ± 1
L. monocytogenes	LSD 531	+	18.5 ± 1
L. monocytogenes	LSD 532	+	20.0 ± 0.5[b]
L. monocytogenes	LSD 535	+	19.0 ± 0.5
L. monocytogenes	LSD 538	+	20.0 ± 0.5
L. monocytogenes	ATCC[b] 19111	+	ND
L. monocytogenes	ATCC 19112	+	ND
L. monocytogenes	ATCC 19114	+	ND
L. monocytogenes	ATCC 19115	+	ND
L. monocytogenes	ATCC 35152	+	ND
Listeria seeligeri	LSD 11	+	13.5 ± 1

Listeria welshimeri	LSD 12		20 ± 0.5
Listeria grayi	LSD 13		15.0 ± 1
Listeria murayi	LSD 14		17.5 ± 1
Listeria ivanovii	ATCC 19119		NA
Listeria ivanovii	HPB[c]28	-	ND
Listeria innocua	HPB13	-	21 ± 0.5
Carnobacterium divergens	ATCC 385	-	15.0 ± 1
Carnobacterium piscicola	ATCC 386	-	17.5 ± 1
Lactococcus lactis subsp. *lactis*	R[d] 0058		NA
Lactococcus lactis subsp. *lactis* biovar. *diacetylactis*	R 0100		NA
Lactococcus lactis subsp. *lactis* biovar. *diacetylactis*	UL[e] 719		NA
Pediococcus acidilactici	UL 5		NA
Pediococcus acidilactici	R 1001		NA
Pediococcus pentosaceus	R 1044		NA
Lactobacillus salivarius	R 0078		NA
Lactobacillus delbrueckii subsp. *lactis*	R 0187		NA
Lactobacillus acidophilus	R 0052		NA
Lactobacillus plantarum	R 1012		NA
Lactobacillus casei	R R0256		NA
Lactobacillus rhamnosus	R 0011		NA
Streptococcus thermophilus	R 0083		NA
Propionibacterium spp.	P5	-	NA
Propionibacterium freudenreichii	R 0501		NA
Bifidobacterium breve	ATCC 15700	-	NA
Escherichia coli	ATCC 11775		NA
Escherichia coli	ATCC 13883		NA

2.4.3.2 Description of the reasonably expected by-products following introduction

After introduction on food, the strain, adapted to the environment of smoked fish can survive and multiply to some extend (see figure 10). In trials conducted on smoked salmon and as shown in figure 10, we found that *C. divergens* M35 (a psychrotroph) survived very well at 4 °C, with counts reaching about $1x10^9$ cfu/g after 21 days, while total counts (including total lactic acid bacteria) were about $5x10^8$ cfu/g. Divergicin M35 was not detected in the samples, possibly due to decomposition by proteases of endogenous lactic bacteria in salmon, since bacteriocins from class II are very sensitive to proteolytic activity.

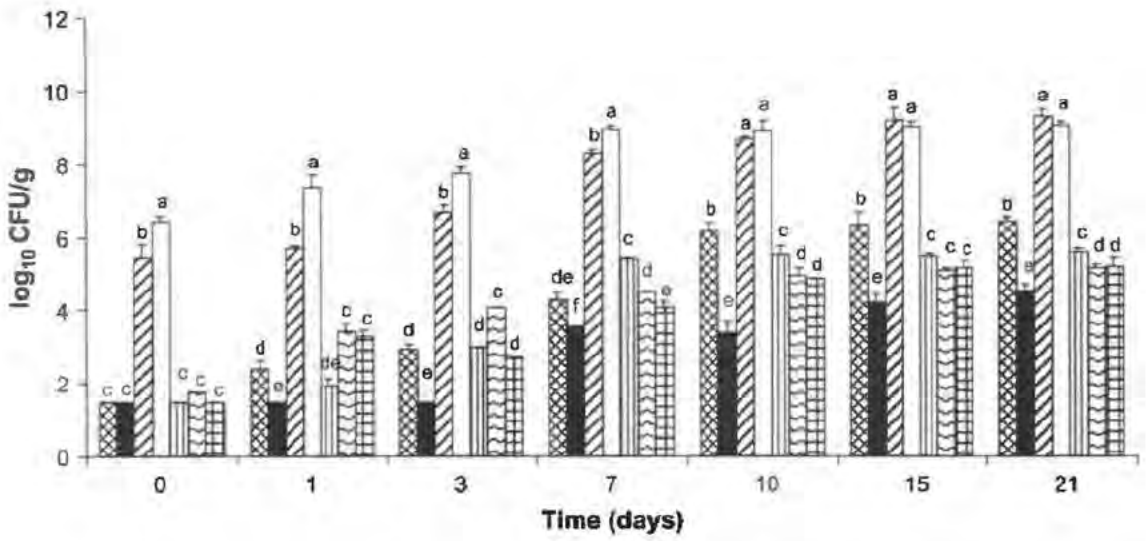


Figure 10. Evolution of *Carnobacterium* spp. count in cold-smoked salmon inoculated with L. monocytogenes and stored at 4 °C for 21 days. ▨ Control (treatment A), ■ L. monocytogenes alone (treatment B), ▨ L. monocytogenes as a co-culture with C. divergens M35 (treatment C), □ L. monocytogenes as a co-culture with C. divergens ATCC 35677 (treatment D), ▥ L. monocytogenes with purified divergicin M35; 50 mg/g (treatment E), ▨ L. monocytogenes with divergicin M35 bio-ingredient (treatment F) and ▦ L. monocytogenes with MRS culture supernatant concentrated 10-fold (treatment G). Error bars indicate standard deviations of treatments and duplicate enumerations per treatment. Different letters at the same time point indicate significant difference ($P < 0.05$). From Tahiri et *al.* 2009 (6).

2.4.3.3 Data on the Efficiency of the Antimicrobial Bio-preservative

Carnobacterium divergens M35 shall be used on fish products for the antimicrobial effect of the culture against *Listeria monocytogenes*. Such an inhibitory effect will be demonstrated through data generated on a period of 21 days on cold-smoked Coho salmon, since this product represents a high risk of contamination. Cold-smoked Coho salmon fillets (Fumoir Grizzly inc., Saint-Augustin, Québec) divided into 216 slices of 25 g each were distributed into treatment groups as indicated below in table 14.

Table 14. Number of samples (slices) of cold-smoked Coho salmon per treatment group.

	***Listeria monocytogenes* added**			
	10 CFU per g		1,000 CFU per g	
Bio-preservative added ↓	4 °C	−20 °C	4 °C	−20 °C
None	24	12	24	12
10^6 CFU per g	24	12	24	12

The experimental treatments were inoculation with *L. monocytogenes* at 10 or 1000 CFU per gram of fish and treatment (or not) of the bio-preservative was done at a concentration of 10^6 CFU of *C. divergens* M35 per gram (suspended at 1.67×10^7 CFU/ml and sprayed onto the slices in two volumes of 750 µL).

Forty-eight slices from each group were stored at 4 °C and the 24 remaining slices were stored at –20 °C. Treated samples (all except negative control) were left for 10 minutes under the laminar flow hood to dry off excess liquid then vacuum-packed individually in plastic packaging material (bags) provided by Fumoir Grizzly inc. and stored for 20 days. Negative controls were not sprayed, as to not modify the composition of the raw fish. Incubation in a laminar hood was done to dry off the excess liquid after the application of the product onto the sliced fish, before the inoculation with a determined concentration of *Listeria monocytogenes*. This allows all the viable cells to be present at the surface of the smoked salmon slice and thus having a reproducible count on every slice.

Two samples stored at 4 °C were processed on days 0, 4, 8, 12, 16 and 20 by homogenizing for 10 minutes with 50 mL of 0.5 % peptone water in filter bags in a Lab Blender 400 Stomacher (Seward Medical, London, UK) at maximal speed. A volume of 50 µL of filtered homogenate was used to count *L. monocytogenes* colonies (black with a dark halo) on PALCAM agar (Oxoid) incubated at 37 °C for 72–96 h. Samples stored at –20 °C were thawed at 4 °C and analyzed likewise on days 2, 4 and 7. Figure 11 shows *L. monocytogenes* viable counts on cold-smoked Coho salmon treated or not with the bio-preservative and stored at 4 °C for 20 days. Inhibition of growth was total for the first 12 days in the case of samples inoculated with 10 CFU/g and treated with the bio-preservative, while counts reached 10^3 CFU/g by the fourth day on samples not treated. Although *L. monocytogenes* counts on the treated slices reached 10^2 CFU/g by the end of the storage period, they were always at least 4Log lower than on untreated slices. The inhibitory effect of the bio-preservative was significant also on slices stored at –20 °C for 20 days, thawed and kept at 4 °C for 7 days. This indicates that the bio-preservative is stable at deep-freeze temperatures and active in the thawed product, preventing the proliferation of *L. monocytogenes* for at least a week at proper refrigerator temperature, in the event of the presence of this pathogen in the smoked fish prior to freezing.

These results show clearly that applying a protective bio-ingredient (i.e., bio-preservative) derived from a culture of *C. divergens* M35 suppresses significantly the proliferation of *L. monocytogenes* in cold-smoked Coho salmon. Since the level of *L. monocytogenes* contamination that occurs in the food-processing industry is typically about 10 CFU/g, we suggest that its proliferation on Coho salmon slices stored at 4 °C is completely inhibited for 12 days. Growth of *L. monocytogenes* did occur subsequently, but much less than on slices not treated with the bio-preservative. Comparable results were obtained in other similar studies (6). When the initial level of *L. monocytogenes* was higher (10^3 CFU/g), the bio-preservative did not provide total inhibition of growth but did slow it considerably, keeping the counts 2Log lower than on untreated slices. It should be noted that contamination with *L. monocytogenes* at levels as high as 10^3 CFU/g is rarely, if never, seen in the industry.

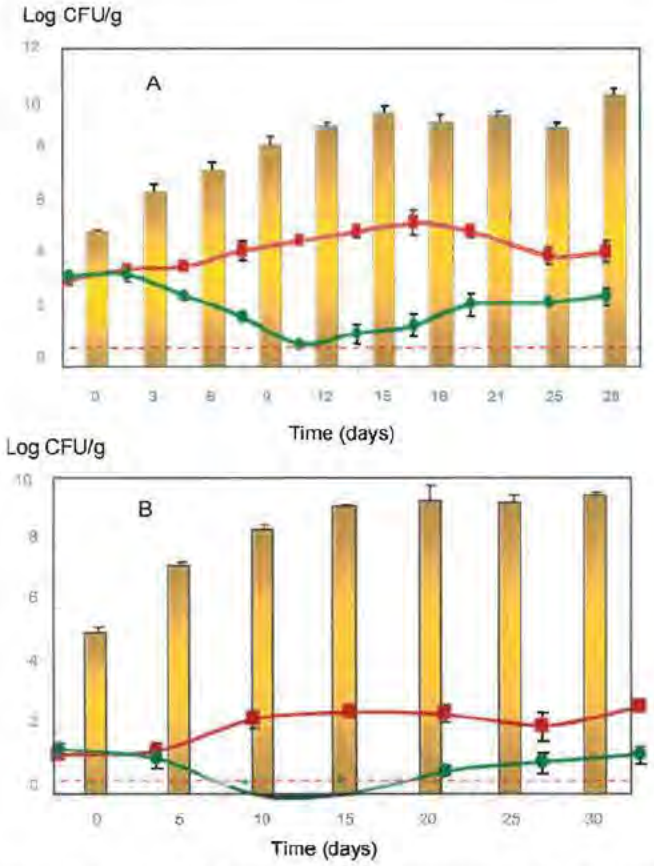


Figure 11. Inhibition of *Listeria monocytogenes* by *C. divergens* M35 bio-preservative applied to cold-smoked salmon. *L. monocytogenes* counts in the absence (red) or presence (green) of the bio-preservative (initial counts were 10e3 CFU/g (A) or 10 CFU/g(B)); yellow – *C. divergens* M35 counts.

We demonstrated the efficacy of the culture on fresh tuna also because this product represents a high risk of contamination, but also a high risk of biogenic amine poisoning due to its high free amino acid content. For fresh tuna, the experimental procedure was divided into three batches (batch 1: negative control, batch 2: positive control-10^3 CFU/g of *Listeria monocytogenes*, batch 3: contamination with *Listeria monocytogenes* (same of batch 2) with treatment with 10^6 CFU/g of bio-ingredient). The concentration of the ingredient applied was determined by the biogenic amine analysis for fresh tuna. Since no variation of biogenic amine is produced with the addition of BacM35 and considering that the strain is not very competitive with the endogenous flora, the highest amount (10^6 CFU of viable *Carnobacterium divergens* M35 cells per gram of tuna) was tested to ensure an optimal inhibition. Each batch was inoculated with 10^3 CFU/g of *Listeria monocytogenes* (except batch 1) and batch 3 was treated with the associated concentration of the bio-ingredient. The samples were then frozen overnight, thawed and kept at 4°C in triplicate for 21 days, which is seven times longer than the conservation time of 3 days. The detection of *L. monocytogenes* was done at key points during the process (days 0, 1, 3, 7,10, 14, 17, and 21) to evaluate the inhibitory activity of the ingredient. Table 15 shows the counts obtained for each batch and day.

Table 15. *Listeria monocytogenes* counts for fresh tuna non-treated (batch 1), fresh tuna artificially inoculated with 10^3 CFU/g of *Listeria monocytogenes* (batch 2) and fresh tuna artificially inoculated with 10^3 CFU/g of *Listeria monocytogenes* and treated with 10^6cfu/g of BacM35 (batch 3) for 21 days.

Day	Batch 1 (CFU/g)	Batch 2 (CFU/g)	Batch 3 (CFU/g)
0	0 (<30)	383 ± 2	145 ± 5
1	0 (<30)	376 ± 7	84 ± 10
3	1.6 ± 0.2 (<30)	293 ± 5	43 ± 2
7	0 (<30)	271 ± 10	20 ± 2 (<30)
10	0 (<30)	116 ± 5	6 ± 2 (<30)
14	0.8 ± 0.1 (<30)	375 ± 88	5 ± 1 (<30)
17	0 (<30)	270 ± 17	0 (<30)
21	1.6 ± 0.2 (<30)	245 ± 15	10 ± 2 (<30)

As shown in table 15, the count of *Listeria monocytogenes* is stable for 21 days, so the concentration is not increasing nor decreasing. Based on that fact, it is possible to assess that the diminished quantity of *L. monocytogenes* of batch 3 is caused by the addition of the protective culture and not by natural mortality of the pathogen through time.

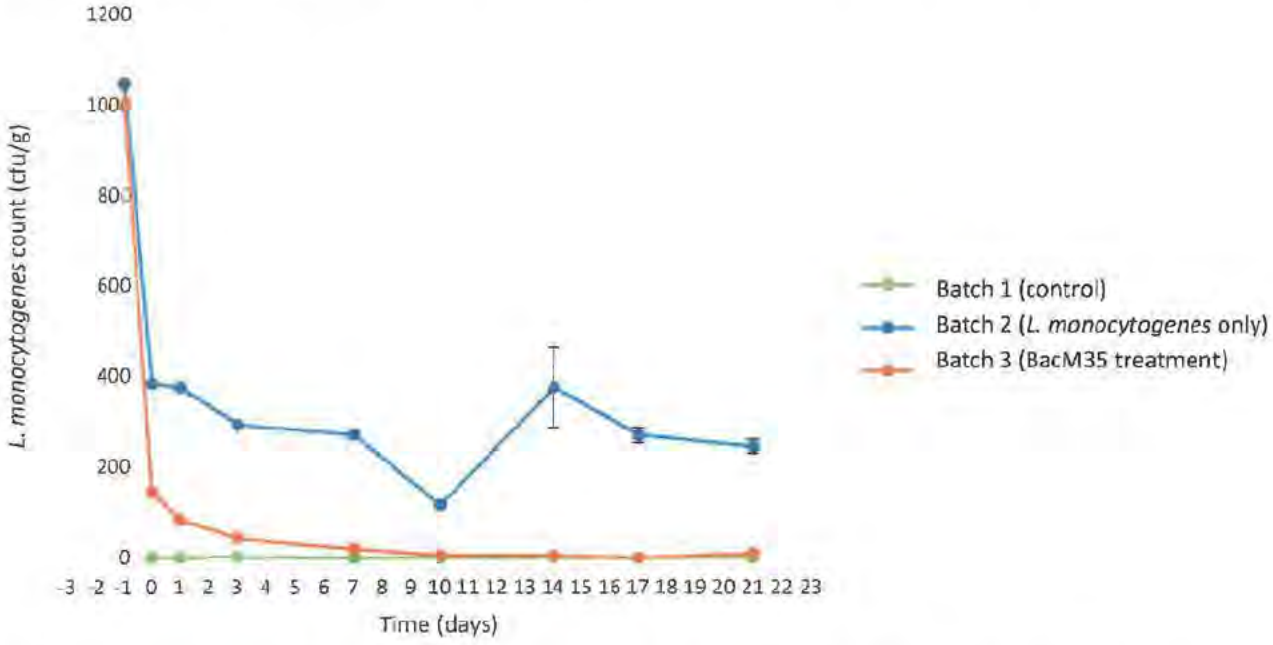


Figure 12. Bacterial counts of *Listeria monocytogenes* (CFU/g of tuna) with error bars during a period of storage at 4°C for 21 days. In green: control batch; in blue: artificially inoculated tuna with 10^3 CFU of *Listeria monocytogenes* per gram; in orange: artificially inoculated tuna with 10^3 CFU of *Listeria monocytogenes* per gram treated with 10^6 CFU/g of BacM35.

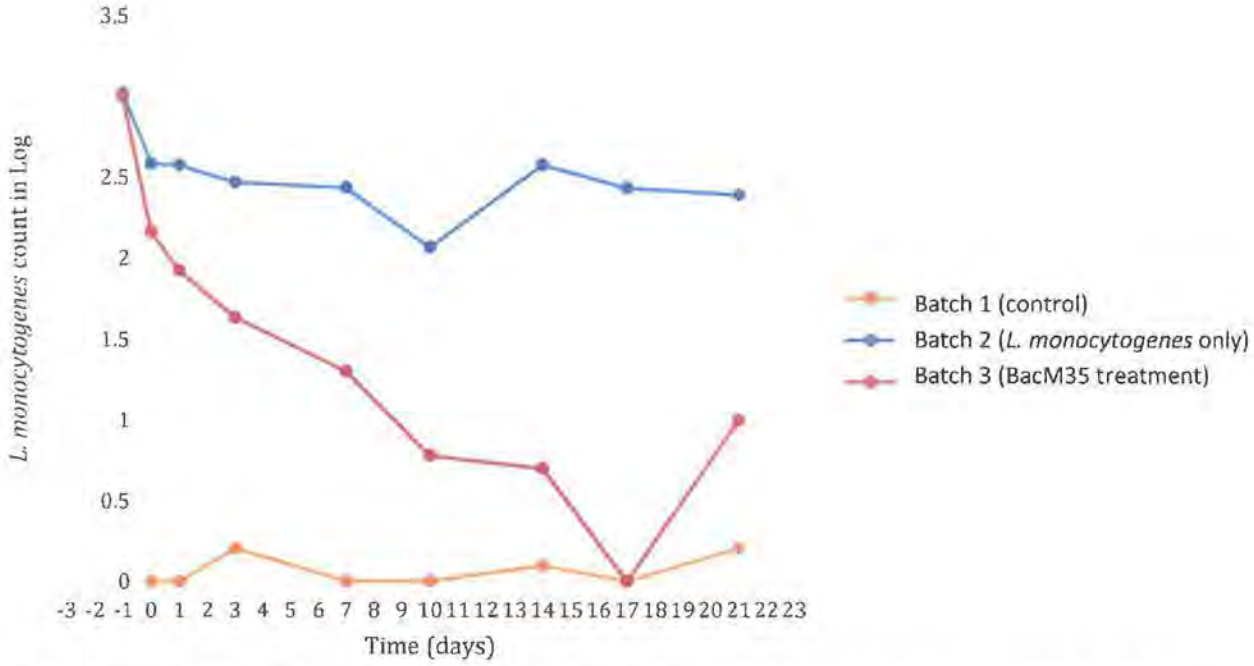


Figure 13. Bacterial counts of *Listeria monocytogenes* in Log with error bars during a period of storage at 4°C for 21 days. In orange: control batch; in blue: artificially inoculated tuna with 10^3 CFU of *Listeria monocytogenes* per gram; in orange: artificially inoculated tuna with 10^3 CFU of *Listeria monocytogenes* per gram treated with 10^6 CFU/g of BacM35.

Those results show that the addition of a *Carnobacterium divergens* M35 based treatment reduces significantly the amount of viable *Listeria monocytogenes* present in fresh tuna, as in cold-smoked salmon also (Figure 12 and 13). There is a reduction below the limit of detection (30 CFU/g) after 7 days, a reduction for up to 2.5Log (day 17) and an immediate reduction of about 0.5Log upon thawing (Figure 13). Here, there is no subsequent growth of *L. monocytogenes* and we hypothesize that considering the general amount of 10 CFU of pathogen per gram present in the industry, there is a complete inhibition after 12 days.

Part 3: Dietary exposure

3.1 Exposure to *Carnobacterium* from the environment and the food

Lactic Acid Bacteria (LAB), including species of the genus *Carnobacterium*, have been used for centuries for the production of fermented foods (2, 10). Several published reports have documented the presence of *Carnobacterium* spp. in food, including meat and meat products, vegetables, fruits, cheeses, and seafood, at concentrations from 5.0×10^5 to 1.0×10^9 CFU/g (22, 76–78). They are able to grow in foods that contain little free carbohydrate even at low temperatures. Unlike other lactic acid bacteria, the relatively weak acidification by *Carnobacterium* suggest the possibility of adding them directly to cold-smoked salmon and other non-fermented or non-marinated ready-to-eat fish and seafood without risking undesirable changes to the organoleptic and sensory characteristics (22, 64, 76–78).

Carnobacteria can tolerate both temperate and cold temperature since they have been isolated from tropical fish and the Arctic sea (79, 80). Microorganisms belonging to the *Carnobacterium* genus are rod-shaped, gram-positive and heterofermentative. Strains can grow at 0°C, with an optimal growth rate around 30°C and are able to survive multiple freeze-thaw cycles (49, 81). Those characteristics make the species *C. divergens* and *C. maltaromaticum* an ideal candidate for a bio-preservative compound.

Strain *Carnobacterium divergens* M35 has been isolated in from mussels from the Atlantic. *Carnobacterium spp.* is frequently found in salt water, because it is frequently isolated from marine products as being one of the first flora to populate this type of product (82).It is therefore a habitat already populated by this strain, but whose original flora is also already regulated by the surrounding flora. Also, *C. divergens* natural colonization of meat is hypothesized to come from the colonization of the intestinal track of animal, thus its presence on meat. It has not been associated with plants, fermented vegetables or the gastrointestinal system of birds (10). Also, as mentioned before, the species is naturally found in healthy human guts so no adverse effect from this species is expected.

Currently, the only abundant reservoirs for this strain are animals, as well as water. The study by Dalgaard *et al.* (10), summarizes the status of *Carnobacterium* spp. in the environment. This genus is omnipresent in the various wet or water reservoirs, but is rarely the dominant genus, because it is not very competitive against the various lactic acid bacteria and other flora. In the majority of these reservoir, it is not possible to carry out an account of this kind, because always in presence only. It is therefore necessary to proceed with enrichment in order to have a countable population.

The population becomes significant when it is implanted in a living organism. In the case of *Carnobacterium divergens* M35, it has been shown that its implantation in a food or in an organism does not cause infection or denaturation, because this strain has a very low proteolytic capacity (1). It is for this reason that it was chosen as a preservative, as it is not competitive and has marginal nutrient consumption. This ability has been studied on many foods and is not expected to differ in one environment.

In water, it is a flora frequently present in different reservoirs. Very few data exist concerning the concentration of this flora, because it varies greatly depending on the water points but once again, it always remains in presence too weak to be countable. A Norwegian study was able to determine that the majority of the bacterial flora found in the gills of Atlantic salmon consisted of *Carnobacterium* spp. at a level around 4Log (CFU/g) (78). As the gills act as a filter, it can be assumed that the native flora in the water is below this level. *Carnobacterium* spp. Was able to become the dominant species in the gills because the high concentration allowed the action of its bacteriocins against the rest of the flora present. But once again, we cannot say that this strain proliferates in this environment, only that it is found there in traces.

In soil, a Canadian study demonstrated its presence in soil in the polar regions of Canada, however, their presence was not quantified (81).

No data or studies currently demonstrate the presence of *Carnobacterium* spp. in the air.

It is impossible to estimate the exact quantity at which humans are already exposed to the genus and this species, but it is safe to assume that through water, meat and fish-based food, humans are already consuming this bacterium daily.

3.2 Human exposure to *Carnobacterium* and to their bacteriocins

Carnobacteria are often present and isolated in the environment and food, from which the species *C. divergens* and *C. maltaromaticum* are the most abundant. As mentioned previously, *Carnobacterium divergens* is frequently isolated from dairy, meat, fish and shrimp products (9, 47, 79, 83). Despite this relative abundance in food products, Carnobacteria are not known members of the human gastrointestinal microbial community, unlike several other Lactic Acid Bacteria. They are mostly studied for the production of bacteriocins that inhibit the human pathogen *Listeria monocytogenes* (10). *Carnobacterium sp.* is ubiquitous to the environment, but mostly found in water and food. As for *C. divergens* itself, this strain is mostly found in food and on meat product, because it naturally colonizes various food (44, 47, 48). There is no global geographic distribution of the microorganism, but the following figure represents the relative abundance of *C. divergens* compared to other *Carnobacteria* in different ecological niches.

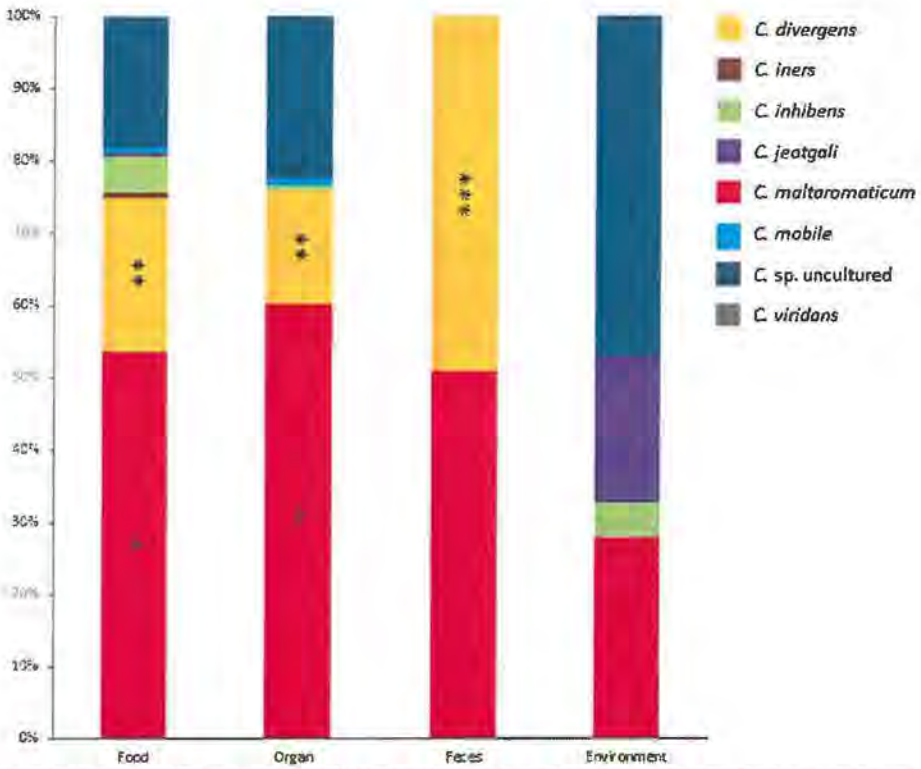


Figure 14. Relative abundance of each *Carnobacterium* species obtained from 16S metagenomic analyses of 681 samples originating from food, organs, feces, and environment. One asterisk indicates significant difference ($P < 0.0001$) compared to all other *Carnobacterium* sp, two asterisks indicate significant difference ($P < 0.01$) compared to *C. iners, C. inhibens, C. jeotgali, C. mobile*, and *C. viridans*. Three asterisks indicate significant difference ($P < 0.0001$) compared to all *Carnobacterium* sp. except *C. maltaromaticum* (44).

Since *Carnobacterium* spp. is a part of the endogenous microflora of several foods, it can be obvious that humans have already been exposed to this species and more specifically those producing bacteriocins such as *C. divergens* M35. However, no case of acute or chronic toxicity directly associated with long term exposure to bacteriocin-producing *C. divergens* or to other *Carnobacterium* species has been reported to date. In addition, as for many other LAB strains, *C. divergens* do not seem to be able to survive the different physiological barriers of the digestive tract (gastric acidity, bile salts, etc.), to implant themselves at the colic level or to produce their bacteriocins *in situ* or to translocate through the digestive barrier. It is also well established that bacteriocins are only active towards a narrow group of specific micro-organisms, it is then not expected to cause adverse effects to biological diversity when released into the digestive tract.

On the other hand, bacteriocins which have been isolated from several foods such as beef, ham, fish, vegetable, and cheese, have probably been consumed for centuries without any reported negative side effects. Indeed, due to the protein nature of the compound, the bacteriocins produced in foods are therefore degraded by the proteases and the other enzymes encountered in the mammalian digestive system and are thus unlikely to make their way into the digestive system. This phenomenon has already been clearly described for nisin A, nisin Z and pediocin using in vitro gastrointestinal model (Fernandez et al. 2012). The notifier also demonstrated scientifically that bacteriocins produced by *C. maltaromaticum*

CB1 are readily digestible and unstable in the mammalian digestive system and are thus unlikely to be toxic or allergenic towards animals. It should be emphasized that Nisin, originally isolated in the late 1930s and produced since the 1950s, was approved as an additive for food use in the USA and more than 50 other countries in the late 1960s.
https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=65

Carnobacterium divergens is already part of the natural gut microbiota of humans (45). As it is already found on meat, fish, dairy product and water, humans and animals have been extensively exposed to the species and it part of the healthy gut microbiota. This probiotic will not have any negative effect from its consumption and since the start of its use in 2007, no infection or increase of infection has been reported in Canada.

So, all these highly suggested that ingestion of bacteriocins would not have any deleterious effect on the human microbiota and would not induce any allergenic or toxic effect for humans.

3.3 The estimate number of persons that may become exposed and degree of their exposure to the microorganism and its by-products.

The degree of exposure to the microorganism is somewhat stable. Any person consuming cold-smoked salmon or cold-smoked trout would consume a maximal level of 10^6 colony forming unit (cfu) per gram of food. The estimate number of people that may become exposed would be the consumers of those products but an exact estimation is very difficult since marketing can influence sales and the number of consumers. No economical analysis can predict the number of persons exposed, so it is safer to assume that any consumer of cold smoked fish may become exposed in the general population.

From an operational point of view, any employee on the production line using the bioingredient is susceptible and employees producing the culture will be exposed. Employee preparing and monitoring the culture during the production and quality control staff as well as employees handling and diluting the powder will be exposed to the concentrated product from up to 10^9 cfu/g and other employee on the production line will be exposed to lower levels of the microorganism.

3.3.1 Estimation of total intake of *C. divergens* M35

As mentioned in section 2.4.3.2, after introduction on food, the strain, adapted to the environment of smoked fish can survive and multiply to some extend (see figure 15). In trials conducted on smoked salmon and as shown in figure 15, we found that *C. divergens* M35 (a psychrotroph) survived very well at 4 °C, with counts reaching about $1x10^9$ cfu/g after 21 days, while total counts (including total lactic acid bacteria) were about $5x10^8$ cfu/g. Divergicin M35 was not detected in the samples, possibly due to decomposition by proteases of endogenous lactic bacteria in salmon, since bacteriocins from class II are very sensitive to proteolytic activity.

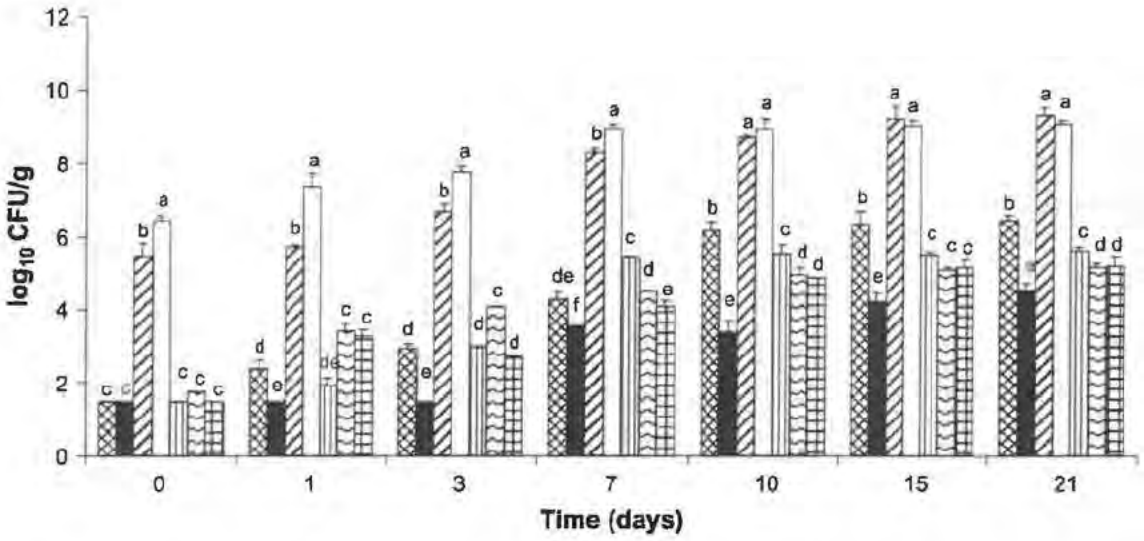


Figure 15. Evolution of *Carnobacterium* spp. count in cold-smoked salmon inoculated with L. monocytogenes and stored at 4 °C for 21 days. ⊠ Control (treatment A), ■ L. monocytogenes alone (treatment B), ▨ L. monocytogenes as a co-culture with C. divergens M35 (treatment C), □ L. monocytogenes as a co-culture with C. divergens ATCC 35677 (treatment D), ▣ L. monocytogenes with purified divergicin M35; 50 mg/g (treatment E), ▩ L. monocytogenes with divergicin M35 bio-ingredient (treatment F) and ⊞ L. monocytogenes with MRS culture supernatant concentrated 10-fold (treatment G). Error bars indicate standard deviations of treatments and duplicate enumerations per treatment. Different letters at the same time point indicate significant difference ($P < 0.05$). From Tahiri et *al.* 2009 (6).

According to the Dietary Guideline for Americans (2020-2025) chapter 4, page 96, adults should consume a weekly amount of 8 to 10 ounces of fish/seafood, which is equivalent to about 225 to 285 grams of salmon (https://www.dietaryguidelines.gov/, consulted January 10th, 2022). Considering that the smoked fish is inoculated at $1x10^6$ CFU/g with growth up to $1x10^9$ CFU/g after 14 days at 4°C (6), then the amount consumed of viable cells varies from $2.25x10^8$ to $2.85x10^{11}$ CFU weekly. Most of the lactic acid bacteria won't survive the passage through the digestive system and around 3% to 12% will make it to the intestine, representing about $6.75x10^6$ CFU to $3.4x10^{10}$ CFU, if we take into account the lowest amount with the lowest percentage and the highest amount with the highest percentage. But, as discussed in the safety part of the submission, *Carnobacterium divergens* M35 is already found in human feces and in the gastrointestinal track of mammals, also it is not a very competitive strain and the microorganism is unlikely become a major colonizer of the gut microflora if humans are already exposed to this species.

The amount consumed is also similar to that of another species, *Carnobacterium maltaromaticum*. The strain *C. maltaromaticum* CB1 has been approved as a food additive in meat by the FDA (GRAS Notice No. GRN 000159
https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=159&sort=GRN_No&order=DESC&startrow=1&type=basic&search=305 , consulted January 12th, 2022 and No. 000305

https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=305&sort=GRN_No&order=DESC&startrow=1&type=basic&search=305 consulted January 12th, 2022) and Health Canada (Reference No NOM/ADM-0097, https://www.canada.ca/en/health-canada/services/food-nutrition/public-involvement-partnerships/notice-modification-list-permitted-preservatives-enable-use-carnobacterium-maltaromaticum-cb1-antimicrobial-preservative-certain-meat-poultry-meat.html, consulted January 12th, 2022).

The maximum amount accepted for consumption of viable cells was $8.6x10^9$ CFU per person per day, with the 90th percentile of $1.6x10^{10}$ CFU per person per day, which is similar than the amount consumed for *C. divergens* M35. Considering that strain CB1 and M35 are very similar, we see no issue with the number of viable cells consumed weekly. It is also stated in the literature that almost no phenotypic distinction is possible between *C. divergens* and *C. maltaromaticum* and molecular methods are necessary to distinguish them. Those two species are proven to be very similar and safe to consume and one of them is already considered GRAS (83). They are frequently isolated from food, without any selection or enrichment and are a major part of some food microbiota with and without modified atmosphere packages, like poultry, pork, fish and milk (2, 9, 10, 47, 76, 79). *Carnobacterium divergens* can even reach 50% of the total lactic acid bacteria present on raw meat (pork, beef and poultry) (48, 83–85). There is no precise count of the exposure to this bacterium, but we can estimate that there is already a frequent and high amount of *C. divergens* consumed naturally by the general population, with no risk health risk associated with it.

3.3.2 Estimation of intake of bacteriocins and other by-products.

As for the consumption of divergicin, due to the presence of proteases already present on food (see section 2.4.3.2), as discussed in section 2.4.2 of the safety of bacteriocins, divergicin M35 was never detected in samples of cold-smoked salmon analyzed (6). The strain grows and has its antimicrobial effect but the bacteriocin is quickly degraded on the surface of food and humans will not consume it.

C. divergens M35 is a non-motile gram-positive rod lactic acid bacterium, non-spore forming, growing in short chains, catalase and oxidase negative. It produces only L-lactic acid and very little gas. Other by-products expected from the fermentation process, excluding divergicin, is L-lactic acid in very small quantities. L-lactic acid is an organic acid naturally present in food and already considered GRAS by the FDA as mentioned in the Code of Federal regulation Title 21, Chapter 1, Subchapter B, Part 184, Subpart B, Section 184.1061 (https://www.govinfo.gov/app/details/CFR-2011-title21-vol3/CFR-2011-title21-vol3-sec184-1061, consulted January 12th, 2022) and represents no risks for consumption.

Part 4: Self-limiting level of use

4.1 Quantity Used, Proposed uses and Purposes Thereof, Detailed Directions for Use, and Recommendations

It appears that the growth of the strain is self-limited at 10^9 CFU in MRS broth at 30°C for 24h and thus the concentration of cell in food will not surpass this amount. Other than this limitation, the concentration applied has to be optimal for inhibition of Lis*teria monocytogenes* and to maintain a safe level of consumption.

4.1.1 Data on the Microflora Alteration

Figure 16 shows the evolution of total lactic acid bacteria in cold smoked salmon stored at 4 °C. The final concentration of the protective culture of *C. divergens* M35 added would then correspond to the maximum concentration found after 15 days of storage. The addition of this concentration allows a significant inhibition of *Listeria monocytogenes* without alternating the intrinsic sensory and organoleptic characteristics of the products. Compared to the control group, groups with *Carnobacterium divergens* M35 have significantly more LAB because the amount of the strain is taken into account, but figure 16 shows that addition of BacM35 does not compromise or modifies the growth of the natural microflora present on the strain. Those results were expected because the genus is not known to be very competitive against other LABs.

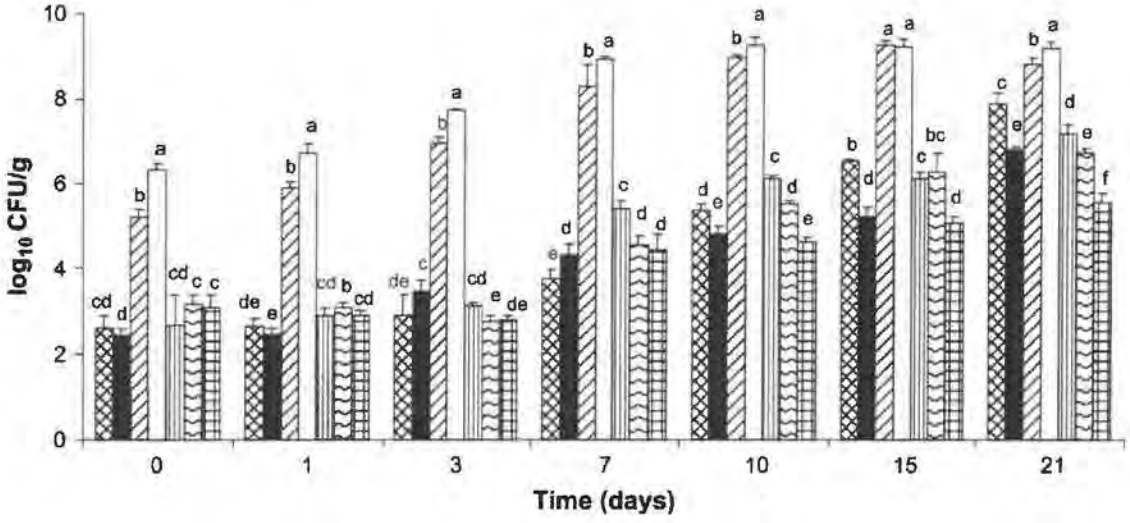


Figure 16. Evolution of total lactic acid bacteria count in cold-smoked salmon inoculated with L. monocytogenes and stored at 4 °C for 21 days. ☒ Control (treatment A), ■ L. monocytogenes alone (treatment B), ▨ L. monocytogenes as a co-culture with C. divergens M35 (treatment C), □ L. monocytogenes as a co-culture with C. divergens ATCC 35677 (treatment D), ▥ L. monocytogenes with

purified divergicin M35; 50 mg/g (treatment E), ▨ L. monocytogenes with divergicin M35 bio-ingredient (treatment F) and ⊞ L. monocytogenes with MRS culture supernatant concentrated 10-fold (treatment G). Error bars indicate standard deviations of treatments and duplicate enumerations per treatment. Different letters at the same time point indicate significant difference ($P < 0.05$). From Tahiri et *al.* 2009 (6).

4.1.2 Data on the Amount Recommended for Use

Different concentrations were tested in order to determine the optimal concentration of use, in this case, the amount appropriated for the inhibition of *Listeria monocytogenes* on smoked salmon and tuna. A quantity of 10^6 CFU/g, $10^{5.5}$ CFU/g, and 10^5 CFU/g were tested to assess to optimal concentration necessary for inhibitory activity. A total of 5 batches were divided, batch 1 is the negative control, batch 2 is the artificially inoculated smoked salmon with 10^3 CFU of *Listeria monocytogenes* per gram of product, batches 3 to 5 are also inoculated with the same quantity of L. monocytogenes but batch 3 was treated with 10^6 CFU of *Carnobacterium divergens* M35 per gram, batch 4 was treated with $10^{5.5}$ CFU of *Carnobacterium divergens* M35 per gram and batch 5 was treated with 10^5 CFU of *Carnobacterium divergens* M35 per gram. Each batch was analyzed in duplicates for 21 days to count the number of viable *L. monocytogenes* cells. Table 16 shows the results associated with that experiment.

Table 16. *Listeria monocytogenes* counts (CFU/g of smoked salmon) for 21 days of smoked salmon (1 A-B), smoked salmon inoculated with 10^3 CFU/g of *L. monocytogenes* (2 A-B), smoked salmon inoculated with *L. monocytogenes* treated with 10^6 CFU/g of *Carnobacterium divergens* M35 (3 A-B), smoked salmon inoculated with *L. monocytogenes* treated with $10^{5.5}$ CFU/g of *C. divergens* M35 (4 A-B) and smoked salmon inoculated with *L. monocytogenes* treated with 10^5 CFU/g of *C. divergens* M35 (5 A-B).

Batch	***Listeria monocytogenes* count (CFU/g)**			
	Day 0	Day 7	Day 14	Day21
1-A	<300	<300	<300	<300
1-B	<300	<300	<300	<300
2-A	$8.95X10^3$	$2.04X10^4$	$5.44X10^4$	$1.10X10^5$
2-B	$8.85x10^3$	$1.57x10^4$	$1.23x10^5$	$1.15x10^5$
3-A	$4.10x10^3$	<300	<300	<300
3-B	$3.15x10^3$	<300	<300	<300
4-A	$5.65x10^3$	<300	<300	<300
4-B	$4.30x10^3$	<300	<300	<300
5-A	$6.25x10^3$	$5.10x10^2$	<300	<300
5-B	$5.15x10^3$	$8.60x10^2$	<300	<300

From this data, we can assume that the quickest protection against *Listeria monocytogenes* in smoked salmon is 10^6 CFU/g of *Carnobacterium divergens* M35. At day 0, the lowest count of bacteria is associated with 10^6 CFU/g, even though $10^{5.5}$ CFU/g is similar. The highest dose will be used to offer optimal protection and a safety net for the processing chain. It has already been demonstrated that this amount does not change the organoleptic properties of the product and that no biogenic amine is produced beyond toxic levels and therefore it is safe for consumption.

The bio-preservative is intended for use on fish (except catfish). Based on the prior determination of its CFU/mL viability, the lyophilized product shall then be re-suspended in water of suitable microbiological quality (drinking water or higher grade). Then, the application will be done by spraying or mixing a certain volume in order to obtain a final concentration of 10^6 CFU per gram or per cm^2 of product. The treated product must then be vacuum-packed and stored at 4 °C or frozen. The supplier guarantees the identity of the bacterial strain and the biological activity of the culture.

4.1.3 Method of Detecting and Monitoring the Bio-preservative in the Food Product

Two methods may be used for the specific detection of *C. divergens* in foods. The first is based on a selective medium developed by Wasney et al. (2001). CTSI agar contains sucrose, manganese sulfate, thallium acetate, inulin, thiamine hydrochloride, vancomycin, and nisin. On this medium, *C. divergens* produces highly characteristic pink colonies. Using a top layer of tryptic soy soft agar inoculated with *Listeria ivanovii* (a non-pathogenic organism), M35 colonies that produce divergicin can be counted specifically. The second method is based on the amplification of a specific DNA sequence of 199 base pairs in length using the primer pair 27F/cdi. The details of this molecular method are described in scientific articles published by Barakat et al. (2000), Tahiri et al. (2004) and Tahiri et al. (2009) (1, 2, 6). These two methods have been successfully used for detection and monitoring *C. divergens* in cold and frozen smoked salmon and tuna.

Part 5: Experience based on common use in food before 1958

To our knowledge, humans have probably been exposed to the microorganism through food before 1958, but the conclusion of the GRAS status is not based on common use prior to 1958. No history of use has been found in the literature.

Part 6: Narrative

The present application relies on the regulatory approval of a bio-ingredient containing *Carnobacterium divergens* M35 cells and its metabolites including the bacteriocin divergicin M35, as generally recognized as safe (GRAS). This bio-ingredient is intended for use as a food ingredient for inhibition of *Listeria monocytogenes* in fish.

The GRAS status claimed is based on literature information and scientific procedures regarding the safety of genus *Carnobacterium* and scientific data generated and evaluated by experts from a research group either in Fumoir Grizzly or at Université Laval, Quebec, Canada through a collaborative research project. The bacterial strain, which is the subject of this application, was identified as *Carnobacterium divergens* by using different state of the art biochemical and molecular methods. Its safety has been confirmed by different in vitro assays that have proven the non-pathogenicity and the non-toxicity of the strain.

6.1 *Carnobacterium* is Part the Endogenous Microbial Ecosystem

The genus *Carnobacterium* includes several bacterial species that are part of the endogenous microbial ecosystem of several food products. *Carnobacterium* species have been isolated from different food products including fish, milk, and meat and have never been associated with food poisoning or infection from oral consumption. It has also been isolated from the intestinal track and feces of mammals and humans. Humans are already exposed to it and have already consumed this microorganism frequently through their lives. *Carnobacterium divergens* is a level 1 biosafety microorganism and a member of the Lactic Acid Bacteria family, that are Generally Recognized as Safe GRAS by the FDA. Bacteria from the Lactic Acid Bacteria are the bacteria the most used in the food industry for fermentation of any type of food (dairy, vegetables, etc.) and the majority or those bacteria are naturally producing bacteriocins as a mechanism of defense targeted against bacteria in an ecological niche.

6.2 *C. divergens* M35 is Sensitive to a Wide Range of Antibiotics, has no hemolytic activity and is considered safe to consume

The antibiotic resistance of *C. divergens* M35 was evaluated using the agar diffusion method based on the inhibition zone diameter around paper discs impregnated with antibiotics. This method was performed according to CLSI M-45A specifications (2006 and 2009) carried out routinely for testing candidates belonging to other genera of LAB. The results indicate that *C. divergens* M35 was sensitive to 11 of the 12 antibiotics tested, under several culture conditions. This resistance profile is consistent with those obtained with other LAB including those belonging to the genera *Carnobacterium* (36, 37, 86). These authors mentioned that resistance to streptomycin and some other antibiotics is intrinsic (non-acquired) and thereof is non-transmissible via plasmids. It is then obvious that there is no risk of transmission of resistance genes by *C. divergens*.

The hemolytic activity of *C. divergens* M35 was evaluated in different media containing defibrinated sheep blood. *C. divergens* M35 did not display any hemolytic activity, under both aerobic and anaerobic conditions.

Finally, extensive data from the literature (see section 2.4 on safety of the strain and bacteriocins), showed the safety of the species *divergens* and the safety of bacteriocins produced by LABs by demonstrating the absence of pathogenicity, acute and subacute toxicity, cytotoxicity and toxigenicity in *in vitro* and *in vivo* models.

6.3 *C. divergens* M35 does not Produce Biogenic Amines

The analysis of the production of biogenic amines in smoked salmon and fresh tuna revealed that the addition of *C. divergens* M35 did not induce any of the following amines: methylamine, tryptamine, putrescine, spermidine, spermine, tyramine, histamine, and cadaverine above the limit of toxicity. The analyses also revealed that tyramine and tryptamine was produce at concentrations of respectively 63 µg/g and 80 µg/g or 21.5 µg/g and 13 µg/g after 16 and 28 days of storage respectively. These concentrations are below the current regulatory allowed limit and are very low compared to those produced by other species of *Carnobacterium* in salmon (up to 4000 µg/g). For fresh tuna, the biogenic amines tyramine and cadaverine were produced at a concentration of 80 µg/g and 378 µ/g respectively, which is not enough to induce any symptoms or toxicity, even for sensitive individuals with decreased monoamine oxidase activity (10, 28). Through scientific procedures, we showed that the addition of the strain on food did not increase the amount of biogenic amines normally found on salmon and tuna throughout the conservation time and posed no safety risk for consumption.

6.4 Conclusion for GRAS determination

Finally, optimized manufacturing conditions and quality control procedures have been developed and described to ensure a high quality, viability, purity and active preparation of *C. divergens* M35 in accordance with current good manufacturing practices (cGMP). Based on a thorough literature review and characterization of the strain by expert scientists, antibiotic sensitivity, biogenic amine production, hemolytic activity and a natural frequent exposure from meat, fish, poultry and milk reported above, it appears that *C. divergens* M35 is not hazardous to humans and will not produce any significant adverse effects in the general population.

Considering that the behavior of the strain was studied in the worst conditions possible: (1) raw fish (tuna and salmon) that both represent a high risk of contamination and that have a high potential for biogenic amine content due to the free amino acids of those histidine-rich scombroid fish (87, 88) and (2) smoked fish (cold smoked salmon) that represent a food product with a reduced bacterial flora. For these two cases, the data obtained proved the safety of *Carnobacterium divergens* M35 from each key characteristic analyzed, we can assess that that use of this strain provides an ideal mean of protection in all fish products. All this information, taken together, allows us to claim with certainty a GRAS status for *C. divergens* M35 and its metabolites.

Part 7: List of supportive data and information in the GRAS notice

7.1 Literature review

1. Tahiri I, Desbiens M, Benech R, Kheadr E, Lacroix C, Thibault S, Ouellet D, Fliss I. 2004. Purification, characterization and amino acid sequencing of divergicin M35: A novel class IIa bacteriocin produced by Carnobacterium divergens M35. Int J Food Microbiol 97:123–136.
2. Barakat RK, Griffiths MW, Harris LJ. 2000. Isolation and characterization of Carnobacterium, Lactococcus, and Enterococcus spp. from cooked, modified atmosphere packaged, refrigerated, poultry meat. Int J Food Microbiol 62:83–94.
3. Cotter PD, Hill C, Ross RP. 2005. Food microbiology: Bacteriocins: Developing innate immunity for food. Nat Rev Microbiol 3:777–788.
4. «d Ennahar S, Sashihara T, Sonomoto K, Ishizaki A. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol Rev 24:85–106.
5. CFIA. Marine biotoxins in bivalve shellfish: Paralytic shellfish poisoning, amnesic shellfish poisoning and diarrhetic shellfish poisoning.
6. Tahiri I, Desbiens M, Kheadr E, Lacroix C, Fliss I. 2009. Comparison of different application strategies of divergicin M35 for inactivation of Listeria monocytogenes in cold-smoked wild salmon. Food Microbiol 26:783–793.
7. Wasney MA, Holley RA, Jayas DS. 2001. Cresol Red Thallium Acetate Sucrose Inulin (CTSI) agar for the selective recovery of Carnobacterium spp . 64:167–174.
8. Bergey DH. 2009. Bergey's Manual of Systematic Bacteriology - Volume 3 : The Firmicutes, 2nd ed. Springer, New-York.
9. Rieder G, Krisch L, Fischer H, Kaufmann M, Maringer A, Wessler S. 2012. Carnobacterium divergens - a dominating bacterium of pork meat juice. FEMS Microbiol Lett. England.
10. Leisner JJ, Laursen BG, Prévost H, Drider D, Dalgaard P. 2007. Carnobacterium: positive and negative effects in the environment and in foods. FEMS Microbiol Rev2007/08/. 31:592–613.
11. Galkin A, Kulakova L, Ashida H, Sawa Y, Esaki N. 1999. Cold-adapted alanine dehydrogenases from two antarctic bacterial strains: gene cloning, protein characterization, and comparison with mesophilic and thermophilic counterparts. Appl Environ Microbiol 65:4014–4020.
12. Coombs J, Brenchley JE. 2001. Characterization of two new glycosyl hydrolases from the lactic acid bacterium Carnobacterium piscicola strain BA. Appl Environ Microbiol 67:5094–5099.
13. Dallaire L, Fliss I, Groleau D. 2017. Optimisation des conditions de fermentation et de stabilisation pour la production de bio-ingrédients fonctionnels à base de Carnobacterium divergens M35. Université Laval.
14. Smati M, Palacios C, Cohen Y, Méchaï F, Tankovic J, Le Flèche-Mateos A, Picard B, Gonzalez F. 2015. Carnobacterium divergens Bacteremia in woman. Emerg Infect Dis 21:1081–1082.
15. Sook Woo G. 2020. Isolation of Carnobacterium divergens from Blood Culture in Korea : A Case Report and Literature Review. Ann Clin Microbiol 23:209–213.
16. Lo CKL, Sheth PM. 2021. Carnobacterium inhibens isolated in blood culture of an immunocompromised, metastatic cancer patient: a case report and literature review. BMC Infect Dis 21:1–5.
17. Giraffa G, Pepe G, Locci F, Neviani E, Carminati D. 1995. Hemolytic activity, production of thermonuclease and biogenic amines by dairy enterococci. Ital J Food Sci 7:341–349.
18. Lorenzo JM, Munekata PE, Dominguez R, Pateiro M, Saraiva JA, Franco D. 2018. Main Groups of Microorganisms of Relevance for Food Safety and Stability: General Aspects and Overall

Description. Innov Technol food Preserv Inact spoilage Pathog Microorg 53–107.
19. Humphrey JD, Lancaster CE, Gudkovs N, Copland JW. 1987. The disease status of Australian salmonids: bacteria and bacterial diseases. J Fish Dis 10:403–410.
20. Hiu SF, Holt RA, Sriranganathan N. 1984. Lactobacillus piscicola, a new species from salmonid fish. Int J Syst Bacteriol 34:393–400.
21. Pastorino P, Colussi S, Pizzul E, Varello K, Menconi V, Mugetti D, Tomasoni M, Esposito G, Bertoli M, Bozzetta E, Dondo A, Acutis PL, Prearo M. 2021. The unusual isolation of carnobacteria in eyes of healthy salmonids in high-mountain lakes. Sci Rep 11:2314.
22. Spanggaard B, Huber I, Nielsen J, Sick EB, Pipper CB, Martinussen T, Slierendrecht WJ, Gram L. 2001. The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. Environ Microbiol 3:755–765.
23. Puvanendran V, Rud I, MSW B, Arnesen JA, Axelsson L. 2021. Probiotic Carnobacterium divergens increase growth parameters and disease resistance in farmed Atlantic cod (Gadus morhua) larvae without influencing the microbiota. Aquaculture 532:736072.
24. Ringø E, Wesmajervi MS, Bendiksen HR, Berg A, Olsen RE, Johnsen T, Mikkelsen H, Seppola M, Strøm E, Holzapfel W. 2001. Identification and characterization of Carnobacteria isolated from fish intestine. Syst Appl Microbiol 24:183–191.
25. Et V. 1997. Optimization of a Liquid Chromatographic Method for Determination of Amines in Fish. J AOAC Int 80:49.
26. Biji KB, Ravishankar CN, Venkateswarlu R, Mohan CO, Gopal TKS. 2016. Biogenic amines in seafood: a review. J Food Sci Technol2016/05/29. 53:2210–2218.
27. Til HP, Falke HE, Prinsen MK, Willems MI. 1997. Acute and subacute toxicity of tyramine, spermidine, spermine, putrescine and cadaverine in rats. Food Chem Toxicol 35:337–348.
28. McCabe BJ. 1986. Dietary tyramine and other pressor amines in MAOI regimens: a review. J Am Diet Assoc 86:1059–1064.
29. ten Brink B, Damink C, Joosten HMLJ, Huis in 't Veld JHJ. 1990. Occurrence and formation of biologically active amines in foods. Int J Food Microbiol 11:73–84.
30. Pilet M-F, Dousset X, Barré R, Novel G, Desmazeaud M, Piard J-C. 1995. Evidence for Two Bacteriocins Produced by Carnobacterium piscicola and Carnobacterium divergens Isolated from Fish and Active Against Listeria monocytogenes. J Food Prot 58:256–262.
31. Connil N, Plissoneau L, Onno B, Pilet M-F, Prevost H, Dousset X. 2002. Growth of Carnobacterium divergens V41 and production of biogenic amines and divercin V41 in sterile cold-smoked salmon extract at varying temperatures, NaCl levels, and glucose concentrations. J Food Prot 65:333–338.
32. Masson F, Lebert A, Talon R, Montel MC. 1997. Effects of physico-chemical factors influencing tyramine production by Carnobacterium divergens. J Appl Microbiol 83:36–42.
33. Bover-Cid S, Holzapfel WH. 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. Int J Food Microbiol 53:33–41.
34. Masson F, Talon R, Montel MC. 1996. Histamine and tyramine production by bacteria from meat products. Int J Food Microbiol 32:199–207.
35. Charteris WP, Kelly PM, Morelli L, Collins JK. 2001. Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. J Food Prot 64:2007–2014.
36. Brillet A. 2005. Sélection et caractérisation de souches de Carnobacterium pour la biopréservation du saumon fumé. Université de Nantes.
37. Duffes F, Corre C, Leroi F, Dousset X, Boyaval P. 1999. Inhibition of Listeria monocytogenes by In Situ Produced and Semipurified Bacteriocins of Carnobacterium spp . Refrigerated Cold-smoked Salmon. J Food Prot 62:1394–1403.
38. Ringø E, Seppola M, Berg A, Olsen RE, Schillinger U, Holzapfel W. 2002. Characterization of

Carnobacterium divergens strain 6251 isolated from intestine of Arctic charr (Salvelinus alpinus L.). Syst Appl Microbiol 25:120–129.
39. Walsh D, Duffy G, Sheridan JJ, Blair IS, McDowell DA. 2001. Antibiotic resistance among Listeria, including Listeria monocytogenes, in retail foods. J Appl Microbiol 90:517–522.
40. Li W, Jin D, Shi C, Li F. 2017. Midgut bacteria in deltamethrin-resistant, deltamethrin-susceptible, and field-caught populations of Plutella xylostella, and phenomics of the predominant midgut bacterium Enterococcus mundtii. Sci Rep 7:1–13.
41. Kinoshita H. 2019. Biosorption of Heavy Metals by Lactic Acid Bacteria for Detoxification. Methods Mol Biol 1887:145–157.
42. Rial D, Vázquez JA, Murado MA. 2011. Effects of three heavy metals on the bacteria growth kinetics: a bivariate model for toxicological assessment. Appl Microbiol Biotechnol 90:1095–1109.
43. George F, Mahieux S, Daniel C, Titécat M, Beauval N, Houcke I, Neut C, Allorge D, Borges F, Jan G, Foligné B, Garat A. 2021. Microorganisms Assessment of Pb(II), Cd(II), and Al(III) Removal Capacity of Bacteria from Food and Gut Ecological Niches: Insights into Biodiversity to Limit Intestinal Biodisponibility of Toxic Metals. Microorganisms 9.
44. Iskandar CF, Borges F, Taminiau B, Daube G, Zagorec M, Remenant B, Leisner JJ, Hansen MA, Sørensen SJ, Mangavel C, Cailliez-Grimal C, Revol-Junelles AM. 2017. Comparative genomic analysis reveals ecological differentiation in the genus Carnobacterium. Front Microbiol 8.
45. Ronholm J, Botta C, Dieter Joerger R, Borges F, Remenant B, Iskandar CF, Taminiau B, Daube G, Zagorec M, Leisner JJ, Hansen MA, Sørensen SJ, Mangavel C, Cailliez-Grimal C, Revol-Junelles A-M. 2017. Comparative Genomic Analysis Reveals Ecological Differentiation in the Genus Carnobacterium. Comp Genomic Anal Reveal Ecol Differ Genus Carnobacterium Front Microbiol 8:357.
46. Jiang F, Zhou K, Ma L, Gressel S, Doudna JA. 2015. A Cas9-guide RNA complex preorganized for target DNA recognition. Science (80-) 348:1477–1481.
47. Woods DF, Kozak IM, Flynn S, O'Gara F. 2018. The Microbiome of an Active Meat Curing Brine. Front Microbiol 9:3346.
48. Jones RJ. 2004. Observations on the succession dynamics of lactic acid bacteria populations in chill-stored vacuum-packaged beef. Int J Food Microbiol 90:273–282.
49. Collins MD, Farrow JAE, Phillips BA, Ferusu S, Jones D. 1987. Classification of Lactobacillus divergens, Lactobacillus piscicola, and Some Catalase-Negative, Asporogenous, Rod-Shaped Bacteria from Poultry in a New Genus, Carnobacterium 37:310–316.
50. (FEEDAP) EP on A and P or S used in AF. 2016. Safety and efficacy of Lavipan® (Lactococcus lactis B/00039, Carnobacterium divergens KKP 2012p, Lactobacillus casei B/00080, Lactobacillus plantarum B/00081 and Saccharomyces cerevisiae KKP 2059p) for weaned piglets, chickens for fattening and turkeys fo. EFSA J 14:e04555.
51. Cotter PD, Hill C, Ross RP. 2005. Food microbiology: Bacteriocins: Developing innate immunity for food. Nat Rev Microbiol.
52. Yang S-C, Lin C-H, Sung CT, Fang J-Y. 2014. Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. Front Microbiol 5:241.
53. Naghmouchi K, Drider D, Fliss I. Action of divergicin M35, a class IIa bacteriocin, on liposomes and Listeria.
54. Eijsink VGH, Skeie M, Middelhoven PH, Brurberg MB, Nes IF. 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl Environ Microbiol 64:3275–3281.
55. Halász A. 2009. Lactic Acid Bacteria, p. 70–82. *In* Lasztity, R (ed.), Food Quality and Standards. EOLSS Publications.
56. Iyer R, Tomar SK, Uma Maheswari T, Singh R. 2010. Streptococcus thermophilus strains:

Multifunctional lactic acid bacteria. Int Dairy J 20:133–141.
57. Abriouel H, Franz CMAP, Omar N Ben, Galvez A. 2011. Diversity and applications of Bacillus bacteriocins. FEMS Microbiol Rev 35:201–232.
58. Swain MR, Anandharaj M, Ray RC, Parveen Rani R. 2014. Fermented fruits and vegetables of Asia: a potential source of probiotics. Biotechnol Res Int 2014:250424.
59. Chen CM, Sebranek JG, Dickson JS, Mendonca AF. 2004. Combining pediocin (ALTA 2341) with postpackaging thermal pasteurization for control of Listeria monocytogenes on frankfurters. J Food Prot 67:1855–1865.
60. Leaflet ASR. 2005. Combining Pediocin (ALTA ™ 2341) with Irradiation for Control of Listeria monocytogenes . 5–6.
61. Fernandez B, Le Lay C, Jean J, Fliss I. 2013. Growth, acid production and bacteriocin production by probiotic candidates under simulated colonic conditions. J Appl Microbiol 114:877–885.
62. Fernandez B, Savard P, Fliss I. 2016. Survival and Metabolic Activity of Pediocin Producer Pediococcus acidilactici UL5: Its Impact on Intestinal Microbiota and Listeria monocytogenes in a Model of the Human Terminal Ileum. Microb Ecol 72:931–942.
63. De Vuyst L, Leroy F. 2007. Bacteriocins from lactic acid bacteria: production, purification, and food applications. J Mol Microbiol Biotechnol 13:194–199.
64. Cleveland J, Montville TJ, Nes IF, Chikindas ML. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol 71:1–20.
65. Soltani S, Hammami R, Cotter PD, Rebuffat S, Said L Ben, Gaudreau H, Bédard F, Biron E, Drider D, Fliss I. 2021. Bacteriocins as a new generation of antimicrobials: Toxicity aspects and regulations. FEMS Microbiol Rev 45:1–24.
66. Reddy KVR, Aranha C, Gupta SM, Yedery RD. 2004. Evaluation of antimicrobial peptide nisin as a safe vaginal contraceptive agent in rabbits: in vitro and in vivo studies. Reproduction 128:117–126.
67. Dreyer L, Smith C, Deane SM, Dicks LMT, van Staden AD. 2019. Migration of Bacteriocins Across Gastrointestinal Epithelial and Vascular Endothelial Cells, as Determined Using In Vitro Simulations. Sci Rep 9:11481.
68. Meade E, Slattery MA, Garvey M. 2020. Bacteriocins, Potent Antimicrobial Peptides and the Fight against Multi Drug Resistant Species: Resistance Is Futile? Antibiot (Basel, Switzerland) 9:32.
69. Dillman AR, Baindara P, Zendo T, Rubén Morones-Ramírez J. 2021. Bacteriocins: An Overview of Antimicrobial, Toxicity, and Biosafety Assessment by in vivo Models.
70. Frazer A, Sharratt J, Hickman J. 1962. The biological effects of food additives - Nisin. J Sci Food Agric 13:32–42.
71. Sahoo TK, Jena PK, Prajapati B, Gehlot L, Patel AK, Seshadri S. 2017. In Vivo Assessment of Immunogenicity and Toxicity of the Bacteriocin TSU4 in BALB/c Mice. Probiotics Antimicrob Proteins 9:345–354.
72. Ketaren B, Marlida Y, Rusmarilin H. 2016. Toxicity Test Pediocin N6 Powder Produced From Isolates Pediococcus Pentosaceus Strain N6 on White Mice. J Food Pharm Sci 4.
73. Dover SE, Aroutcheva AA, Faro S, Chikindas ML. 2007. Safety study of an antimicrobial peptide lactocin 160, produced by the vaginal Lactobacillus rhamnosus. Infect Dis Obstet Gynecol 2007:78248.
74. Chen Y, Ludescher RD, Montville TJ. 1998. Influence of lipid composition on pediocin PA-1 binding to phospholipid vesicles. Appl Environ Microbiol 64:3530–3532.
75. Li J, Chikindas ML, Ludescher RD, Montville TJ. 2002. Temperature- and surfactant-induced membrane modifications that alter Listeria monocytogenes nisin sensitivity by different mechanisms. Appl Environ Microbiol 68:5904–5910.
76. Morea, Baruzzi, Cocconcelli. 1999. Molecular and physiological characterization of dominant

bacterial populations in traditional mozzarella cheese processing. J Appl Microbiol 87:574–582.
77. Leroi F, Joffraud J-J, Chevalier F, Cardinal M. 1998. Study of the microbial ecology of cold-smoked salmon during storage at 8°C. Int J Food Microbiol 39:111–121.
78. Ringø E, Holzapfel W. 2000. Identification and characterization of carnobacteria associated with the gills of Atlantic salmon (Salmo salar L.). Syst Appl Microbiol 23:523–527.
79. Alves VF, De Martinis ECP, Destro MT, Vogel BF, Gram L. 2005. Antilisterial activity of a Carnobacterium piscicola isolated from Brazilian smoked fish (surubim [Pseudoplatystoma sp.]) and its activity against a persistent strain of Listeria monocytogenes isolated from surubim. J Food Prot 68:2068–2077.
80. Groudieva T, Kambourova M, Yusef H, Royter M, Grote R, Trinks H, Antranikian G. 2004. Diversity and cold-active hydrolytic enzymes of culturable bacteria associated with Arctic sea ice, Spitzbergen. Extremophiles 8:475–488.
81. Walker VK, Palmer GR, Voordouw G. 2006. Freeze-thaw tolerance and clues to the winter survival of a soil community. Appl Environ Microbiol 72:1784–1792.
82. Leroi F, Joffraud JJ, Chevalier F, Cardinal M. 1998. Study of the microbial ecology of cold-smoked salmon during storage at 8°C. Int J Food Microbiol 39:111–121.
83. Laursen BG, Bay L, Cleenwerck I, Vancanneyt M, Swings J, Dalgaard P, Leisner JJ. 2005. Carnobacterium divergens and Carnobacterium maltaromaticum as spoilers or protective cultures in meat and seafood: Phenotypic and genotypic characterization. Syst Appl Microbiol 28:151–164.
84. Grant IR, Patterson MF. 1991. A numerical taxonomic study of lactic acid bacteria isolated from irradiated pork and chicken packaged under various gas atmospheres. J Appl Bacteriol 70:302–307.
85. Bjorkroth J, Ristiniemi M, Vandamme P, Korkeala H. 2005. Enterococcus species dominating in fresh modified-atmosphere-packaged, marinated broiler legs are overgrown by Carnobacterium and Lactobacillus species during storage at 6 degrees C. Int J Food Microbiol 97:267–276.
86. Ringø E, Bendiksen HR, Wesmajervi MS, Olsen RE, Jansen PA, Mikkelsen H. 2000. Lactic acid bacteria associated with the digestive tract of Atlantic salmon (Salmo salar L.). J Appl Microbiol 89:317–322.
87. Taylor SL, Stratton JE, Nordlee JA. 1989. Histamine poisoning (scombroid fish poisoning): an allergy-like intoxication. J Toxicol Clin Toxicol 27:225–240.
88. Kanki M, Yoda T, Tsukamoto T, Baba E. 2007. Histidine decarboxylases and their role in accumulation of histamine in tuna and dried saury. Appl Environ Microbiol 73:1467–1473.

7.2 List of targeted species of fish and/or products, but not limited to, for the application of *Carnobacterium divergens* M35

- **Salmonidae**
- **Pleuronectidae**
- **Scophthalmidae**
- **Psettodidae**
- **Anaplomatidae**
- **Gadidae**
- **Hexagrammidae**
- **Pinguipedidae**
- **Moridae**
- **Pleuronectidae**
- **Cynoglossidae**
- **Paralichthyidae**
- **Centrarchidae**
- **Saleidae**
- **Cynoglossidae**
- **Triakidae**
- **Clupeidae**
- **Scombridae**
- **Hexagranmidae**
- **RTE fish products (ex. fried fish, fileted fish etc.)**

7.3 Food grade and allergen free certification of the ingredients

TATE & LYLE

Product Information Sheet
MALTOSWEET™ 12A
300000000501

Contents:

TATE & LYLE

Product Information Sheet
MALTOSWEET™ 12A
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1. Supplier Information

Name:	Tate & Lyle
Address:	919 08 Boleráz 114 Slovakia
Main Phone:	+421 (33) 5561 601
Main Fax:	+421 (33) 5561 602
www.tateandlyle.com	

2. Emergency Contacts

During office hours: +421 33 5561 601
After office hours: +421 33 5561 140
After Hours Emergency: +421 911 726 366

3. Product Information

Common Name:	Maltodextrins
Material Number:	16100012
Specification Number:	300000000501
CAS Number:	9050-36-6
EINECS Number:	232-940-4
21 CFR References:	
Food Grade:	Yes
Appearance:	powder
Color:	White To Light Yellow
Odor:	Bland

4. Regulatory Status

- This product complies with the requirements of EU Directives and Regulations in force on foods and food ingredients. Prospective purchasers are advised to conduct their own

tests, studies and regulatory review to determine the fitness of Tate & Lyle products for their particular purposes, product claims or specific application.

5. Ingredient Statement

Maltodextrin

6. Quality Documents

Quality documents (Specification, Technical Data Sheet, MSDS & Nutritional) available upon Request.

7. Kosher/Halal Status

Certified Kosher: Yes
Certified Kosher for Passover: No

The above-mentioned ingredient or its components is certified Kosher by:

- London Beth Din Kashrut Division (KLBD)

Certified Halal: Yes

8. Allergen Status

If present in this Tate & Lyle ingredient, allergens will be denoted as "Present" in the table below:

Allergen	Status
Absent	

Meaning of Present/Absent/Present-Exempt
Present - Intentionally added during the production process.
Absent - Not intentionally added during the production process.
Present-Exempt - Intentionally added during the production process, however have been given exemption from being required to be labeled on the final package.

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The above list of allergens is in accordance with all applicable EU and US legal requirements.

Additional allergen information:

- Although all reasonable care has been taken in managing allergen risk, the above statement should not be considered as a guarantee or claim of "free of..."

9. Sulfur Dioxide and Sulfite Level

The sulfur dioxide and sulfite level for above-mentioned ingredient or its components is:

Package	Sulfur Dioxide and Sulfite Level
Bulk, Super Sack	< 10 ppm

10. Gluten Status

This product is not either originating from gluten containing cereals or not identified as containing gluten and complies with the applicable EU and US legal requirements based on real-time PCR gluten test methodology. It can be safely used in foodstuffs for people intolerant to gluten.

11. GMO Statement

Tate & Lyle certifies that the above mentioned product is produced from non GMO sources therefore it is not considered to be genetically modified organisms under EC regulations 1829 and 1830/2003.

12. Irradiation, ETO and Sewage Sludge

The above-mentioned ingredient or its components have not been produced and handled with the use of:

1. treatment with ionizing radiation
2. exposure to ethylene oxide
3. sewage or sludge.

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13. Organic Statement

- Products manufactured at this Tate & Lyle facility are not designated as Organic.

14. Diet Suitability

Vegetarian: Suitable
Vegan: Suitable
Ovo-Vegetarian: Suitable
Lacto-Vegetarian: Suitable
Lacto-Ovo-Vegetarian: Suitable

15. Bovine Spongiform Encephalopathies (BSE) /Transmissible Spongiform Encephalopathies (TSE)

1. This ingredient does not contain ingredients of animal origin.
2. If processing aids are contained in this ingredient, the processing aids are not derived from animal origins.
3. Prior to the manufacture of this ingredient, no ingredients of animal origin are present in equipment.

16. Sudan I – IV Dyes Content

- The above-mentioned ingredient or its components do not contain Sudan azo dyes (this includes Sudan I, Sudan II, Sudan III and Sudan IV, which is also known as scarlet red).

17. Country of Origin

The above-mentioned ingredient or its components is manufactured in the following country:
Slovakia

18. Shelf Life

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The expected shelf life for the ingredient based on being stored under the proper storage conditions:

Package	Shelf Life
Bulk, Super Sack	730 Days

19. Transportation Information/Storage Conditions

Storage Conditions

Container	Storage Temperature	Storage conditions to achieve maximum shelf life
Bulk, Super Sack	20 °C	Clean, dry area, ambient temperature. Away from odorous materials.

20. Lot Code Explanation

Batch Numbering Scheme:

PACKED PRODUCTS

PPYYM9NNNN

PP - Plant ID
YY - Last two digits of the year (2012)
M - Month (A, B, C, D, E, F, G, H, I, J, K, L for Jan - Dec)
9NNNN - Plant related sequential number

Example: KO12G91130

KO - Plant ID for Tate & Lyle Netherlands
12 - Last two digits of the year (2012)
G - Month, July
91130 - Plant related sequential number

BULK DELIVERIES

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PPYYMNNNNN

PP - Plant ID
YY - Last two digits of the year (2012)
M - Month (A, B, C, D, E, F, G, H, I, J, K, L for Jan - Dec)
NNNNN - Plant related sequential number

Example: KO12G00001

21. Certificate of Analysis

- Certificates of Analysis are available by request.

22. current Good Manufacturing Practices (cGMP)

- The process used for the production of this product is operated based on Tate & Lyle's procedures, quality guidelines, HACCP programs and Good Manufacturing Practices. This operation complies with the FDA Good Manufacturing Practices detailed in 21 CFR 110 for Food Manufactures, and those referenced in the European Commission Regulation (EC) No. 852/2004 on hygiene of foodstuff.

23. Guarantee (Continuing)

- Available upon request.

24. HACCP/Flowchart

- Available upon request.

25. Audit Information

- Third Party audits are performed annually at Tate & Lyle locations. Information available upon request.

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26. Emergency/Recall Procedures

- Please be assured that Tate & Lyle has a written Recall Procedure. In the event of an actual recall, Tate & Lyle will notify all customers who have receive affected product. Mock Recalls are conducted at least annually.

27. Bioterrorism

- Tate and Lyle is in compliance with 21 CFR Part 1-Registration of Food Facilities, and Maintenance and Inspection of Records for Foods, under Public Health Security and Bioterrorism Preparedness and Response Act of 2002. Due to concerns with security, our legal department limits giving out the plant number unless there is an imperative border crossing issue.

28. Pest Control

- Tate & Lyle employs outside contractors to provide routine pest control service.

29. Pesticides

- Tate & Lyle has an ongoing pesticide monitoring program.

30. Proposition 65

- The State of California enacted the Safe Drinking Water and Toxic Enforcement Act of 1986 ("Proposition 65"), which "prohibits any person in the course of doing business from knowingly and intentionally exposing any individual to a chemical known to the State of California to cause cancer or reproductive toxicity without first giving clear and reasonable warning to such individuals." In our effort to satisfy the requirements of this law, independent laboratory analyses were obtained for those products which we manufacture or formulate for sale to you.The results of this analysis confirmed that there are no chemicals regulated under Proposition 65, other than naturally occurring chemicals, detectable in our products using standard analytical methods.

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TATE & LYLE

Specification Sheet **300000000501**

MALTOSWEET™ 12A

1610001212620, 1610001212600, 16100012

Product Description : This product is a purified spraydried maltodextrin obtained by hydrolysis of starch. It is a white to light yellow powder with a moderate sweet taste and typical odour. This product complies to the Food Chemical Codex and to the European Pharmacopeia monographs on Maltodextrin.

Appearance: powder
Color: White To Light Yellow
Odor: Bland

Quality Inspection Plan (Official Specification Values)

Parameter	Target	Range/ Attribute	Unit	Method
Moisture		0.00 - 5.00	%	Oven - A0102 (Ref. ISO 1741)
DE		11.00 - 14.00		Lane-Eynon - A0201 (Ref. ISO 5377)
Dextrose on DS	1.00		%	HPLC - A0210 (Ref. ISO 10504)
Maltose on DS	4.00		%	HPLC - A0210 (Ref. ISO 10504)
Triose on DS	6.00		%	HPLC - A0210 (Ref. ISO 10504)
Higher sugars on DS	89.00		%	HPLC - A0210 (Ref. ISO 10504)
SO2		<= 10.00	mg/kg	Iodine titration - A0901 (Ref. CRA E-67/
pH		4.00 - 5.50		pH - A0922 (Ref. CRA F-42)
Bulk density compacted		500.0 - 650.0	g/dm3	Gravimetric - A1101
Bulk density loose		450.0 - 600.0	g/dm3	Gravimetric - A1101
Conductivity, 28 Brix		<= 100.00	µS/cm	Conductivity meter - A1202
Yeasts		<= 50	n/10g	Pour plate - A1923
Moulds		<= 50	n/10g	Pour plate - A1923
Total mesophylic count		<= 1000	n/10g	Pour plate - A1924

TATE & LYLE

Product Composition / Labeling
Maltodextrin

Regulatory Status
Compliance Statement:

- This product complies with the requirements of EU Directives and Regulations in force on foods and food ingredients. Prospective purchasers are advised to conduct their own tests, studies and regulatory review to determine the fitness of Tate & Lyle products for their particular purposes, product claims or specific application.

Country of Origin

Country of Manufacture: Slovakia

Kosher Status

Certified Kosher: Yes
Certified Kosher for Passover: No

The above-mentioned ingredient or its components is certified Kosher by:

- London Beth Din Kashrut Division (KLBD)

Halal Status

Certified Halal: Yes

Vegetarian Status

Vegetarian: Suitable
Vegan: Suitable
Ovo-Vegetarian: Suitable
Lacto-Vegetarian: Suitable
Lacto-Ovo-Vegetarian: Suitable

Presence of Allergens

If present in this Tate & Lyle ingredient, allergens will be denoted as "Present" in the table below.

Allergen	Status
Crustacean & Shellfish	Absent
Mollusk	Absent
Fish	Absent
Egg	Absent
Milk (including Lactose)	Absent

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Peanut & Oils	Absent
Soybean & Oils	Absent
Gluten Containing: Wheat, Rye, Barley, Oats, Spelt, Kamut, Triticale	Absent
Seeds, Sesame & Oils	Absent
Celery/Celeriac	Absent
Mustard	Absent
Lupin	Absent
Tree Nuts, Almond	Absent
Tree Nuts, Brazil	Absent
Tree Nuts, Cashew	Absent
Tree Nuts, Chestnut	Absent
Tree Nuts, Coconut and Oils	Absent
Tree Nuts, Hazelnut / Filbert	Absent
Tree Nuts, Hickory	Absent
Tree Nuts, Macadamia	Absent
Tree Nuts, Pine	Absent
Tree Nuts, Pistachio	Absent
Tree Nuts, Queensland	Absent
Tree Nuts, Walnut	Absent
Buckwheat	Absent
Bee Pollen/ Propolis	Absent
Royal Jelly	Absent
Pork	Absent
Peach	Absent
Tomato	Absent
Sulfur Dioxide and Sulfite > 10 ppm	Absent

Meaning of Present/Absent/Present-Exempt
Present - Intentionally added during the production process.
Absent - Not intentionally added during the production process.
Present-Exempt - Intentionally added during the production process, however have been given exemption from being required to be labeled on the final package.

The above list of allergens is in accordance with all applicable EU and US legal requirements.

Additional allergen information is available in the Product Information Sheet for MALTOSWEET™ 12A

GMO Status

The above-mentioned ingredient is considered Non-GMO

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For additional information regarding the GM status of the above-mentioned ingredient, please refer to the Product Information Sheet.

Storage Conditions

Container	Storage Temperature	Storage conditions to achieve maximum shelf life
Bulk, Super Sack	20 °C	Clean, dry area, ambient temperature. Away from odorous materials.

Shelf Life

Package	Shelf Life	Sulfur Dioxide and Sulfite Level
Bulk, Super Sack	730 Days	< 10 ppm

Organic Statement

Certified for Organic Labeling: No

For more information please consult the Product Information Sheet for MALTOSWEET™ 12A

Nutritional Information

Nutritional Information	Nutrients Per 100 Grams
Energy	1.615,0000 kj
Calories	380 kcal
Total Fat	0 g
Saturated Fat	0 g
Carbohydrate	95 g
Sugars	5,2 g
Protein	0 g
Salt	0 g

Supplier Information

Name: Tate & Lyle
Address: 919 08 Boleráz 114

TATE & LYLE

Slovakia
Main Phone: +421 (33) 5561 601
Main Fax: +421 (33) 5561 602
www.tateandlyle.com

Disclaimer

CERTIFICATE OF CONFORMANCE

YEAST EXTRACT - 19512

Origin

The product is manufactured in European Union.
The product is of microbial origin and is natural.

Non G.M.O. Status

This product does not contain G.M.O. and is in accordance with European Regulations (EC) 1829/2003 and 1830/2003, regarding G.M.O. content and labelling of such products.

Non Animal

This product is 100% from non-animal origin raw materials. As a result we can certify that this product is not in the scope of the monograph 1483 of the European Pharmacopoeia current edition regarding TSE/BSE Risk assessment.

Sanitary certificate

A sanitary certificate is supplied with each delivery.

Food Grade

This product is food grade.

Halal

This product is certified Halal (certificate available on demand).

REACH Registration

This product is registered according to REACH regulation (EC) 1907/2006.
EC number : 286-294-5
CAS number : 84604-16-0
Chemical name : Saccharomyces cerevisiae, ext.
Submission number : RR922293-15
Registration number : 01-2119539417-34-0000

Nanomaterials

This product does not meet the definition of nanoparticulate substances or the definition of mixtures containing substances in the nanoparticulate state (regulations 2015/2283 & 2011/696).
As a result, we do not have any substance subject to declaration, according to Decree 2012-232.

CERTIFICATE OF CONFORMANCE

YEAST EXTRACT - 19512

Packaging

The packaging materials in contact with this product comply with the following regulations :

- In accordance with Regulation (EC) 1935/2004 regarding materials and articles intended to come into contact with food,
- In accordance with Regulation (EC) 10/2011 regarding plastic materials and articles intended to come into contact with food.

Contaminants

Residual solvents

No solvent from class I, II or III, as defined in ICH Q3C (R5) guidelines, is used in order to manufacture this product.

Heavy metals

This product is analysed according to a yearly monitoring plan. The result show compliance with the maximum levels set up by European regulation 1881/2006 and 396/2005 eventhough there is no maximum level set out for yeast extract. Therefore, to the best of our knowledge, there is no risk associated with heavy metals.

Dioxins

This product is analysed according to a yearly monitoring plan. The result show compliance with the maximum levels set up by Européean regulation 1881/2006 and 396/2005 eventhough there is no maximum level set out for yeast extract. Therefore, to the best of our knowledge, there is no risk associated with

Pesticides

This product is analysed according to a yearly monitoring plan. The result show compliance with the maximum levels set up by Européean regulation 1881/2006 and 396/2005 eventhough there is no maximum level set out for yeast extract. Therefore, to the best of our knowledge, there is no risk associated with pesticides.

Mycotoxins

This product is analysed according to a yearly monitoring plan. The result show compliance with the maximum levels set up by Européean regulation 1881/2006 and 396/2005 eventhough there is no maximum level set out for yeast extract. Therefore, to the best of our knowledge, there is no risk associated with mycotoxins.

Estelle BOURDIN, Quality Manager

Technical Data Sheet

ORGANO TECHNIE
SOURCE DE VIE

Edition 04/2008
Replace Ed. 04/2005

Organotechnie® S.A.S.
27, avenue Jean Mermoz
93120 La Courneuve, France
Tél : +33 (0) 1 49 92 87 50
Fax : +33 (0) 1 49 92 87 51

e-mail : info@organotechnie.com
web : www.organotechnie.com

Yeast Extract
19512

Definition
Yeast Extract is manufactured by autolysis of *Saccharomyces cerevisiae*.

Description
Fine pale-yellow powder easily soluble in water.
Yeast Extract contains a mix of peptides, free amino acids, purine and pyrimidine bases and hydrosoluble vitamins of B group.

Use
Source of organic nitrogen and growth factors recommended in media for:
- Analytical microbiology
- Industrial fermentation.

Physico-chemical characteristics

	Standard
Solubility in water at 5 %	Complete
pH (5 % solution)	6.4 - 7.4
Loss on drying	≤ 6.0 %
Total nitrogen TN	10.0 - 11.8 %
α-amino nitrogen AN	4.8 - 6.3 %
AN/TN x 100	41 - 60
Residue on ignition	≤ 18.0 %
Chloride (as NaCl)	≤ 1.0 %

Microbiology

	Standard
Total aerobic microbial count	≤ 5 000 /g
Coliforms	≤ 10 /g
Escherichia coli	Absence / g
Salmonella	Absence / 25 g
Staphylococcus aureus	Absence / g
Yeasts and moulds	≤ 100 /g

afaq ISO 9001 Qualité AFNOR CERTIFICATION

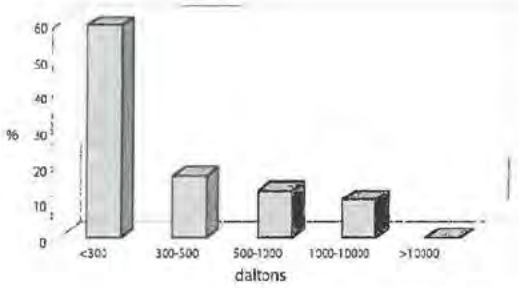
Yeast Extract / 19512

Edition 04/2008
Replace Ed. 04/2005

Organotechnie S.A.

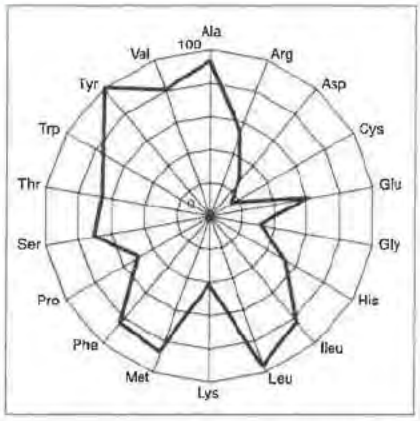
Typical data

Molecular weight distribution	g / 100 g
> 10 000 daltons	0
1 000 - 10 000 daltons	10.7
500 - 1 000 daltons	13.0
300 - 500 daltons	17.2
< 300 daltons	59.1
Average Molecular Weight	**438 daltons**



Molecular weight distribution

Amino Acids		Total - T (g/100 g)	Free - F (g/100 g)	F/T x 100
Alanine	Ala	4.1	3.8	92.7
Arginine	Arg	2.8	1.5	53.6
Aspartic acid	Asp	6.0	1.8	30.0
Cystine	Cys	0.6	0.1	16.7
Glutamic acid	Glu	11.0	6.8	61.8
Glycine	Gly	2.5	1.3	52.0
Histidine	His	1.1	0.6	54.5
Isoleucine	Ileu	2.6	2.2	84.6
Leucine	Leu	3.7	3.6	97.3
Lysine	Lys	4.2	1.8	42.9
Methionine	Met	0.8	0.7	87.5
Phenylalanine	Phe	2.5	2.1	84.0
Proline	Pro	2.2	1.1	50.0
Serine	Ser	2.7	1.9	70.4
Threonine	Thr	2.6	1.7	65.4
Tryptophan	Trp	0.8	0.6	75.0
Tyrosine	Tyr	0.8	0.8	100.0
Valine	Val	3.1	2.5	80.6



Amino Acids F/T x 100

Documentation

The certificate of analysis and the sanitary certificate are supplied with each delivery.

Packing and storage

25 kg net corrugated board box with inner polyethylene bags.
Keep in original packaging closed when not in use,
at room temperature in a dry area.
Hygroscopic product.

Best before: 3 years.

Health and safety information

Dusty powder.
Avoid inhalation.

Sigma-Aldrich
Flavors & Fragrances
6000 N. Teutonia Avenue
Milwaukee, WI 53209 USA
Tel. 800-227-4563 / 414-438-2608
Fax 414-438-4216
Visit us at www.sigma-aldrich.com

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March 27, 2020

Food Grade Statement

W291708 Polysorbate 80-FG

To the best of our knowledge, this product is manufactured under current Good Manufacturing Practices (cGMP) for food facilities and conforms to Hazard Analysis and Critical Control Points (HACCP) principles. The product is intended for use in manufacturing food and/or designated Generally Recognized As Safe (GRAS) by:

E # E 433
FEMA # 2917
EU Regulation 1333/2008 & 178/2002
FDA 21 CFR (172.515)
FDA 21 CFR (172.836)
FDA 21 CFR (172.838)
FDA 21 CFR (172.840)
FDA 21 CFR (172.842)
FDA 21 CFR (173.340)
FDA 21 CFR (175.105)
FDA 21 CFR (176.180)
FDA 21 CFR (178.3400)
FDA 21 CFR (573.860)
FDA 21 CFR (73.1)
FDA 21 CFR (73.1001)

To the best of our knowledge, this product was not adulterated or misbranded as defined by the Federal Food, Drug, and Cosmetic Act, state, or municipal ordinances in which the definition of adulteration and misbranding is substantially the same as defined by the Act. This product is allowed under the provision of Section 404 or 505 of the Act, to be introduced into interstate commerce.

Sigma-Aldrich warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product for their particular use.

Best Regards,

Sigma-Aldrich Flavors & Fragrances

Sigma-Aldrich
Flavors & Fragrances
6000 N. Teutonia Avenue
Milwaukee, WI 53209 USA
Tel. 800-227-4563 / 414-438-2608
Fax 414-438-4216
Visit us at www.sigma-aldrich.com

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March 27, 2020

Food Allergen Statement

W201703 Polysorbate 80-FG

To the best of our knowledge, this product does not contain nor is derived from any food allergens, including:

- Buckwheat
- Celery
- Crustaceans / Shellfish
- Egg
- Fish & Fish products including Roe
- Lupine, legumes/pulses, carrot
- Milk including Lactose
- Mollusc
- Mustard
- Peanuts
- Sesame seeds
- Soybeans
- Sulfur dioxide & Sulfites
- Tree nuts[1]
- Wheat and other Cereals containing gluten[2]
- Coriander / Cilantro
- Glutamate
- Maize
- Natural Rubber latex

[1]Tree nuts include: almonds, brazil nuts, cashews, coconuts chest nuts, hazelnuts, macadamia or Queensland nuts, pecan nuts, pine nuts, pistachio nuts, walnuts
[2]Cereals containing gluten include: wheat, rye, barley, oats, spelt, kamut or their hybridized strains

Best Regards,

Sigma-Aldrich Flavors & Fragrances

SIGMA-ALDRICH

SIGMA-ALDRICH

Sigma-Aldrich
Flavors & Fragrances
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Milwaukee, WI 53209 USA
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March 27, 2020

Food Allergen Statement

W302430 Sodium Acetate Anhydrous->99%, FCC, FG

To the best of our knowledge, this product does not contain nor is derived from any food allergens, including:

- Buckwheat
- Celery
- Crustaceans / Shellfish
- Egg
- Fish & Fish products including Roe
- Lupine, legumes/pulses, carrot
- Milk including Lactose
- Mollusc
- Mustard
- Peanuts
- Sesame seeds
- Soybeans
- Sulfur dioxide & Sulfites
- Tree nuts[1]
- Wheat and other Cereals containing gluten[2]
- Coriander / Cilantro
- Glutamate
- Maize
- Natural Rubber latex

[1]Tree nuts include: almonds, brazil nuts, cashews, coconuts chest nuts, hazelnuts, macadamia or Queensland nuts, pecan nuts, pine nuts, pistachio nuts, walnuts
[2]Cereals containing gluten include: wheat, rye, barley, oats, spelt, kamut or their hybridized strains

Best Regards,

Sigma-Aldrich Flavors & Fragrances

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Flavors & Fragrances
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Milwaukee, WI 53209 USA
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Fax 414-438-4216
Visit us at www.sigma-aldrich.com

March 27, 2020

Food Grade Statement

W302406 Sodium Acetate Anhydrous->99%, FCC, FG

To the best of our knowledge, this product is manufactured under current Good Manufacturing Practices (cGMP) for food facilities and conforms to Hazard Analysis and Critical Control Points (HACCP) principles. The product is intended for use in manufacturing food and/or designated Generally Recognized As Safe (GRAS) by:

E # E 262
FEMA # 3024
EU Regulation 1223/2009
EU Regulation 1333/2008 & 178/2002
FCC
FDA 21 CFR (150.141)
FDA 21 CFR (150.161)
FDA 21 CFR (173.310)
FDA 21 CFR (182.70)
FDA 21 CFR (184.7121)

To the best of our knowledge, this product was not adulterated or misbranded as defined by the Federal Food, Drug, and Cosmetic Act, state, or municipal ordinances in which the definition of adulteration and misbranding is substantially the same as defined by the Act. This product is allowed under the provision of Section 404 or 505 of the Act, to be introduced into interstate commerce.

Sigma-Aldrich warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product for their particular use.

Best Regards,

Sigma-Aldrich Flavors & Fragrances

7.4 Results from batch analysis (microbiological contaminants and heavy metals)

AGAT Laboratories

9770 ROUTE TRANSCANADIENNE
ST. LAURENT, QUEBEC
CANADA H4S 1V9
TEL (514)337-1000
FAX (514)333-3046
http://www.agatlabs.com

CLIENT NAME: LABORATOIRE INNODAL
5-835 AVE DES JESUITES
QUEBEC CITY, QC G1S 3N2
514-916-3176

ATTENTION TO: Ariane Renaud

PROJECT: Analyse BacM35

AGAT WORK ORDER: 20M581278

FOOD CHEMISTRY REVIEWED BY: Olivier Lachance, Method Development Supervisor

MICROBIOLOGY ANALYSIS REVIEWED BY: Samara Brookman, Lab Manager

SOIL ANALYSIS REVIEWED BY: Amar Bellahsene, Chimiste

DATE REPORTED: Mar 17, 2020

PAGES (INCLUDING COVER): 8

VERSION*: 2

Should you require any information regarding this analysis please contact your client services representative at (514) 337-1000

Notes

VERSION 2:preliminary sent 2020/03/10

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CANADA H4S 1V9
TEL (514)337-1000
FAX (514)333-3046
http://www.agatlabs.com

Certificate of Analysis

AGAT WORK ORDER: 20M581278

PROJECT: Analyse BacM35

CLIENT NAME: LABORATOIRE INNODAL

ATTENTION TO: Ariane Renaud

SAMPLED BY:Tomy Faubert

SAMPLING SITE:Divers,Divers

Metals

DATE RECEIVED: 2020-03-05

DATE REPORTED: 2020-03-17

SAMPLE DESCRIPTION:				01082018 (2/3)	02112017 (3/3)	20022020 (1/3)
SAMPLE TYPE:				Food	Food	Food
DATE SAMPLED:						
Parameter	Unit	G / S	RDL	1004664	1004665	992909
Arsenic (Food)	ppm		0.1	<0.1	<0.1	<0.1
Cadmium (Food)	ppm		0.1	<0.1	<0.1	<0.1
Lead (Food)	ppm		0.1	<0.1	<0.1	<0.1

Comments: RDL - Reported Detection Limit; G / S - Guideline / Standard

Certified By:

AGAT Laboratories

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FAX (514)333-3046
http://www.agatlabs.com

Certificate of Analysis

AGAT WORK ORDER: 20M581278

PROJECT: Analyse BacM35

CLIENT NAME: LABORATOIRE INNODAL

ATTENTION TO: Ariane Renaud

SAMPLED BY:Tomy Faubert

SAMPLING SITE:Divers,Divers

Food Microbiology

DATE RECEIVED: 2020-03-05

DATE REPORTED: 2020-03-17

		SAMPLE DESCRIPTION:		01082018 (2/3)	02112017 (3/3)	20022020 (1/3)
		SAMPLE TYPE:		Food	Food	Food
		DATE SAMPLED:				
Parameter	Unit	G / S	RDL	1004664	1004665	992909
Aerobic Bacteria Count - Food	CFU/g		5	27 000 000	52 000 000	>300 000 000 E
Yeast count - Food	CFU/g		20	<20	<20	<20
Mold count (Food)	CFU/g		20	20 E	<20	<20
Staphylococcus aureus - Food	CFU/g		25	<25	<25	<25
Enterobacteria 3M - Food	CFU/g		10	<10	<10	<10
Salmonella Detection by MDS - Food	/25g		NA	Not detected	Not detected	Not detected
Listeria monocytogenes by MDS - Food	/25g		NA	Not detected	Not detected	Not detected
E.coli O157 by MDS - Food	/25g		NA	Not detected	Not detected	Not detected
Temperature upon receipt	°C		N/A	Ambient	Ambient	Ambient

Comments: RDL - Reported Detection Limit; G / S - Guideline / Standard

1004664 Results are preliminary and subject to change if they are not certified.
The laboratory is not accredited for the parameters with an asterisk.
E: Estimated result

1004665 Results are preliminary and subject to change if they are not certified.
The laboratory is not accredited for the parameters with an asterisk.

992909 Results are preliminary and subject to change if they are not certified.
The laboratory is not accredited for the parameters with an asterisk.
E: Estimated result

Certified By:

AGAT Laboratories
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CANADA H4S 1V9
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FAX (514)333-3046
http://www.agatlabs.com

Certificate of Analysis

AGAT WORK ORDER: 20M581278

PROJECT: Analyse BacM35

CLIENT NAME: LABORATOIRE INNODAL

ATTENTION TO: Ariane Renaud

SAMPLED BY:Tomy Faubert

SAMPLING SITE:Divers,Divers

Total Extractable metals

DATE RECEIVED: 2020-03-05

DATE REPORTED: 2020-03-17

		SAMPLE DESCRIPTION:		01082018 (2/3)	02112017 (3/3)	20022020 (1/3)
		SAMPLE TYPE:		Food	Food	Food
		DATE SAMPLED:				
Parameter	Unit	G / S	RDL	1004664	1004665	992909
Mercury	mg/kg		0.2	<0.2	<0.2	<0.2

Comments: RDL - Reported Detection Limit; G / S - Guideline / Standard

Certified By:

CHIMISTE
Amar Bellahsene
2011-214

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CANADA H4S 1V9
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FAX (514)333-3046
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Quality Assurance

CLIENT NAME: LABORATOIRE INNODAL

PROJECT: Analyse BacM35

SAMPLED BY:Tomy Faubert

AGAT WORK ORDER: 20M581278

ATTENTION TO: Ariane Renaud

SAMPLING SITE:Divers,Divers

Food Chemistry Analysis

RPT Date: 2020-03-17			DUPLICATE			REFERENCE MATERIAL				METHOD BLANK SPIKE			MATRIX SPIKE		
PARAMETER	Batch	Sample Id	Dup #1	Dup #2	RPD	Method Blank	Measured Value	Acceptable Limits		Recovery	Acceptable Limits		Recovery	Acceptable Limits	
								Lower	Upper		Lower	Upper		Lower	Upper
Metals															
Arsenic (Food)	310	NA	< 0.1	< 0.1	0.0%	< 0.1	99%	80%	120%	109%	80%	120%	109%	80%	120%
Cadmium (Food)	310	NA	< 0.1	< 0.1	0.0%	< 0.1	96%	80%	120%	104%	80%	120%	108%	80%	120%
Lead (Food)	310	NA	< 0.1	< 0.1	0.0%	< 0.1	92%	80%	120%	104%	80%	120%	110%	80%	120%

Certified By:

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http://www.agatlabs.com

Quality Assurance

CLIENT NAME: LABORATOIRE INNODAL

PROJECT: Analyse BacM35

SAMPLED BY:Tomy Faubert

AGAT WORK ORDER: 20M581278

ATTENTION TO: Ariane Renaud

SAMPLING SITE:Divers,Divers

Soil Analysis

RPT Date: 2020-03-17			DUPLICATE			REFERENCE MATERIAL				METHOD BLANK SPIKE			MATRIX SPIKE		
PARAMETER	Batch	Sample Id	Dup #1	Dup #2	RPD	Method Blank	Measured Value	Acceptable Limits		Recovery	Acceptable Limits		Recovery	Acceptable Limits	
								Lower	Upper		Lower	Upper		Lower	Upper
Total Extractable metals															
Mercury	1010027		< 0.2	< 0.2	0.0%	< 0.2	103%	70%	130%	82%	80%	120%	97%	70%	130%

Certified By:

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ST. LAURENT, QUEBEC
CANADA H4S 1V9
TEL (514)337-1000
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http://www.agatlabs.com

Method Summary

CLIENT NAME: LABORATOIRE INNODAL

AGAT WORK ORDER: 20M581278

PROJECT: Analyse BacM35

ATTENTION TO: Ariane Renaud

SAMPLED BY:Tomy Faubert

SAMPLING SITE:Divers,Divers

PARAMETER	DATE PREPARED	DATE ANALYZED	AGAT S.O.P	LITERATURE REFERENCE	ANALYTICAL TECHNIQUE
Food Chemistry Analysis					
Arsenic (Food)	2020-03-10	2020-03-12	FC-102-15012F	AOAC 985-01; MET-101-6107F	ICP/OES
Cadmium (Food)	2020-03-10	2020-03-12	FC-102-15012F	AOAC 985-01; MET-101-6107F	ICP/OES
Lead (Food)	2020-03-10	2020-03-12	FC-102-15012F	AOAC 985-01; MET-101-6107F	ICP/OES
Microbiology Analysis					
Aerobic Bacteria Count - Food	2020-03-09	2020-03-09	MFHPB-18		INCORPORATION
Yeast count - Food	2020-03-05	2020-03-09	MFHPB-22		SPREAD PLATE
Mold count (Food)	2020-03-05	2020-03-09	MFHPB-22		SPREAD PLATE
Staphylococcus aureus - Food	2020-03-05	2020-03-09	MFHPB-21		SPREAD PLATE
Salmonella Detection by MDS - Food	2020-03-05	2020-03-09	MFLP-06		SELECTIVE ENR.
Enterobacteria 3M - Food	2020-03-05	2020-03-09	MFLP-09		SPREAD PLATE
Listeria monocytogenes by MDS - Food	2020-03-05	2020-03-09	MFLP-72		SELECTIVE ENR.
E.coli O157 by MDS - Food	2020-03-05	2020-03-09	MFLP-73		SELECTIVE ENR.
Temperature upon receipt	2020-03-05	2020-03-05	N/A		N/A
Soil Analysis					
Mercury	2020-03-12	2020-03-12	MET-101-6102F	MA. 200 Hg 1.1	COMBUSTION

7.5 Strain information and product specification

INNODAL
innocuity - naturally

Strain information

Carnobacterium divergens M35

Identification	Based on morphological characteristics, carbohydrate fermentation profile (API50CH) and genetic identification of 16SrRNA.
Isolation source	Smoked mussels from Québec, Canada.
Colony morphology	1mm to 1.5 mm round white colonies. Convex shape, smooth surface and soft consistency (after 2 days on STYA media à 30°C aerobic incubation).
Cell morphology	Short rods, non motile, non spore-forming. Single or in short chain.
Gram stain	Positive
Catalase reaction	Negative
Carbohydrate fermentation profile	API50CH
Growth temperature	5°C to 37°C, optimal at 30°C
Acid type production	L-lactic acid
GMO status	No genetic modifications were made to this strain.
Plasmid identification	None identified
Safety of strain	Level 1 microorganism. Safety Assessment by Health Canada (Notice No. NOP/AVP-0018) for use in smoked salmon and smoked trout.
Cultivation method	STYA or TBSYE media, aerobic incubation at 30°C.
Long term storage	STYA or TSBYE media supplemented with 20% glycerol stored at -80°C.
Legal affiliations	Strain was filed at the International Depository Authority of Canada in Winnipeg, Manitoba, under the number ADI 050404-01. Patent application number CA2507566A1 (*Carnobacterium divergens* M35 and bacteriocin produced therefrom).

INNODAL
innocuity · naturally

Product Information

BacM35

Description

BacM35 is a dried culture for cold-smoked salmon, cold-smoked trout and fresh tuna. Its application can inhibit the growth of *Listeria monocytogenes* for a period of 14 days at 4°C. The culture survives well at low temperature such as 4°C and can tolerate multiple freeze-thaw cycles.

Composition

Viable *Carnobacterium divergens* cells and fermentation metabolites.

Application

Usage:	Recommended for cold-smoked salmon, cold-smoked trout vacuumed-packed and fresh tuna. Works on fresh and frozen product.
Dosage:	Recommended dosage is 10^{5-7} viable cells per gram of fish. Culture works best by spraying onto the product. Please contact us for specific information.

Physical Appearance

Free flowing powder, pale yellow color. Soluble in water.

Packaging

Material:	BOPA/PE (Polyethylene)
Size:	500g
Type:	Vacuumed-packed pouches.

Storage and Handling

Keep at 4.0 ± 1.0 °C in a dark dry place.

Shelf Life

12 months if stored in recommended conditions

Dietary Information

Kosher:	Yes. Approved by the Kashruth Council of Canada
Halal:	No

GMO Status

BacM35 does not contain GMOs and is not composed of genetically modified raw material.

INNODAL
innocuity - naturally

Product Specification Sheet

BacM35 Batch No. 01082018

Product Description

BacM35 is a dried culture for cold-smoked salmon, cold-smoked trout and fresh tuna. Its application can inhibit the growth of *Listeria monocytogenes* for a period of 14 days at 4°C. The culture survives well at low temperatures such as 4°C and can tolerate multiple freeze-thaw cycles.

Appearance

Free flowing yellow powder. Do not inhale.

Ingredients

Food grade fermentation metabolites and viable *Carnobacterium divergens* cells.

Country of Origin

Canada

Presence of Allergens

To the best of our knowledge, this product does not contain nor is derived from any food containing allergens.

Microbiological Contaminants

Microorganisms	Detection Status
Yeast and Moulds	20 E
Staphylococcus aureus	< 20 cfu/g
Enterobacteria	< 10 cfu/g
Salmonella spp.	Absent (not detected)
Listeria monocytogenes	Absent (not detected)
Escherichia coli O157:H7	Absent (not detected)

Heavy metal

This product does not contain any heavy metals (As, Cd, Pb, Hg).

Food Grade Statement

This product is manufactured under current Good Manufacturing Practices (cGMP). To the best of our knowledge this product is food grade and is only made with food grade ingredients.

GMO Status

BacM35 does not contain GMOs and is not composed of genetically modified raw material.

Manufacturer's Information

Laboratoire Innodal
201 Monseigneur Bourget
Lévis,Qc, Canada
G6V 6Z3
info@innodal.com

Fire and Explosion Hazards Data

Not flammable

Reactivity Data

Unstable? No☒ Yes ☐

INNODAL
innocuity - naturally

Product Specification Sheet

BacM35 Batch No. 02112017

Product Description

BacM35 is a dried culture for cold-smoked salmon, cold-smoked trout and fresh tuna. Its application can inhibit the growth of *Listeria monocytogenes* for a period of 14 days at 4°C. The culture survives well at low temperatures such as 4°C and can tolerate multiple freeze-thaw cycles.

Appearance

Free flowing yellow powder. Do not inhale.

Ingredients

Food grade fermentation metabolites and viable *Carnobacterium divergens* cells.

Country of Origin

Canada

Presence of Allergens

To the best of our knowledge, this product does not contain nor is derived from any food containing allergens.

Microbiological Contaminants

Microorganisms	Detection Status
Yeast and Moulds	< 20 cfu/g
Staphylococcus aureus	< 20 cfu/g
Enterobacteria	< 10 cfu/g
Salmonella spp.	Absent (not detected)
Listeria monocytogenes	Absent (not detected)
Escherichia coli O157:H7	Absent (not detected)

Heavy metal

This product does not contain any heavy metals (As, Cd, Pb, Hg).

Food Grade Statement

This product is manufactured under current Good Manufacturing Practices (cGMP). To the best of our knowledge this product is food grade and is only made with food grade ingredients.

GMO Status

BacM35 does not contain GMOs and is not composed of genetically modified raw material.

Manufacturer's Information

Laboratoire Innodal
201 Monseigneur Bourget
Lévis,Qc, Canada
G6V 6Z3
info@innodal.com

Fire and Explosion Hazards Data

Not flammable

Reactivity Data

Unstable? No☒ Yes ☐

INNODAL
innocuity - naturally

Product Specification Sheet

BacM35 Batch No. 20022020

Product Description

BacM35 is a dried culture for cold-smoked salmon, cold-smoked trout and fresh tuna. Its application can inhibit the growth of *Listeria monocytogenes* for a period of 14 days at 4°C. The culture survives well at low temperatures such as 4°C and can tolerate multiple freeze-thaw cycles.

Appearance

Free flowing yellow powder. Do not inhale.

Ingredients

Food grade fermentation metabolites and viable *Carnobacterium divergens* cells.

Country of Origin

Canada

Presence of Allergens

To the best of our knowledge, this product does not contain nor is derived from any food containing allergens.

Microbiological Contaminants

Microorganisms	Detection Status
Yeast and Moulds	< 20 cfu/g
Staphylococcus aureus	< 20 cfu/g
Enterobacteria	< 10 cfu/g
Salmonella spp.	Absent (not detected)
Listeria monocytogenes	Absent (not detected)
Escherichia coli O157:H7	Absent (not detected)

Heavy metal

This product does not contain any heavy metals (As, Cd, Pb, Hg).

Food Grade Statement

This product is manufactured under current Good Manufacturing Practices (cGMP). To the best of our knowledge this product is food grade and is only made with food grade ingredients.

GMO Status

BacM35 does not contain GMOs and is not composed of genetically modified raw material.

Manufacturer's Information

Laboratoire Innodal
201 Monseigneur Bourget
Lévis,Qc, Canada
G6V 6Z3
info@innodal.com

Fire and Explosion Hazards Data

Not flammable

Reactivity Data

Unstable? No☒ Yes ☐

Health Canada's Proposal to Enable the Use of a New Food Additive, *Carnobacterium divergens* M35, as an Antimicrobial Preservative in Sliced Ready-to-Eat Cold-Smoked Salmon and Sliced Ready-to-Eat Cold-Smoked Trout

Notice of Proposal – *Lists of Permitted Food Additives*

Reference Number: [NOP/AVP-0018]

June 07, 2016

Bureau of Chemical Safety
Food Directorate
Health Products and Food Branch





Canada

Summary

Food additives are regulated in Canada under Marketing Authorizations (MAs) issued by the Minister of Health and the *Food and Drug Regulations*. Approved food additives and their permitted conditions of use are set out in the *Lists of Permitted Food Additives* that are incorporated by reference in the MAs and published on Health Canada's website. A petitioner can request that Health Canada approve a new additive or a new condition of use for an already approved food additive by filing a food additive submission with the Department's Food Directorate. Health Canada uses this premarket approval process to determine whether the scientific data support the safety of food additives when used under specified conditions in foods sold in Canada.

Health Canada received a food additive submission seeking approval for the use of a live culture preparation of the bacterium *Carnobacterium divergens* M35 to limit or inhibit the growth of the foodborne pathogen *Listeria monocytogenes* on sliced ready-to-eat cold-smoked salmon and sliced ready-to-eat cold-smoked trout.

Marine food products in general can be susceptible to the growth of several pathogens, and fish can provide an environment for the growth of the pathogen *L. monocytogenes*. This bacterium is capable of surviving the cold-smoking process that is used to make ready-to-eat cold-smoked fish products and it can grow at refrigeration temperatures. As a result, sliced ready-to-eat cold-smoked salmon and trout, which are sold refrigerated, are food products that could potentially harbour *L. monocytogenes*.

Certain bacteria produce antimicrobial peptides (proteins) known as bacteriocins. *Carnobacterium divergens* M35 produces divergicin M35, which is a bacteriocin to which *L. monocytogenes* is susceptible.

The *C. divergens* M35 product that is proposed for use on sliced ready-to-eat cold-smoked salmon and trout is a suspension, in water, of live *C. divergens* M35 cells and the fermentation medium containing divergicin M35. The petitioner who filed the food additive submission indicated that in order for it to be effective as an antimicrobial treatment, the culture of *C. divergens* M35 must be applied to the sliced ready-to-eat cold-smoked salmon or trout and allowed to grow and produce its bacteriocin *in situ*. The aqueous suspension can be sprayed onto cold-smoked salmon or trout by the cold-smoked fish manufacturer prior to packaging of the sliced products.

The results of Health Canada's evaluation of available scientific data support the safety and efficacy of *C. divergens* M35 when used as requested by the petitioner. Therefore, it is the intention of Health Canada to modify the *List of Permitted Preservatives* by adding the following entry to the list:

Proposed Modification to Part 2 of the *List of Permitted Preservatives*

Item No.	Column 1 Additive	Column 2 Permitted in or upon	Column 3 Maximum Level of Use and Other Conditions
C.1A	*Carnobacterium divergens* M35	Sliced ready-to-eat cold-smoked salmon; Sliced ready-to-eat cold-smoked trout	Good Manufacturing Practice

Rationale

Health Canada's Food Directorate completed a premarket safety and efficacy assessment of the bacterium *C. divergens* M35. The assessment considered microbiological, toxicological, chemical and technical aspects relevant to *C. divergens* M35 when used as described above.

C. divergens M35 is a lactic acid bacterium. Lactic acid bacteria have a long history of food use and they do not pose a health risk to the general population. *Carnobacterium divergens* species are found naturally in the environment and have been isolated from dairy, meat, and fish products. *Carnobacterium divergens* M35 was isolated from mussels.

Bacteriocins produced by lactic acid bacteria are likely already consumed as part of a normal diet. Since they are easily broken down by proteolytic enzymes in the human gastrointestinal tract, and the resulting single amino acids would be normal dietary constituents, they do not pose a food safety concern.

Carnobacterium species are known to produce biogenic amines. From a food safety perspective, histamine and tyramine are probably the two most important biogenic amines of bacterial origin in food. The potential production of biogenic amines by *C. divergens* M35 when used as a food additive was assessed. Only tyramine showed a potential to increase over time. However, the levels of tyramine observed over the shelf-life of fish products treated with *C. divergens* M35 were well below levels found in many commonly consumed foods, such as cheddar cheese, beer, and aged chicken livers.

The food additive submission included efficacy data demonstrating that sliced ready-to-eat cold-smoked salmon treated with a *C. divergens* M35 preparation did not support the growth of *L. monocytogenes* during the food product's intended shelf-life, whereas growth of this pathogen increased during the shelf-life of untreated controls. The efficacy data provided were judged acceptable for sliced ready-to-eat cold smoked trout as well.

Based on the results of the premarket evaluation, Health Canada's Food Directorate considers that the data support the safety and efficacy of *C. divergens* M35 when used under the conditions

set out in the table above. The Department is therefore proposing to enable the use of *C. divergens* M35 as described in the above table.

Other Relevant Information

As of the time the food additive submission was filed in Canada, the petitioner had not filed any requests with other jurisdictions for the same use of *C. divergens* M35.

The Canadian *Food and Drug Regulations* require that food additives meet the food-grade specifications set out in the most recent edition of the *Food Chemicals Codex* (FCC) if there are no specifications in the Regulations. The FCC is a compendium of standards for purity and identity for food ingredients, including food additives, which is published by the United States Pharmacopeial Convention.

The FCC contains an appendix with information about food uses of live microbial cultures, but there are no monographs with requirements for these cultures. However, the petitioner provided specifications used to control the purity of the *C. divergens* M35 preparation and Health Canada found these specifications to be acceptable from a microbiological perspective.

Implementation and Enforcement

The proposed change will be effective the day on which it is published in the *List of Permitted Preservatives*. This will be announced via a Notice of Modification which will be published on Health Canada's Website.

The Canadian Food Inspection Agency is responsible for the enforcement of the *Food and Drugs Act* and its associated regulations with respect to foods.

Contact Information

For additional information or to submit comments related to this proposal, please contact:

Bureau of Chemical Safety, Food Directorate
251 Sir Frederick Banting Driveway
Tunney's Pasture, PL: 2202C
Ottawa, Ontario K1A 0L2
E-mail: bcs-bipc@hc-sc.gc.ca
If communicating by e-mail, please use the words "***Carnobacterium divergens* M35**" in the subject line of your e-mail. Health Canada is able to consider information received by **August 20, 2016**, 75 days from the date of this posting.

Health Canada Santé Canada

Canada

We're revising Canada's food guide. Participate in the consultation process to help revise Canada's food guide.

Food and Nutrition

Notice of Modification to the *List of Permitted Preservatives* to Enable the Use of *Carnobacterium divergens* M35 as an Antimicrobial Preservative in Sliced Ready-to-Eat Cold-Smoked Salmon and Sliced Ready-to-Eat Cold-Smoked Trout - Document Reference Number: NOM/ADM-0079

Background

Health Canada received a food additive submission seeking approval for the use of a live culture preparation of the bacterium *Carnobacterium divergens* M35 to limit or inhibit the growth of the foodborne pathogen *Listeria monocytogenes* on sliced ready-to-eat cold-smoked salmon and sliced ready-to-eat cold-smoked trout.

The results of Health Canada's evaluation of available scientific data support the safety and efficacy of *C. divergens* M35 when used as requested by the petitioner.

Health Canada published a proposal on June 7, 2016, which was open for public comment for 75 days. Health Canada received one comment in response to the proposal but no new scientific information was submitted that changed the outcome of the safety evaluation. Since the conclusion of the evaluation remains as described in the proposal, Health Canada has modified the *List of Permitted Preservatives*, effective **October 13, 2016**.

The purpose of this communication is to publically announce the Department's decision in this regard and to provide the appropriate contact information for any inquiries or for those wishing to submit any new scientific information relevant to the safety of this food additive.

Information Document

To obtain an electronic copy of the *Notice of Modification to the List of Permitted Preservatives to Enable the Use of Carnobacterium divergens M35 as an Antimicrobial Preservative in Sliced Ready-to-Eat Cold-Smoked Salmon and Sliced Ready-to-Eat Cold-Smoked Trout - Document Reference Number: NOM/ADM-0079*, please contact our publications office or send an e-mail to publications@hc-sc.gc.ca with the subject heading "hpfb BCS nom-adm-0079-eng".

Contact Information

Health Canada's Food Directorate is committed to reviewing any new scientific information on the safety in use of any food additive, including *Carnobacterium divergens* M35. Anyone wishing to submit new scientific information on the use of this food additive or to submit any inquiries may do so in writing, by regular mail or electronically. If you wish to contact the Food Directorate electronically, please use the word "***Carnobacterium divergens* M35**" in the subject line of your e-mail.

Bureau of Chemical Safety, Food Directorate

Supporting Information

Food Additives

Date Modified: 2016-10-13

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 07/31/2022
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
001066	April 13, 2022
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*
   ☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____
2. ☐ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*
3. Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: 2021-11-12
4. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Laura Boivin	president
Organization *(if applicable)*	
Fumoir Grizzly	
Mailing Address *(number and street)*	
159 D'Amsterdam	

City	State or Province	Zip Code/Postal Code	Country
St-Augustin-De-Desmaures	Quebec	G3A 2V5	Canada

Telephone Number	Fax Number	E-Mail Address
418-878-8941	418-878-8942	lb@grizzly.qc.ca

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
Organization *(if applicable)*	
Mailing Address *(number and street)*	

City	State or Province	Zip Code/Postal Code	Country

Telephone Number	Fax Number	E-Mail Address

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Carnobacterium divergens M35 viable culture

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway ☐ Electronic files on physical media

☒ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes 4

Total number of pages 95

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8) and 170.250(d) and (e))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

The bio-preservative preparation containing Carnobacterium divergens M35 cells and its metabolites including the bacteriocin divergens M35, is intended for use as protective culture for inibition of Listeria monocytogenese in fish product. The lyophylized bio-preservative shall be re-suspended in water of suitable microbiological quality and apply by spraying to obtain a final concentration of 10e6 cfu(colony forming unit) per gram of product. The bio-preservative can be applied to fresh or frozen fish products. The treaded product must be vacuum-packed or not and stored at 4oC.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Laura Boivin
(name of notifier)

has concluded that the intended use*(s)* of Carnobacterium divergens M35 viable culture
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Laura Boivin *(name of notifier)* agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

159 D'Amsterdam St-Augustin-De-Desmaures, Qc, Canada, G3A 2V5
(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
	Laura Boivin, president	04/07/2022

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form FDA 3667- submission	1-4
	Carnobacterium divergens M35 culture use as a bio-preservative in fish product	1-85
	Health Canada's proposal to enable the use of new food additive Carnobacterium divergens M35 , as an antimicrobial preservative in sliced ready to eat cold smoked salmon and sliced ready to eat cols smoked	1-4
	Notice of modification to the list of permitted preservatives to enable the use of carnobacterium divergens M35 as an antimicrobial preservative in sliced ready-to-eat cold smoked salmon and sliced ready-to-eat cold smoked trout	1-2