Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Road
Woodbridge, VA 22192-5755
703 590 7337 (Fax 703 580 8637)
smedley@cfr-services.com

November 26, 2018

#824

Dr. Susan Carlson
Director, Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Dear Dr. Carlson:

SUBJECT: Transmittal of the NOMAD BIOSCIENCE GmbH –
GRAS Notice for SALMOCIN
For use as antimicrobial agent

Enclosed you will find the GRAS notice for SALMOCIN as an antimicrobial used for meats, poultry, fish, and egg products as submitted by NOMAD BIOSCIENCE GmbH.

I have provided a CD of the GRAS notice and all the cited references.

Should you have any questions on this filing, please contact me, at your convenience.

Sincerely,

(b) (6)

Kristi O. Smedley, Ph.D.
Consultant to NOMAD BIOSCIENCE GmbH

Attachments

FDA Form 3667 (Hard Copy and CD-Copy)
SALMOCIN GRN NARRATIVE of Notice (CD-Copy)
Appendices (CD-copy)
Full Complement of References (CD-copy)

cc: Yuri Gleba, Nomad

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: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000824	Nov 27, 2018
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):* 2017/12/05

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
Yuri Gleba, Ph.D.	Chief Executive Officer

Organization *(if applicable)*: Nomad Bioscience GmbH

Mailing Address *(number and street)*: Biozentrum Halle, Weinbergweg 22

City	State or Province	Zip Code/Postal Code	Country
Halle/Saale	Saxony-Anhalt	D-06120	Germany

Telephone Number	Fax Number	E-Mail Address
49 345 1314 2424	49 345 1314 2601	gleba@nomadbioscience.com

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

Name of Contact Person	Position or Title
Kristi O. Smedley, Ph.D.	Sponsor's US Regulatory Representative

Organization *(if applicable)*: Center for Regulatory Services, Inc.

Mailing Address *(number and street)*: 5200 Wolf Run Shoals Rd.

City	State or Province	Zip Code/Postal Code	Country
Woodbridge	Virginia	22192	United States of America

Telephone Number	Fax Number	E-Mail Address
703-590-7337	703-580-8637	smedley@cfr-services.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

SALMOCIN antimicrobial for control of Salmonella enterica in food.

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

Submission consists of CD containing electronic files of GRAS Notice

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

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 775

☐ 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)* GRN 593, GRN 676

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))

☐ 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.

SALMOCIN is a food processing aid consisting of salmocin antimicrobial proteins applied to control *Salmonella enterica* on red meats, poultry (skin-on and skin-off), fish, and whole (broken) egg products, at an application rate not to exceed 3 mg SALMOCIN (total salmocin protein) per kg or liter of treated food product (approximately ≤1.4 mg/lb). SALMOCIN is intended for use in food processing facilities.

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

Did you include this other information in the list of attachments?

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Yuri Gleba, Ph.D.
(name of notifier)

has concluded that the intended use(s) of SALMOCIN antimicrobial for control of Salmonella enterica in food.
(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. Nomad Bioscience GmbH
(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.

Center for Regulatory Services Inc., 5200 Wolfe Run Shoals Rd, Woodbridge, VA 22192, USA
(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)
(b) (6)	Kristi O. Smedley, Ph.D.	11-26-2018

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)

NOMAD BIOSCIENCE GmbH
Biozentrum Halle
Weinbergweg 22
D-06120 Halle/Saale
Germany
Tel. 49 345 1314 2606
Fax. 49 345 1314 2601

25 November 2018

Antonia Mattia, Ph.D.
Director, Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRAS Notice for SALMOCIN Antimicrobial for Controlling *Salmonella* Pathovars in Food

Dear Dr. Mattia,

Nomad Bioscience GmbH ("Nomad"; "Notifier") is submitting this GRAS Notice for its **SALMOCIN antimicrobial for controlling *Salmonella* pathovars** in food products. NOMAD has concluded, under FDA's Final Rule pertaining to 21 CFR 170 (August 17, 2016), that the naturally occurring proteins comprising SALMOCIN are Generally Recognized as Safe (GRAS) for use as an antimicrobial treatment to reduce the levels of *Salmonella* bacteria (bactericidal) on meat, poultry, seafood and egg products susceptible to *Salmonella* contamination. Consequently, this Notice is submitted for **dual review** by FDA and USDA's Food Safety and Inspection Service personnel.

Nomad previously submitted GRAS notices to the Center for Food Safety and Applied Nutrition (CFSAN) for its COLICIN product as an antimicrobial for controlling pathogenic *E. coli* on food. Notifier received No Questions letters for COLICIN's application to fruits and vegetables (GRN 593) and meat products (GRN 676); the latter being approved for use on meat by USDA and listed in FSIS Directive 7120.1 Rev 42, Aug 22, 2017 (USDA FSIS 2017). These products were manufactured in plants using food species hosts such as spinach, lettuce and leafy beets. On Oct 26, 2018 FDA also issued a No Questions letter for Notifier's COLICIN product when manufactured in the non-food plant host *Nicotiana benthamiana* (GRN 775). The manufacturing process and use pattern for COLICIN to control *E. coli* and the current SALMOCIN product to control *Salmonella* are very similar, as described here and in prior notices; therefore, we reference those GRNs when appropriate for added perspective.

In the present Notice, we define the use of SALMOCIN to prevent or reduce contamination of food with pathogenic strains of *Salmonella*. *Salmonella enterica* is the type species and is further divided into six subspecies that include over 2500 serovars. *Salmonella enterica* infections are common and are the leading cause of gastroenteritis worldwide. *Salmonella* causes an estimated 1.2 million illnesses in the United States each year, resulting in an estimated 20,000 hospitalizations and 450 deaths (CDC 2017a). During the 5-year

period 2012-2017, 51 *Salmonella* outbreaks were recorded in USA. Most of the food poisonings were due to contaminated poultry or vegetables and fruits, but also red meats, fish and eggs (CDC 2017b).

In this Notice, we claim the safe use of *Salmonella*-derived antimicrobial proteins of the bacteriocin class to control pathogenic *Salmonella* serovars in food. We have named these proteins "salmocins" (*Salmonella* bacteriocins) because they are related in function to *E. coli*-produced bacteriocins (colicins) that we described in prior notices (GRN 593, GRN 676 and GRN 775). We have cloned and expressed salmocins in plants for manufacturing safety and ease of scale-up and found that all expressed salmocins showed high antibacterial activity. Two salmocins in particular showed broad and highly potent activity against all 105 *Salmonella* serovars evaluated.

We have verified salmocins' efficacy and suitability singly and as mixtures. The product SALMOCIN therefore can consist of a single salmocin protein or a mixture of two or more salmocins. The intended application rate to food products susceptible to *Salmonella* contamination, such as poultry, beef, tuna and raw egg, is 0.1 – 3 mg SALMOCIN per kg of treated food. Nomad has concluded that SALMOCIN should be Generally Recognized as Safe (GRAS) under 21 CFR 170.36, and exempt from pre-market approval requirements as specified in Section 201(s) of the Federal Food, Drug, and Cosmetic Act.

In the current Notice, Nomad documents the identity, manufacturing process, product quality, safety, dietary exposure and potential risks from consumption of its SALMOCIN product, based on the company's own results and from publicly available sources of information. In addition, we provide suitability and residual technical effect information for using SALMOCIN during food processing, including the methodology used for such assessments. The latter information is meant to be reviewed with assistance from FSIS and any other departments or agencies, at FDA's discretion.

Our submission complies with the 7-part format prescribed by FDA in its Final Rule for the GRAS Notice process (August 17, 2016), and includes a CD containing PDFs of the following documents:

1. FDA Form 3667 Nomad Bioscience GRN for SALMOCIN antimicrobial
2. GRN for SALMOCIN antimicrobial (Parts 1-7), which includes:
   APPENDIX A: SALMOCIN Safety Data Sheet
   APPENDIX B: SALMOCIN Manufacturing Process
   APPENDIX C: SALMOCIN Characterization
   APPENDIX D: Standard Operating Procedure for SALMOCIN Efficacy Determination
3. Copies of references cited in the GRN

If the Agency has any questions or requires additional information to aid their review of Nomad's findings and conclusions, please contact us at the address listed above. For convenience, you may also contact our regulatory and product development representatives in the USA, Dr. Kristi Smedley at Center for Regulatory Services Inc., Woodbridge, VA (Tel 703-590-7337; Email smedley@cfr-services.com), or Dr. Daniel Tusé at DT/Consulting Group, Sacramento, CA (Tel 707-290-9528; Email daniel@dt-cg.com).

Sincerely,

(b) (6)

Yuri Gleba, Ph.D.
Chief Executive Officer

Table of Contents

Table of Figures

Table of Tables

1 General Introduction and Claim of Exemption from Premarket Approval Requirements for SALMOCIN to Control *Salmonella* in Food

Nomad Bioscience GmbH ("Nomad"; Notifier) SALMOCIN product is produced recombinantly using a plant-based manufacturing process to match the amino acid sequence of naturally occurring salmocin-family antimicrobial proteins. Salmocins (*Salmonella*-derived bacteriocins) destroy bacterial cells by hydrolyzing essential nucleic acids enzymatically (DNase, RNase) or by compromising bacterial cell membrane integrity by inducing formation of pores. Specificity against target pathogenic strains of *Salmonella* is conferred by specific cell wall receptors/translocation systems. From the standpoint of safety, salmocins share up to 99-100% amino acid sequence identity with many colicins, the safety of which was shown in GRN 593.

Although humans and other animals are more highly exposed to *E. coli* commensally than they are to *Salmonella*, species from the latter are carried in the gastrointestinal tract without causing disease or disease symptoms. Analyses of the human colon microbiome show that up to 5% of the US population (3.9% of children) are asymptomatic yet test positive for non-typhoidal *Salmonella* serotypes (Gunn 2014; Morpeth 2009). Due to bacterial growth and lysis, it can be expected that humans are exposed to salmocins naturally. Further, the pathogenesis of non-typhoidal *Salmonella* serovars has been extensively studied. Pathology results from expression of several virulence factors that trigger epithelial inflammation and intestinal fluid loss. We found no reports in the public literature linking *Salmonella* pathogenesis to the presence of salmocin genes or the expression of salmocin proteins. Similarly, we had found no causal relationship reported for colicin synthesis and *E. coli* gastroenteritis (GRN 593).

Being bacteriocin-class proteins, salmocins share many of the same safety attributes of *E. coli* colicins for use as food safety interventions, including potent but short-lived technical effect, heat denaturation (destruction by cooking), instability to low pH environment of the stomach and susceptibility to hydrolysis by enzymes in the stomach (pepsin) and the upper gastrointestinal tract (trypsin and chymotrypsin), as detailed in GRN 593 for colicins and in Section 6 of this Notice for salmocins.

The SALMOCIN product is applied to red meats, poultry, seafood and egg products at a rate of 0.1 – 3 mg SALMOCIN/kg food to prevent the growth of or kill contaminating *Salmonella* pathovars. This Notice provides exposure estimates from consumption of food products treated with SALMOCIN and includes a corresponding risk assessment. Notifier concludes that under the conditions of use described herein, SALMOCIN is generally recognized as safe and therefore should be exempt from premarket approval procedures under 21 CFR 170.36(a)(I). This Notice includes descriptions of the manufacturing process and the quality of the product, efficacy *in vitro* and on sample food matrices, methods used to assess suitability as well as residual technical effect, and the results obtained.

1.1 Submission of Notice

This Notice is submitted in compliance with Subpart E of FDA's Final Rule of the GRAS Notification process (August 17, 2016) 21 CFR 170.203-170.285.

1.2 Name and Address of Notifier

NOMAD BIOSCIENCE GmbH
Biozentrum Halle
Weinbergweg 22
D-06120 Halle/Saale, Germany
Office: 49 345 1314 2424
Fax: 49 345 1314 2601

Notifier's US Representative
Kristi O. Smedley, Ph.D.
Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192
Office: 703-590-7337; Mobile: 703-786-7674
Fax: 703-580-8637
eMail: smedley@cfr-services.com

Notifier's US Representative
Daniel Tusé, Ph.D.
DT/Consulting Group
2695 13th Street
Sacramento, CA 95818
Telephone: 707-290-9528
Fax: 916-822-4124
Email: daniel@dt-cg.com

1.3 Common or Usual Name of the Notified Substance

SALMOCIN

1.4 Conditions of Use

What is **claimed as GRAS in this Notice** is:

> A SALMOCIN antimicrobial product consisting of one or more plant-produced salmocin proteins to include SalE1a, SalE1b, SalE2, SalE3 or SalE7, mixed or blended in any combination to meet Specification and applied to foods susceptible to *Salmonella* contamination, specifically red meat, poultry, fish and egg products, at a rate not exceeding 3 mg total salmocins per kg or liter of food product treated.

This Notice concerns salmocins and salmocin mixtures designed to control pathogenic *Salmonella enterica* serovars in food. SALMOCIN is comprised of one or more bacteriocin proteins natively encoded by *Salmonella enterica* serovars to gain ecological competitiveness against other *Salmonella* strains. By selecting which salmocins are included in an antimicrobial formulation at optimized ratios, the host-specificity of the product and its potency can be controlled.

The product SALMOCIN can thus be formulated to contain one or a mixture of two or more individual salmocin proteins, depending on the breadth of application needed in various food products. Details on salmocins, including their range of biological activities, are provided in Section 2-4 of this Notice. The overall safety of these proteins is summarized in Section 6. A summary list of salmocins' safety attributes when used as intended, including the sources of supporting information, is provided in Table 7-1.

Specifically, the intended use of SALMOCIN is as a spray to be applied to solid food matrices (e.g. meats), as a solution into which solid foods are temporarily dipped, or as a powder or solution to be added to liquid or semisolid foods (e.g. egg products), to control *S. enterica* on fresh or processed foods at application rates of 0.1 – 3 mg SALMOCIN per kg (or liter) of treated food (approximately 0.05 – 1.4 mg/lb).

The **subpopulations** potentially exposed to SALMOCIN are comprised of individuals of all ages who consume meat, poultry and egg products susceptible to *S. enterica* contamination and treated with Notifier's antimicrobial.

1.5 Statutory Basis for Notifier's GRAS Conclusion

The statutory basis of the GRAS status in through **scientific procedures** in accordance with 21 CFR 170.30(b): GRAS Conclusion. In accordance with the information provided in the Notice, it is Nomad Bioscience's conclusion that SALMOCIN is generally recognized as safe when used to minimize contamination of meat and egg products by *Salmonella enterica* at an application rate of 0.1 – 3 mg SALMOCIN per kg or liter of treated food.

1.6 Not Subject to Preclearance

Notifier has concluded that SALMOCIN as manufactured via its plant-based process is generally recognized as safe, and as such the substance is not subject to pre-market approval requirements of the Federal Food Drug and Cosmetic Act.

1.7 Availability of Information for FDA and USDA Review

All data and information that serve as a basis for the GRAS and suitability conclusions are included in this Notice.

1.8 Public Disclosure

The information provided in this Notice is publicly available and not subject to exception under 170.225(c)(8). All information contained in this Notice can be shared without restriction.

1.9 Certification

On behalf of Nomad Bioscience GmbH (Notifier), I certify that to the best of my knowledge, this GRAS Notice is complete, representative, and balanced with respect to the information provided, favorable or unfavorable, known to me and pertinent to the evaluation of the safety and GRAS status of our SALMOCIN antimicrobial product.

(b) (6)

Yuri Gleba, Ph.D.
Chief Executive Officer
NOMAD BIOSCIENCE GmbH
Biozentrum Halle
Weinbergweg 22
D-06120 Halle/Saale
Germany

2 Identity, Method of Manufacture, Specifications, Technical Effect

2.1 Identity, Structural and Functional Information

Identity

Table 2-1 lists the candidate active components of the SALMOCIN product that may be used singly or in combination to achieve the desired antibacterial suitability in food. *Salmonella* bacteriocins are similar to *E. coli*-derived colicins. All salmocin gene sequences were obtained from non-typhoidal, salmocinogenic *Salmonella* strains that naturally attack susceptible strains of *S. enterica*. The putative bacteriocin genes from *Salmonella* were selected from the public NCBI database on the basis of homology to the enzymatic domains of *E. coli* colicins but with differences in amino acid composition in translocation and receptor protein domains of Class A colicins, as we detailed in Schneider et al. (2018).

Five *Salmonella* bacteriocins (salmocins) representing 3 activity groups (DNase, RNase, pore-forming) of analogous colicin proteins and most likely with different translocation mechanisms, were selected and designated Sal (for *Salmonella*) followed by additional letters designating the highest similarity to its corresponding *E. coli* GRAS colicin (e.g. *Salmonella* SalE7 is most similar to GRAS *E. coli* ColE7).

The table lists each component salmocin by the common name we use herein, together with its mode of action (type of antibacterial effect), molecular mass, and collection accession number.

Table 2-1. Candidate active components of SALMOCIN product formulation

No.	Salmocin	Mode of Action	MW (Da)	GenBank Accession No.
1	SalE2	DNase	61960	KTM78572.1
2	SalE3	RNase	61710	GAS18013.1
3	SalE7	DNase	62260	KSU39545.1
4	SalE1a	Pore-forming	52812	OIN35410.1
5	SalE1b	Pore-forming	57584	OIN32443.1

Structural Information on SALMOCIN Components

The structures and modes of action of salmocins are related to those of colicins; this may be a consequence of co-evolution of the two genera of enterobacteria, *Salmonella* and *E. coli*, in the same hosts. The two genera, *Escherichia* and *Salmonella*, are closely related, and *Salmonella* strains have been known to harbor colicin genes (Barker 1980; Campos 1988; Nedialkova 2014; Vicente 1984). Activity against *Salmonella* was confirmed by Notifier for colicins M, Ia, Ib, 5, 10 and S4, in agreement with literature reports. However, *Salmonella* serovar Typhimurium was reported to be insensitive to Group A colicins E1, E2 and E3 (Graham 1977; Guterman 1975), yet some *Salmonella* strains are sensitive to colM (Graham 1977).

NCBI database searches showed that analogues of *E. coli* colicins Ia, Ib, M, and B seem to be widely distributed in *Salmonella* with 99-100% identity in amino acid sequence. This allowed us to select five

salmocins with potentially higher activity and specificity against *Salmonella* pathovars. The sequences of the selected salmocins are described below.

Individual Salmocin Amino Acid Sequences

Five (5) recombinant salmocins are described as candidate components of the SALMOCIN product, to be used either singly or in combination. Additional salmocins may be included in the product at a future date provided they meet specification. The amino acid sequence of each of the current salmocins listed in Table 2-1 is provided below, together with the GenBank accession number.

Additional structural information and the methods used to characterize the proteins, including amino acid verification, are described in APPENDIX C.

SalE2 (GenBank: KTM78572.1)

```
MSGGDGIGHN SGAHSTGGVN GSSSGRGGSS SGGGNNPNSG PGWGTTHTPD GHDIHNYNPG EFGGGGHKPG GNGGNHSGGT
GDGQPPGAAM AFGFPALVPA GAGGLAVTVS GDALAAAIAD VLAVLKGPFK FGAWGIALYG ILPTEIAKDD PRMMSKIVTS
LPADAVTESP VSSLPLDQAT VSVTKRVTDV VKDERQHIAV VAGVPASIPV VDAKPTTHPG VFSVSVPGLP DLQVSTVKNA
PAMTALPRGV TDEKDRTVHP AGFTFGGSSH EAVIRFPKES GQAPVYVSVT DVLTPEQVKQ RQDEENRRQQ EWDATHPVEV
AERNYRLASD ELNRANVDVA GKQERQIQAA QAVAARKGEL DAANKTFADA KEEIKKFERF AHDPMAGGHR MWQMAGLKAQ
RAQNEVNQKQ AEFNAAEKEK ADADAALNVA LESRKQKEQK AKDASDKLDK ENKRNHPGKA TGKGQPVGDK WLEDAGKEAG
APVPDRIADK LRDKEFKNFD DFRKKFWEEV SKDPELSKQF IPGNKKRMSQ GLAPRARNKD TVGGRRSFEL HHDKPISQDG
GVYDMDNIRV TTPKLHIDIH RGK
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SalE3 (GenBank: GAS18013.1)

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MSGGDGRGHN TGAHSTSGNI NGGPTGLGVS GGASDGSGWS SENNPWGGGS GSGIHWGGGS GRGNGGGNGN SGGGSGTGGN
LSAVAAPVAF GFPALSTPGA GGLAVSISAS ELSAAIAGII AKLKKVNLKF TPFGVVLSSL IPSEIAKDDP NMMSKIVTSL
PADDITESPV SSLPLDKATV NVNVRVVDDV KDERQNISVV SGVPMSVPVV DAKPTERPGV FTASIPGAPV LNISVNNSTP
AVQTLSPGVT NNTDKDVRPA GFTQGGNTRD AVIRFPKDSG HNAVYVSVSD VLSPDQVKQR QDEENRRQQE WDATHPVEVA
EREYENARAE LEAENKNVHS LQVALDGLKN TAEGLALSDA GRHPLTSSES RFVAVPGYSG GGVHFDATAT VDSRDRLNSL
LSLGGAAYVN NVLELGEVSA PTEDGLKVGN AIKNAMIEVY DKLRQRLITR QNEINHAQVS LNTAIESRNK KEEKKRSAEN
KLNEERNKPR KGTKDYGHDY HPAPETEEIK GLGDIKKGIP KTPKQNGGGK RKRWIGDKGR KIYEWDSQHG ELEGYRASDG
QHLGSFDPKT GKQLKGPDPK RNIKKYL
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SalE7 (GenBank: KSU39545.1)

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MSGGDGIGHN SGAHSTGGVN GSSSGSGGSS SGSGNNPNSG PGWGTTHTPN GDIHNYNPGE FGGGGNKPGG HGGNSGNHDG
SSGNGQPSAA PMAFGFPALA PAGAGSLAVT VSGEALSAAI ADIFAALKGP FKFGAWGIAL YGIMPTEIAK DDPNMMSKIM
TSLPADTVTD TPVSSLPLDQ ATVSVTKRVA DVVKDERQHI AVVAGVPMSV PVVDAKPTTR PGIFSATVPG LPALEVSTGK
SIPASTALPR GITEDKDRTE HPAGFTFGGS SHDAVIRFPK ESGQAPVYVS VTDVLTPEQV KQRQDEESRR QQEWDATHPV
EVAERNYRLA SDELNRVNAD VAGKQERQAQ AGQAVAARKG ELDAANKTFA DAKEEIKKFE HFARDPMAGG HRMWQMAGLK
AQRAQNEVNQ KQAEFDAAEK EKADADAALN AALESRKQKE QKAKDTKERL DKENKRNQPG KATGKGQPVS DKWLEDAGKE
SGSPIPDSIA DKLRDKEFRN FDDFRKKFWE EVSKDPELSK QFIKGNRDRM QVGKAPKSRK KDAAGKRTSF ELHHDKPVSQ
DGGVYDMDNL RITTPKRHID IHRGQ
```

SalE1a (GenBank: OIN35410.1)

```
MADNTIAYYE DGVPHSADGK VVIVIDGKMP VDTGAGGTGG GGGGKVGGTS ESSAAIHATA KWSTAQLKKT LAEKAARERE
TAAAMAAAKA KRDALTQHLK DIVNDVLRHN ASRTPSATDL AHANNMAMQA EAQRLGRAKA EEKARKEAEA AELAFQEAER
QREEAVRQLA ETERQLKQAE EEKRLAALSD EARAVENARK NLDTAKSELA NVDSDIERQR SQLSSLDADV KKAEENLRLT
MRIKGRIGRK MQAKSQAIVD DKKRIYSDAE NVLNTMTVNR NLKAQQVTDA ENELKVAIDN LNSSQMKNAV DATVSFYQTL
TEKYGEKYSL IAQELAEKSK GKKIGNVDEA LAAFEKYKDV LDKKFSKADR DAIVNALKSF NYDDWAKHLD QFAKYLKITG
HVSFGYDVVS DVLKASETGD WKPLFITLEQ KVLDTGMSYL VVLMFSLIAG TTLGIFGVAI ITAILCSFVD KYILNALNDA
LGI
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SalE1b (GenBank: OIN32443.1)

MSDNTIAYYE DGVPYSADGQ VVIVIDGKMP VDTGAGGTGG GGGGKVGGTS ESSAAIHATA KWSKAQLQKS LEEKAARERE
TAAAMAAAKA KRDALTQHLK DIVNDVLRYN ASRTPSATDL AHANNMAMQA EAQRLGRAKA EEKARKEAEA AEKSLQEAER
QREEAARQRA EAERQLKQAE AEEKRLAALS EEARAVEITQ KNLAAAQSEL SKMDGEIKSL NVRLSTSIHA RDAEMNSLSG
KRNELAQESA KYKELDELVK KLEPRANDPL QNRPFFDATS RRARAGDTLA EKQKEVTASE TRINELNTEI NQVRGAISQA
NNNRNLKVQQ VTETENALKV AIDNLNSSQM KNAVDATVSF YQTLTAKYGE KYSLIAQELA EQSKGKKISN VDEALAAFEK
YKDVLDKKFS KADRDAIVNA LKSVDYADWA KHLDQFSRYL KISGRVSTGY DIYSDIRKGM DTNDWRPLFL TLEKLAVDAG
VGYIVALGFS VIASTALGIW GVAIITGVIC SFVDKKDLEK LNEALGI

Quantitative Composition

SALMOCIN is prepared in bulk as a concentrated solution or as a dry (freeze-dried or spray dried) powder. The concentrated solution can be diluted for application as a spray or dip. The dry formulation can be dissolved in water or other compatible aqueous medium or added directly to food.

SALMOCIN is preferentially manufactured using the plant host *Nicotiana benthamiana*, although food species plants can also be used for production. When using *N. benthamiana*, the salmocin extract is purified to reduce host alkaloid impurities prior to product formulation. Such purification is not needed when using food species plants. The salmocin manufacturing summary is found in APPENDIX B; purification details are found in APPENDIX C. The Composition per target Specification is shown in Table 2-2.

SALMOCIN is dissolved/diluted in water/aqueous medium to a concentration of 5 – 150 mg/L for spray application to solid foods at a rate not to exceed 20 mL solution/kg (9 mL/lb) of food product. This equates to an application rate of 0.1 – 3 mg/kg. Alternatively, food products can be dipped in a solution of SALMOCIN at a concentration of 5 – 150 mg/L.

When adding SALMOCIN to liquid or semisolid foods (e.g. egg products), either as a dry powder or aqueous solution, the total application rate should be $\leq$3 mg/kg or $\leq$3 mg/L of food.

Salmocins can be prepared singly or in combination with other salmocins. For mixtures, each salmocin protein is manufactured separately and then combined in defined ratios. The decision to formulate a single salmocin or mixtures of salmocins depends on the food application and the strain or serotype of *Salmonella* targeted for control.

That is, a SALMOCIN formulation consisting of a single component comprises only the specified salmocin as the active ingredient. A SALMOCIN formulation consisting of two or more different salmocins comprises two or more active ingredients having synergistic or additive potency or expanded host range.

Regardless of formulation or mode of application of the SALMOCIN product, the highest amount of salmocin protein(s) to be applied to food should be $\leq$3 mg/kg or /L ($\leq$~1.4 mg/lb).

Modes of Action

Salmocins can be considered food treatment enzymes restricted to controlling pathogenic *Salmonella enterica* serovars without affecting the properties of the treated food, or the normal commensal intestinal microbiome. As do other bacteriocins, including colicins, salmocins have a catalytic or pore-forming domain and an outer membrane cell surface receptor binding/targeting domain (Cascales 2007; Schneider 2018).

Currently, there are three types of activities (modes of action) that characterize the salmocins under development, specifically:

1. DNase (DNA degradation) – SalE2 and SalE7
2. RNase (RNA degradation) – SalE3
3. Pore formation (destruction of transmembrane ion gradient; osmotic lysis) – SalE1a and SalE1b

Entry of the salmocins to the cells' interior is mediated by transporter-translocation mechanisms and lead to rapid degradation of nucleic acids (nuclease salmocins) or pore formation and depletion of energy generation capacity (pore-forming salmocins), both leading to cell stasis followed by cell death (bactericidal effect).

The host range and potency of SALMOCIN can be optimized by mixing salmocins with complementary translocation mechanisms and antibacterial modes of action (e.g. nucleolytic + pore-forming salmocins).

2.2 Method of Manufacture

Notifier uses a plant-based manufacturing process for producing SALMOCIN proteins. The method is described in APPENDIX B and is an adaptation of the process used to manufacture biopharmaceuticals, which have been administered in multiple clinical trials under FDA IND. It is also the same GRAS method used by Notifier to produce food safety antimicrobials for controlling enteropathogenic *E. coli* in fruits and vegetables (GRN 593; No Questions) and meats (GRN 676; No Questions and GRN 775; No Questions), *Clostridium perfringens* in cooked meats (GRN 802; Pending), and the GRAS non-caloric sweetener THAUMATIN (GRN 738; No Questions).

The plant-derived biomass remaining after salmocin protein extraction is treated and discarded (disposed) per local regulations and is not used as a human food or animal feed product, additive or supplement.

2.3 Composition and Specification

Characteristic properties

Notifier's SALMOCIN component proteins are produced recombinantly in green plants. Because there are differences in the way plant, microbial and mammalian cells express genes, the codon sequence at the gene level can be optimized for stable, high-yield expression in plants. In some cases, protein maturation among hosts can result in slight differences (e.g. 1 or 2 amino acids) in the final protein post translation, such as in retention or deletion of an N-terminal methionine, or N-terminal acetylation.

Such maturation events are well known, are found in approved pharmaceuticals produced in heterologous hosts, and are not impactful on protein safety, as reviewed by Kamionka (2011). Because Notifier discovered salmocins by genomic analysis of *Salmonella* for colicin-like sequences, the cloned DNA sequences for plant expression encode protein sequences identical to those encoded by bacterial genes, but with different DNA sequences resulting from the codon optimization mentioned above.

Importantly, the full-length amino acid consensus sequences responsible for the structure, activity and safety of the salmocins have been retained in every case. Therefore, the proteins produced recombinantly in plants retain the predicted amino acid sequences of naturally occurring salmocins, as reported in Schneider et al. (2018) and as verified from published gene and protein accession databases. Molecular characterization of plant-made salmocins was accomplished by advanced mass spectrometry, as described in APPENDIX C.

Lack of glycosylation

Native salmocin proteins naturally produced by bacteria are non-glycosylated. None of the salmocins have glycan addition sites along their backbone and therefore the plant-produced proteins are also non-glycosylated polypeptides, as confirmed by MS/MS analysis (Schneider 2018).

Susceptibility to digestion

Being simple polypeptides without a glycan coat or multiple disulphide bridges, salmocins are predicted to be susceptible to environmental conditions including denaturation by heat (cooking) or acid such as stomach acid, and proteolytic digestion by stomach and intestinal enzymes, much as are other bacteriocins (Cascales 2007; Gordon 2006; Murinda 2003) and bacteriophage endolysins (Nelson 2012; Schmelcher 2012).

Susceptibility to digestion in simulated stomach and duodenal environments has been confirmed by Notifier for all salmocins (Section 2.4.2). Hence, only low ingestion levels of salmocins are expected from uncooked foods, and insignificant ingestion of salmocins is expected from properly cooked foods.

Low immunogenicity

Being proteins, intact salmocins are theoretically capable of inducing immune responses through mucosal contact. However, the high degree of amino acid, structural and activity homologies among salmocins and colicins and the very low potential for immunogenicity or allergenicity of colicins (GRN 593), suggest that salmocins, also, would have low probability of inducing immunogenic/allergenic effects.

To comprehensively assess risk, Notifier studied at the molecular level the allergenic potential of candidate salmocins for use in food and determined, from published information, that salmocins have a low potential for inducing immune or allergic responses. Allergenic potential is discussed in Section 6.5.

Formulation

SALMOCIN is preferentially provided as a dry powder for ease of transportation and storage, and for added stability. The bulk product is dissolved in water or other aqueous medium (e.g. raw egg; meat gravy) according to instructions and can be applied as a wash, spray, dip, package fill or marinade depending on the intended use.

Content of potential human toxicants in SALMOCIN

There are two potential human toxicants that may remain in low levels in the SALMOCIN product when it is manufactured using the host plant *N. benthamiana*, namely, the alkaloids nicotine and anabasine.

The host plant *N. benthamiana* shares metabolic pathways with other members of the family Solanaceae, which includes tomato, pepper, potato, eggplant and others. All these plants contain low residual yet measurable levels of pyridine alkaloids.

The purpose of including a chromatographic purification step during downstream processing is to produce salmocins with levels of residual alkaloids that are no higher than the levels consumed in common vegetables in a typical diet. This topic is extensively discussed in the published GRN 775, Section 6.1.4 Host impurities from *Nicotiana* species and their impact on safety; pp 32-37.

Specification

The manufacturing process to produce bulk SALMOCIN product is described in APPENDIX B. Individual salmocins are purified and formulated singly or blended at prescribed ratios into a mixed SALMOCIN product. For ease of transport and storage and added stability, SALMOCIN is typically formulated as a dry powder.

What is claimed as GRAS in this Notice is:

> A SALMOCIN antimicrobial product consisting of one or more plant-produced salmocin proteins to include SalE1a, SalE1b, SalE2, SalE3 or SalE7, mixed or blended in any combination to meet Specification and applied to foods susceptible to *Salmonella* contamination, specifically red meat, poultry, fish and egg products, at a rate not exceeding 3 mg total salmocins per kg or liter of food product treated.

A candidate commercial blend of salmocins includes SalE1b, which is a pore-forming salmocin that is active against all tested *Salmonella enterica* pathovars, plus SalE7, a DNase salmocin added to complement the bactericidal activity while reducing the probability of inducing bacterial resistance. Other salmocin blends based on complementary modes of action are of course possible.

The current **target Specification** for a mixed-salmocin SALMOCIN product is summarized in Table 2-2. For convenience, this same target Specification is also shown in the Manufacturing section of this GRN, (APPENDIX B, Table B-1).

In the target Specification, upper limits for the host-derived impurities nicotine and anabasine of 0.4 μg/mg and 0.1 μg/mg, respectively, were set as defined in Section 6.2 (Low Safety Risk from Consumption of Plant Host Impurities in Salmocins) based on assessed risk and relative to alkaloid levels currently consumed in a typical US diet. Consumption of residual SALMOCIN applied to foods is expected to be $\leq$0.8 mg/person-day; hence, the worst-case exposure levels for nicotine and anabasine assuming no losses during cooking and 100% product market penetration for all treated foods, are estimated to be <0.32 and <0.08 μg/person-day, respectively, or 0.4 μg/person-day total product-derived alkaloids. Current consumption of these alkaloids from common vegetables is approximately 1 μg/person-day (see Section 6.2 for full discussion).

Current results listed in the target Specifications are derived from analyses of salmocin proteins produced in multiple independent developmental batches. Details on the methods used to quantify these parameters are found in APPENDIX C.

Table 2-2. Target specification of SALMOCIN (mixed salmocin) product

SALMOCIN Antimicrobial Dry Powder Formulation			
Parameter	**Method**	**Specification limit**	**Results of analyses[1]**
Appearance	Visual	Powder, white to beige	Conforms
Specific Activity (Potency; salmocin protein basis)[2]	Viability inhibition; *S. enterica* ssp. *enterica* serovar Enteritidis reference strain ATCC® 13076™	Minimum, based on least potent salmocin in blend: $\geq 1 \times 10^6$ AU/g protein	Average potency from salmocin blend: $3.52 \pm 4.14 \times 10^9$ AU/g protein
pH of a 1% solution in water	Potentiometric	6.0-7.5	Average 7.0 ± 0.5
Nicotine (per total salmocin blend)[3]	HPLC/MS	$\leq$ 0.4 μg/mg	Average 0.24 ± 09 μg/mg
Anabasine (per total salmocin blend)[3]	HPLC/MS	$\leq$ 0.1 μg/mg	Average 0.056 ± 0.05 μg/mg
Bioburden	USP32<61>	$\leq$ 10 CFU/25 g sample	0 (absent)
Agrobacterium (CFU/10 g sample)	Selective plate based assay	0 (absent)	0 (absent)
Undesirable microorganisms: *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp.* or coagulase-positive *Staphylococcus spp.*, per 25 g final product	USP32<1111>	0 (absent)	0 (absent)
Stability of dry concentrate product (0-10°C storage)[4]	Specific activity at T_n vs. T_0; plate-based assay	> 6 months	> 6.25 months (average) at time of GRN submission

[1]Results of analyses for a dry SALMOCIN (mixed-salmocin) product are based on average results obtained from analyses of individual salmocin proteins blended at a ratio (dwb) of 1x each SalE1b and SalE7.

[2]Measured potency values for Specification are based on potency averages $\pm$ s.d. of multiple non-consecutive developmental batches of salmocins SalE1b (4 batches) and SalE7 (5 batches).

[3]Alkaloid impurity values are averages derived from a minimum of 3 non-consecutive developmental batches of protein.

[4]Stability results are interim; all salmocins are in a continuing stability program. Stability is calculated from potency vs. time of storage for dry salmocins and salmocin solutions under different storage conditions and are expressed as averages from a minimum of 3 non-consecutive developmental batches.

2.4 Technical Effect and Suitability of Use

In this section, we describe the efficacy and suitability of SALMOCIN as an antimicrobial processing aid for food, as well as the duration of SALMOCIN's technical effect.

2.4.1 Biological activity of SALMOCIN on target pathogenic *S. enterica* serotypes

Structure and mechanism of action underlying potency

Salmocins are bacteriocins produced by some *Salmonella* strains that specifically target other *Salmonella* strains, including pathogenic strains of *S. enterica* that are associated with food-borne gastroenteritis. Salmocins share a high degree of similarity to colicins, similar bacteriocins produced by some *E. coli* strains to control the growth of other *E. coli* strains for competitive ecological advantage. The field of bacteriocins and their applications, including the colicinogenicity of *Salmonella*, has been extensively reviewed (Barker 1980; Callaway 2004; Campos 1988; Cascales 2007; Nedialkova 2014; Schulz 2015; Vicente 1984; Yang 2014) and others and comparative characterizations can be made.

Salmocins share with other bacteriocins the mode of action underlying potency and selectivity, namely, (a) they use a receptor/translocation system to gain access to a susceptible cell's interior, leading to (b) destruction of nucleic acids, other macromolecules including cell wall components, or disruption of membrane integrity and polarity via formation of pores. The modes of action of the five salmocins in the SALMOCIN product include three types of antibacterial activity, namely, DNase, RNase and pore formation.

Notifier's SALMOCIN product consists of one or more (blend) individually produced salmocins that act alone or synergistically to destroy pathogenic serotypes of *S. enterica*. Because simultaneous attack of multiple components within the target cell can lead to more rapid destruction of the cell, the potency of salmocin mixtures against certain pathovars of *S. enterica* can be higher than the potency of individual salmocins. Also, salmocins with overlapping activity optima (e.g. pH, temperature and salinity) can be expected to work best as mixtures and offer more consistent protection of a wider range of foods in more diverse preparation and storage environments. In addition, a combination of salmocins targeting different receptor/translocation systems will reduce the probability for the emergence of salmocin-resistant mutants and expand the breadth of activity of the mixture compared to individual salmocins.

Range of activity of plant-produced salmocins against pathogenic serovars of *S. enterica*

Salmocin proteins were evaluated for bactericidal activity first in a series of screens *in vitro* to determine the range of activity across *S. enterica* serovars as well as their potency. Subsequently, the biological activity of plant-produced salmocins was determined in simulated intended applications to assess suitability. These latter studies involved suboptimal cold-storage incubations on-matrix with contaminated food samples. Representative results from each series of studies are presented in this Notice. Selected results have also been published in Schneider et al. (2018).

Specifically, plant-produced salmocins were evaluated for activity against *S. enterica* serovars relevant to food contamination, using laboratory settings as well as treatment of intended foods (e.g. poultry, beef, seafood and raw whole eggs). To determine the salmocin antimicrobial activity spectrum, 109 strains that included 105 *S. enterica* ssp. *enterica* serotypes were selected and screened.

The strains screened are listed in Table 2-3 and included one strain each of all serotypes (except serotypes Typhi and I4,5:12:r:-) that have been documented at the U.S. Centers for Disease Control and Prevention as having caused at least 100 incidences of human *Salmonella* infection from 2003-2012 (CDC 2016), two

strains each of serotypes Typhimurium, Enteritidis and Javiana, and 6 serotypes causing <100 incidences or not reported to CDC.

Table 2-3. Human pathogenic strains of *S. enterica* used to evaluate SALMOCIN efficacy

No.	Culture Collection Reference No.	Serotype	Serotype Antigenic Formula	Source of Supply	Number of Incidences
1“	ATCC® 13076™*	Enteritidis	I 1,9,12:g,m:-	1	74450
2“	ATCC® 49223™*		I 9,12:g,m	1	
3“	ATCC® 14028™*	Typhimurium	I 4,5,12:i:1,2	1	70251
4“	ATCC® 13311™*		I 4,5,12:i:1,2	1	
5“	ATCC® 6962™*	Newport	I 6,8:e,h:1,2	1	44675
6“	ATCC® 10721™*	Javiana	I 1,9,12:I,z28:1,5	2	22868
7“	ATCC®BAA-1593™		I 9,12:-:1,5	2	
8“	ATCC® 8326™*	Heidelberg	I 4,5,12:r:1,2	1	15912
9	17-00918	-	I 4,[5],12:i:-	3	13567
10“	ATCC® 8387™*	Montevideo	I 6,7:g,m,s:-	2	11377
11“	ATCC® 8388™*	Muenchen	I 6,8:d:1,2	2	9589
12“	ATCC® 9712™*	Saintpaul	I 1,4,5,12:e,h:1,2	2	9420
13“	ATCC®BAA-1675™	Infantis		2	8106
14“	ATCC® 9239™*	Oranienburg	I 6,7:m,t:-	2	7514
15“	ATCC® 700136™*	Braenderup	I 6,7:e,h:e,n,z15	2	7371
16“	ATCC®BAA-2739™	Mississippi	I 13,23:b:1,5	2	5693
17“	ATCC® 8391™*	Thompson	I 6,7:k:1,5	2	5660
18“	ATCC® 51957™*	Agona	I 4,12:f,g,s:-	1	5072
19	16-04932	Paratyphi B var. L(+) tartrate +	I 4,5:b:1,2	3	4624
20“	ATCC® 9115™*	Bareilly	I 6,7:y:1,5	2	3704
21“	NCTC 4840	Poona	I 13,22:z:1	1	2977
22	16-4909	Hadar	I 6,8:z10:e,n,x	3	2857
23	16-05099	Schwarzengrund	I 4:d:1,7	3	2835
24“	ATCC® 8392™*	Berta	I 9,12:f,g,t:-	2	2779
25“	ATCC® 9270™*	Anatum	I 3,10:e,h:1,6	1	2753
26	16-04966	Stanley	I 4,5:d:1,2	3	2438
27	15-04731	Litchfield	I 6,8:e,v:1,2	3	2386
28	10-03610	Hartfort	I 6,7:y:e,n,x	3	2312
29“	ATCC® 51958™*	Mbandaka	I 6,7:z10:e,n,z15	2	2286
30	16-03044	Panama	I 9:e, v:1,5	3	1903
31	16-04172	-	I 4,[5],12:b:-	3	1860
32	14-03918	Sandiego	I 4,5:e,n:e,n,z15	3	1759
33“	ATCC® 9150™*	Paratyphi A	I 1,2,12:a:-	1	1731
34“	DSM 10062	Senftenberg	I 1,3,19:g,s,t:-	4	1594
35	NCTC 7077	Norwich	I 6,7:e, h:1,6	5	1481
36	16-05141	Tennessee	I 6,7:z29:-	3	1476
37	16-05288	Rubislaw	I 11:r:e,n,x	3	1394
38“	ATCC® 6960™*	Derby	I 1,4,12:f,g:-	2	1392
39	07-06267	-	I 13,23:b:-	3	1275
40	16-05246	Give	I 3,10:l,v:1,7	3	1250
41	16-05252	Paratyphi B	I 4,5:b:1,2	3	1249
42	14-04905	Miami	I 9:a:1,5	3	1087
43“	ATCC® 15480™*	Dublin	I 1,9,12:g,p:-	2	1086
44“	ATCC® 9263™*	Kentucky	I (8),20:i:z6	2	984

No.	Culture Collection Reference No.	Serotype	Serotype Antigenic Formula	Source of Supply	Number of Incidences
45	16-05080	Brandenburg	I 4:l,v:e,n,z15	3	963
46	16-04827	Virchow	I 6,7:r:1,2	3	961
47	16-02846	Gaminara	I 16:d:1,7	3	953
48	17-00031	Weltevreden	I 3,10:r:z6	3	876
49	16-05006	Bovismorbisficans	I 6,8:r:1,5	3	839
50	17-00039	Manhattan	I 6,8:d:1,5	3	836
51	14-05486	Adelaide	I 35:f,g:-	3	820
52	16-05394	Uganda	I 3,10:e,z13:1,5	3	817
53	15-03669	Pomona	I 28:Y:1,7	3	781
54	16-04580	Muenster	I 3,10:e,h:1,5	3	756
55	15-01597	Kiambu	I 4:z:1,5	3	699
56	15-02141	Blockley	I 6,8:k:1,5	3	688
57	16-04687	Ohio	I 6,7:b:e,w	3	656
58	16-05313	Hvittingfoss		3	620
59	16-01351	Reading	I 4,5:e,h:1,5	3	619
60	11-00574	Inverness	I 38:k:1,6	3	587
61	13-02698	Urbana	I 30:b:e,n,x	3	565
62	16-05172	London	I 3,10:e,v:1,6	3	480
63	14-05710	Johannesburg	I 40:b:e,n,x	3	443
64	16-05303	Chester		3	435
65	16-02928	Havana	I 13,23:f,g:-	3	395
66	16-01712	Bredeney	I 4:l,v:1,7	3	383
67	15-01962	-	I6,7:-:1,5	3	366
68	15-02251	Telelkebir	I 13,23:d:e,n,z15	3	361
69“	ATCC® 10723™*	Cerro	I 18:z4,z23:-	2	346
70	16-04988	Albany	I 8,20:z4:z24	3	344
71	16-02205	Agbeni	I 13,23:g,m:-	3	343
72	14-02295	Minnesota	I 21:b:e,n,x	3	337
73	14-01914	Worthington	I 13,23:z:e,w	3	336
74	16-05041	Rissen	I 6,7:f,g:-	3	312
75	16-02392	Oslo	I 6,7:a:e,n,x	3	306
76	11-06323	Baildon	I 9,46:a:e,n,x	3	278
77	16-02147	Cotham	I 28:i:1,5	3	253
78	15-03689	Ealing	I 35:g,m,s	3	237
79	418	Lomalinda	I 9, 12:a:e, n, x	3	232
80	15-01471	Cubana	I 13,23:z29	3	213
81	09-01912	Carrau	I 6,14,24:y:1,7	3	209
82	16-02464	Eastbourne	I 9:e,h:1,5	3	203
83	17-00172	Monschaui	I 35:m,t:-	3	201
84	15-01577	Alachua	I 35:z4,z23:-	3	193
85	16-03390	Corvallis	I 8,20:z4, z23	3	189
86	16-00455	Potsdam	I 6,7:e,v:e,n,z15	3	187
87	17-00107	Meleagridis	I 3,10:e,n:e,w	3	169
88	16-05286	Indiana		3	158
89	15-02982	Concord	I 6,7:l,v:1,2	3	157
90	03-08607	-	I 6,7:k:-	3	149
91“	ATCC® 10708™*	Cholerasius	I 6,7:C:1,5	1	148
92	16-03583	Altona	I 8,20:r:z6	3	145
93	11-07920	Pensacola	I 9:m,t:-	3	143

No.	Culture Collection Reference No.	Serotype	Serotype Antigenic Formula	Source of Supply	Number of Incidences
94	01-02501	Othmarschen	I 6,7:g,m:-	3	134
95	12-02378	-	I 4,[5],12:-:1,2	3	130
96	16-05338	Lovingstone	I 6,7:d:e,w	3	123
97	15-03273	Grumpensis	I 13,23:d:1,7	3	122
98	15-04797	Wandsworth	I 39:b:1,2	3	118
99	13-04865	Kintambo	I 13,23:m,t:-	3	114
100	13-05516	Edinburgh		3	113
101	16-04965	Kottbus	I 6,8:e,h:1,5	3	109
102	15-00740	Durban	I 9:a:e,n,z15	3	104
103“	NCTC 6017	Abony	I 4,12,27:b:e,n,x	1	60
104“	ATCC® 9842™*	Bispebjerg	I 4,12:a:enx	1	1
105“	ATCC® 15611™*	Vellore	I 1,4,12,27:z10:z35	1	-
106“	ATCC® 13036™*	Pullorum	I 9,12:-:-	1	-
107	ATCC® 12002™*	Tallahassee	I 6,8:z4,z32:-	1	67
108“	DSM 4883	Gallinarum	I 9:-:-	4	-
109“	DSM 13674	-	I 9,12:-:-	4	

List of *Salmonella enterica* ssp. *enterica* strains analyzed for antimicrobial susceptibility. Serotype antigenic formula is given in (Subspecies [space] O antigens [colon] Phase 1 H antigens [colon] Phase 2 H antigens) as provided by the supplier. Numbers in source of supply correspond to 1 - Microbiologics, Inc. (St. Cloud, USA), 2 – LGC Standards (Teddington, UK), 3 – Robert Koch Institute, national reference center for salmonellosis and other enteric pathogens (Wernigerode, Germany), 4 – Leibnitz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), 5 - National Collection of Type Cultures (Salisbury, UK). Strains marked with the symbol “ in the first column were used for antimicrobial susceptibility testing in triplicate experiments. The number of incidences (last column) refers to laboratory-confirmed human *Salmonella* infections (US) reported to CDC 2003-2012 published in National Enteric Disease Surveillance: *Salmonella* Annual Report, 2013 (CDC 2016).

A. Breadth of antibacterial activity *in vitro* of plant-expressed salmocins

Protein extracts of plant-expressed salmocins were evaluated for antimicrobial activity first. Results of this evaluation demonstrate that plant-produced salmocins are antibacterial against *S. enterica in vitro*.

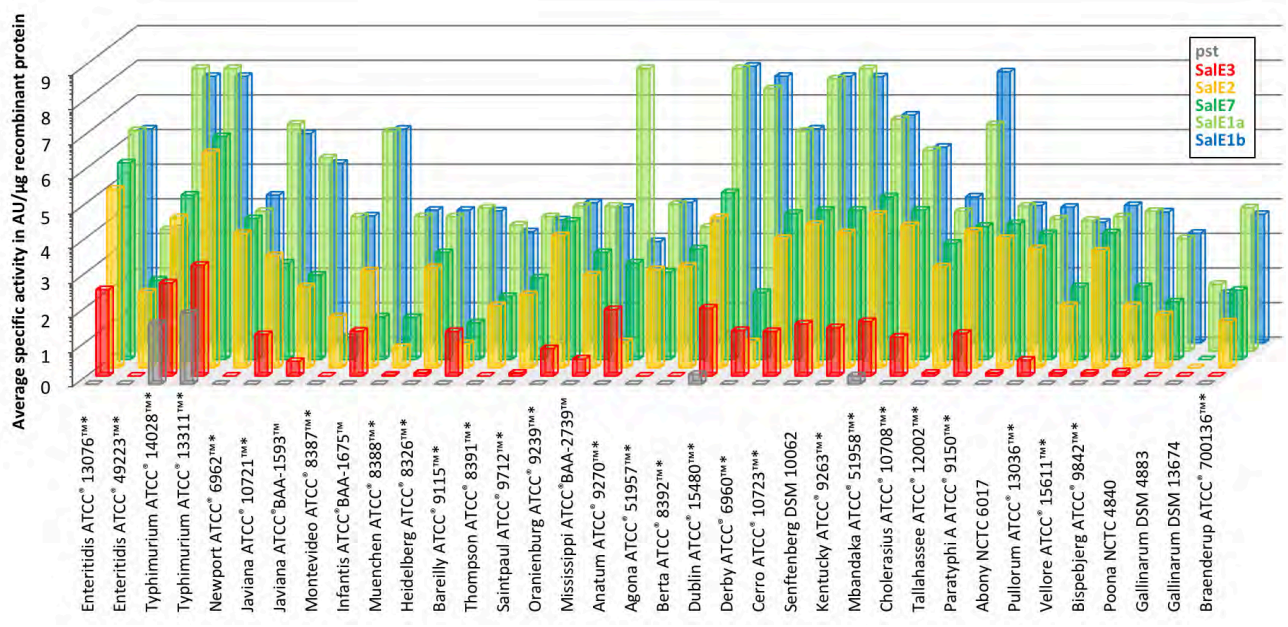
In order to estimate the breadth of the activity spectrum, all 109 non-Typhi strains obtainable by Notifier and listed in Table 2-3 were tested at least once, yielding positive activity for all salmocin-containing plant extracts (data not shown). Subsequently, 36 representative *S. enterica* spp. enterica food-borne illness-related strains were re-screened in triplicate experiments with salmocins to verify activity.

Figure 2-1 shows the average (from triplicate experiments) composite antibacterial activity of six salmocins evaluated against the indicated *S. enterica* serovars. Salmocin pst showed uniformly lower activity than the other salmocins and was not pursued further as a candidate.

The broadest spectrum of antimicrobial activity was identified for salmocins SalE1a and SalE1b, which showed positive antibacterial activity against 100% and 99% of all strains evaluated, respectively.

Significant breadth of activity was also observed for salmocins SalE7 (95%), SalE2 (94%) and SalE3 (70%) as reflected by their activity on the subset of 36 *S. enterica* spp. *enterica* strains represented in Figure 2-1. These data have also been published in Schneider et al. (2018).

Figure 2-1. Activity of salmocins against *S. enterica* ssp. *enterica* serovars



Semi-quantitative screen of antimicrobial activity of serial dilutions of salmocin-containing plant extracts determined by radial diffusion assay via spot-on-lawn-method. Thirty-six (36) diverse serovars of *S. enterica* spp. enterica known as the causal agents of food-borne illnesses were used in the screen. Activities of 6 candidate salmocins are shown as log differential inhibition ($\Delta\log_{10}$ CFU salmocin vs. control) against salmocin-free plant extract background (control). Results are averages of 3 independent experiments for all serovars screened, except for serotype Braenderup (average of two independent experiments).

Conclusion from breadth of activity studies

Although many of the 6 salmocin proteins evaluated for bactericidal activity against *Salmonella enterica* pathovars show control of multiple strains, SalE1a, SalE1b and SalE7 show particularly high potency against many of the pathovars relevant to food contamination. Consequently, although we present results for individual salmocins and salmocins mixtures in comparative studies *in vitro*, our antibacterial studies on food matrices focus on SalE1a, SalE1b and SalE7 as the most likely candidates for use singly or as blends in a SALMOCIN product.

B. Antibacterial efficacy *in vitro* of individual salmocins

Individual plant-made salmocins were evaluated for antimicrobial activity (purification methods are described in APPENDIX C). Results demonstrate that plant-produced salmocins are active against *S. enterica* spp. *enterica in vitro*. The results also demonstrated the possibility of using individual salmocins to control *S. enterica* in food.

Results show average activity and standard deviations from triplicate experiments (higher values = higher potency). Figure 2-2 summarizes results for salmocin SalE2. In this series, the strain numbers of *S. enterica* spp. *enterica* serovars are found in the first column of Table 2-3. Subsequent figures show efficacy of SalE3 (Figure 2-3), SalE7 (Figure 2-4), SalE1a (Figure 2-5) and SalE1b (Figure 2-6).

Figure 2-2. Activity of salmocin SalE2 against *S. enterica* ssp. *enterica* serovars

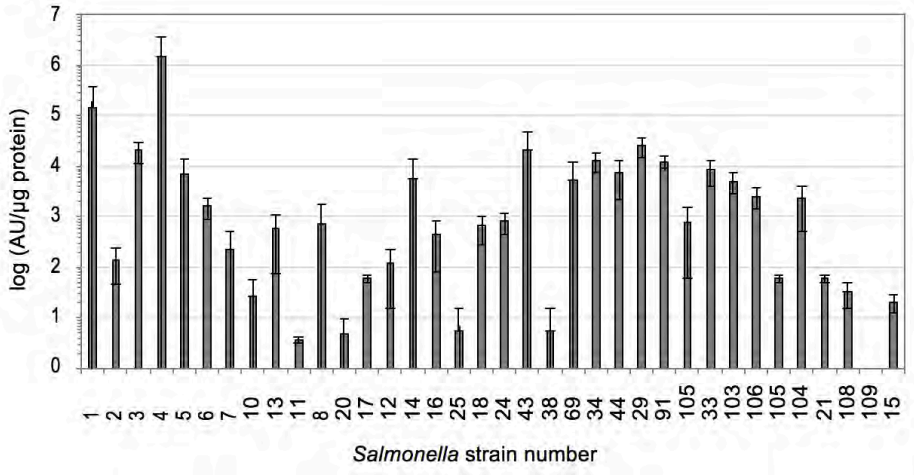


Figure 2-3. Activity of salmocin SalE3 against *S. enterica* ssp. *enterica* serovars

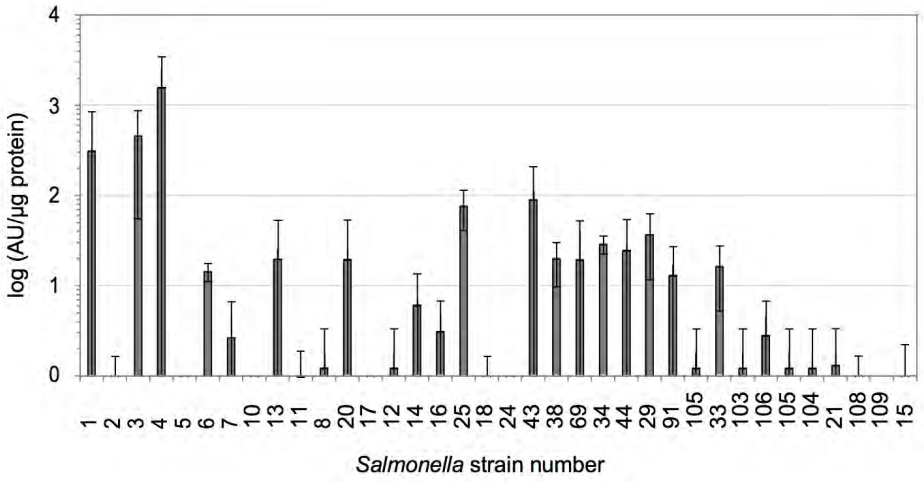


Figure 2-4. Activity of salmocin SalE7 against *S. enterica* ssp. *enterica* serovars

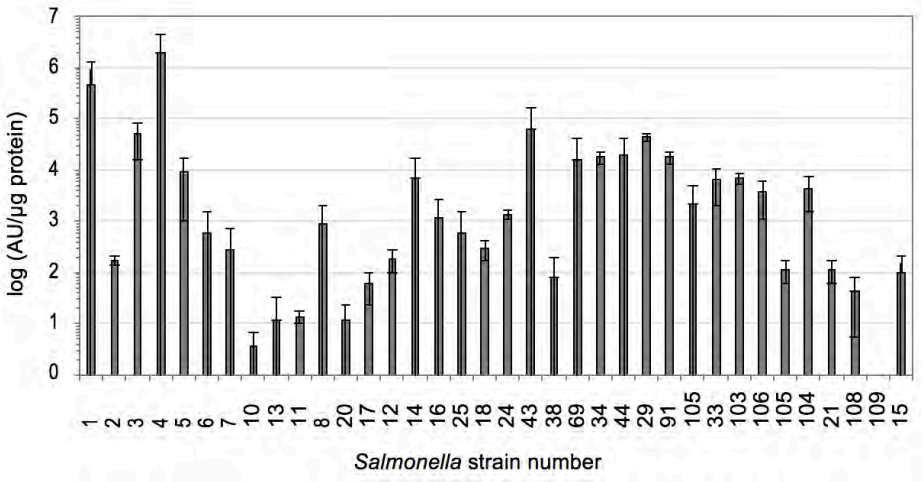


Figure 2-5. Activity of salmocin SalE1a against *S. enterica* ssp. *enterica* serovars

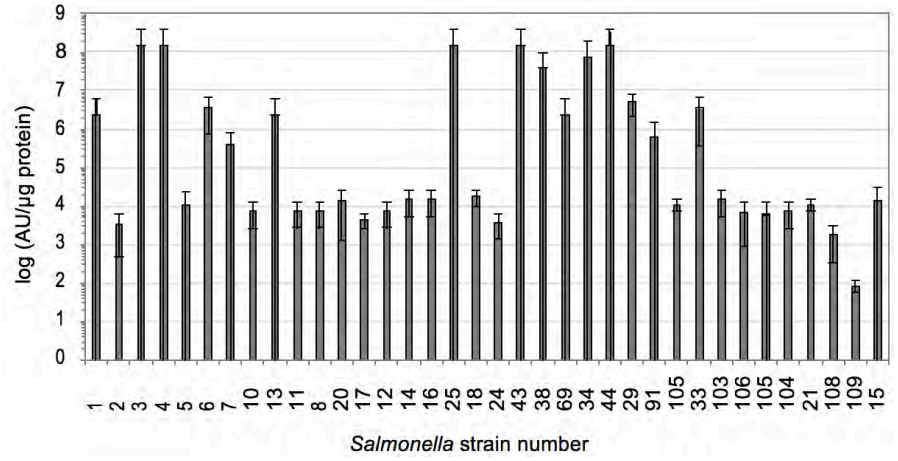
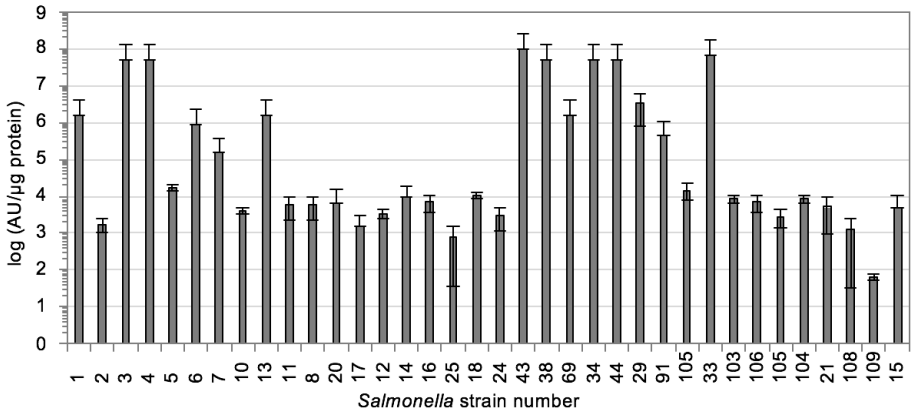


Figure 2-6. Activity of salmocin SalE1b against *S. enterica* ssp. *enterica* serovars



Conclusion from selectivity screens *in vitro* with individual salmocins

Although strain susceptibility to various salmocins is clearly visible, also evident is the fact that one or more salmocins can inhibit nearly all of the 109 strains (105 pathovars) of the species, including all 36 evaluated *S. enterica* pathovars positively identified as the causative agents of food-borne infections.

C. Potency of selected salmocins *in vitro* in comparison to kanamycin

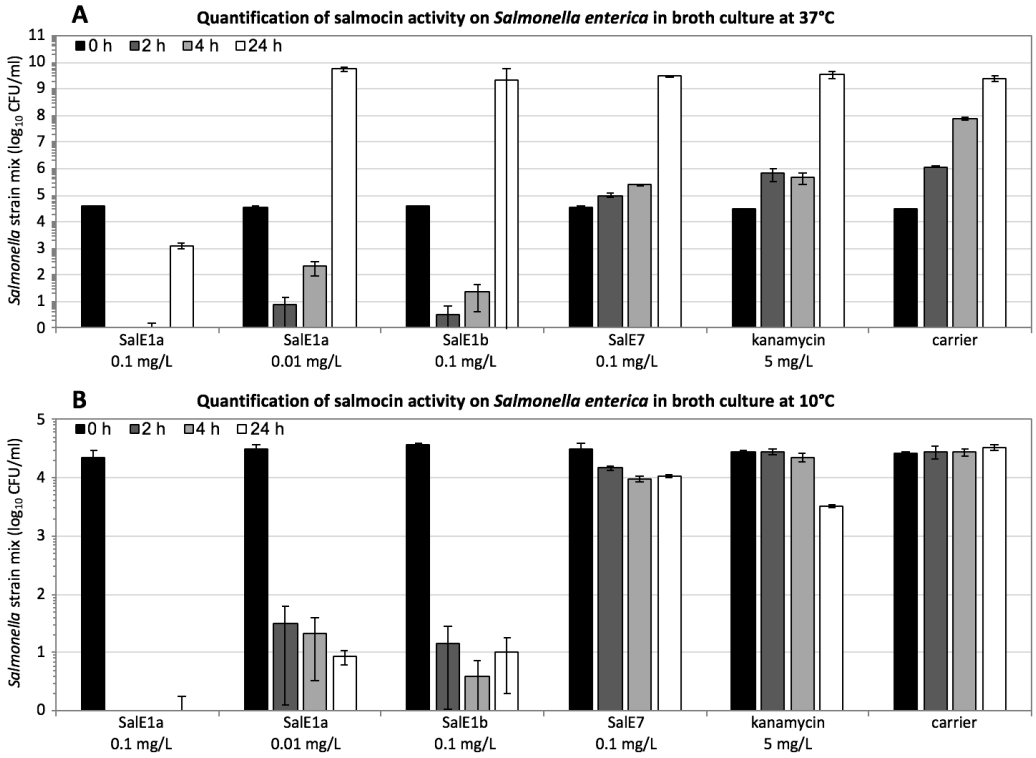
Using **kanamycin** (kanamycin A) as a positive control antibiotic, the relative bactericidal activity of various salmocins as a function of concentration (potency) were compared. Kanamycin is an aminoglycoside antibiotic that stops protein synthesis in Gram– and some Gram+ bacteria, leading to cell death. In this series of studies *in vitro*, protein powders of the most broadly active salmocins, namely, SalE1a, SalE1b and SalE7, were dissolved in water (0.1 mg/ml = 0.01% w/w). The concentration of salmocin proteins in their respective stock solutions was verified by the Bradford assay. Dilutions of salmocin solutions to specified protein concentrations were prepared using sterile 1 x phosphate-buffered saline (PBS), pH 7.4, as the diluent. The carrier or vehicle solution was 1 x PBS, pH 7.4, which also served as the negative control. Kanamycin antibiotic was dissolved and diluted to desired concentration also using 1 x PBS, pH7.4.

As target (indicator) organisms, *Salmonella. enterica* WT (i.e. not nalidixic acid-resistant) strains were used, specifically *S. enterica* ssp. *enterica*, serotype Enteritidis (strain ATCC®13076™) and *S. enterica* ssp. *enterica*, serotype Typhimurium (strain ATCC®14028™). The target pathogens were grown in LB medium to OD_{600}~0.3, then diluted to OD_{600}=0.1 prior to exposure. The bacterial cultures were mixed 1:1 v/v (2 *Salmonella* strains representing 2 different serotypes) and diluted to a final OD_{600}=0.0001 (representing ~$1.5x10^4$ CFU/ml) using LB medium pre-chilled to 4°C.

To quantify salmocin potency, 14-ml aliquots of bacterial cultures in 100 ml Erlenmeyer flask were supplemented with 1 ml salmocin solution, 1 ml carrier solution, or 1 ml antibiotic solution. The treated cultures were incubated at 37°C or 10°C and agitated in an orbital platform at 150 rpm for up to 24 h.

Aliquots of 550 µl each were taken from each flask at T=0 h (before addition of salmocin, carrier or antibiotic solutions) and T=2 h, T=4 h or T=24 h of co-incubation of bacterial cultures and test solutions for enumeration of viable bacteria. Serial 1:10 dilutions of culture aliquots with peptone water were plated on LB medium to allow growth of surviving bacteria. Plates were incubated at 37°C for 16-20 h to determine survivors as colony forming units (CFU). The results of these potency studies are shown in Figure 2-7. In the figure, Panel **A** (top) and Panel **B** (bottom) show results of exposures at 37°C and 10°C, respectively.

Figure 2-7. Potency *in vitro* of broad-spectrum salmocins SalE1a, SalE1b and SalE7 at 37°C and 10°C



Conclusions from potency studies *in vitro* with salmocins SalE1a, SalE1b and SalE7

These data indicate that the weakest broad-spectrum salmocin, SalE7, is approximately 50-times more potent than kanamycin on a concentration basis (w/v). The more potent SalE1a and SalE1b are highly active even at concentrations as low as 0.01 mg/L (10 micrograms/L or 10 parts per billion) against reference pathovars, which make them several thousand-times more potent than the antibiotic.

Note also that kanamycin does not block protein synthesis well unless the bacteria are actively growing, and likewise SalE7 (DNase) is more potent during active bacterial growth. In contrast, pore-forming salmocins SalE1a and SalE1b can act independent of growth and are therefore highly active at suboptimal growth temperatures (e.g. 10°C). These results provide further evidence of the value of using salmocin mixtures.

D. Antibacterial efficacy of salmocin mixtures on poultry meat, skinless matrix

Chicken breast fillet was purchased at a local supermarket (Halle, Germany) for use in studies modeling poultry matrices. Nalidixic acid resistant mutants of strains of *S. enterica* ssp. *enterica* serovars Enteritidis (strain ATCC®13076™*), Typhimurium (strain ATCC®14028™*), Newport (strain ATCC®6962™*), Javiana (strain ATCC®10721™*), Heidelberg (strain ATCC®8326™*), Infantis (strain ATCC®BAA-1675™*) and München (strain ATCC®8388™*) were individually grown in LB medium supplemented with 25 µg/ml nalidixic acid to stationary phase, diluted with fresh LB and grown to exponential phase.

For contamination of poultry, bacterial cultures were diluted with LB medium to OD_{600}=0.001 (~2 x 10^5 CFU/ml) and mixed 1:1:1:1:1:1:1. A pool of raw skinless chicken breast fillets cut into pieces of about 20 g weight was inoculated with 1 ml of a mixture of seven (7) *S. enterica* strains at ~2 x 10^5 CFU/ml density per 100 g of meat at room temperature, resulting in an initial contamination level of meat matrices of about 3 log CFU/g of the 7-serotype mixture of pathogenic *S. enterica*; attachment of bacteria to meat surfaces was allowed for 30 min at room temperature.

Contaminated chicken breast trim were treated by spraying (≤30 ml/kg) with either plant extract control (TSP extract of wild-type host plant material with no salmocins), or salmocin-containing solutions (either individual or mixtures of extracts of containing salmocins SalE1a, SalE1b, SalE2 or SalE7 prepared with the same buffer as the plant extract control) at application rates of 3 mg/kg SalE1a alone, or 3 mg/kg SalE1a, 1 mg/kg SalE1b, 1 mg/kg SalE2, 1 mg/kg SalE7 (high-dose treatment), or 0.3 mg/kg SalE1a, 0.1 mg/kg SalE1b, 0.1 mg/kg SalE2, and 0.1 mg/kg SalE7 (low-dose treatment).

Treated meat samples were further incubated at room temperature for 30 min. Aliquots of meat trim corresponding to ~40 g each were packed into BagFilter®400P sterile bags (Interscience) and stored for 1 h, 24 h or 72 h at 10°C. That temperature was selected because it represents realistic industrial meat processing conditions that are suboptimal yet permissive for bacterial growth.

In total, meat samples were incubated at room temperature for 1.5 h during salmocin treatment before they were sealed and stored at 10°C. For analysis of bacterial populations, poultry aliquots were homogenized with 4 vol. peptone water using Bag Mixer®400CC® homogenizer and colony forming units (CFU) of *S. enterica* were enumerated on XLD medium (Sifin Diagnostics) supplemented with 25 µg/ml nalidixic acid upon plating of serial dilutions of microbial suspensions. Samples were analyzed in quadruplicate. Statistical significance was determined by two-tailed unpaired parametric t-test.

The results of this model on-matrix exposure using skinless chicken breast trim are shown in Figure 2-8 and Figure 2-9. In this series, high doses of salmocins either alone or as mixtures were used to assess the antibacterial effects on a 7-serovar mix of *S. enterica* spp. *enterica* on contaminated skinless poultry meat. Figure 2-8 shows results of SalE1a used singly at an application rate equivalent of 3 mg salmocin/kg treated meat or applied as a mixture of SalE1a (3 mg/kg meat) + SalE1b (1 mg/kg) + SalE2 (1 mg/kg) + SalE7 (1 mg/kg). Inset shows chicken trim pieces used for suitability assessment.

Figure 2-9 shows results of a similar experiment when lower concentrations of the same salmocins were used at one-tenth (1/10^{th}) the application rates (right-most, darkest gray set of bars on figure).

Figure 2-8. Activity of high-dose SalE1a and salmocin mixtures against 7-serovar mix of *Salmonella enterica* on skinless poultry

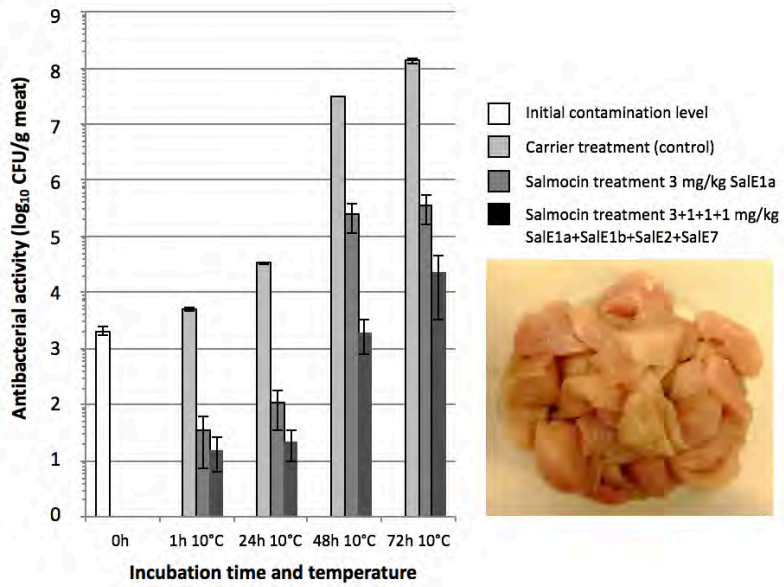
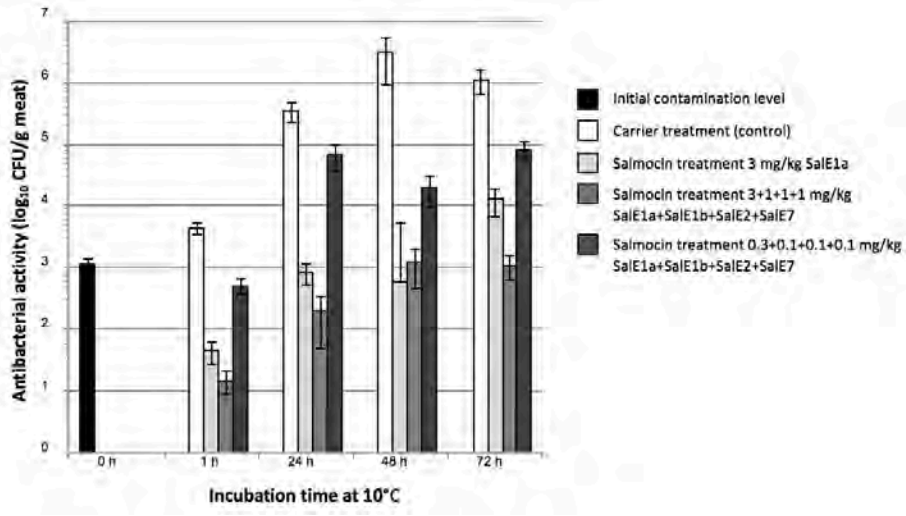


Figure 2-9. Activity of low-dose SalE1a and salmocin mixtures against 7-serovar mix of *Salmonella enterica* on skinless chicken matrix



For Figures 2-8 and Figure 2-9, contaminated skinless poultry meat samples were treated by spraying with solutions of a single broad-spectrum salmocin (SalE1a) or with mixtures of salmocins with complementary modes of action. Error bars indicate standard deviation of biological replicates, N=4. In Figure 2-8, statistically significant reductions in bacterial contamination were found by assessment of viable bacterial counts obtained from **high-dose** salmocin-treated (individual SalE1a at an application rate of 3 mg/kg meat (p-values for comparison at 1 h, 24 h, 48 h and 72 h post treatment were <0.0001, 0.0012, 0.0137 and 0.0037, respectively) or salmocin blend consisting of SalE1a+SalE1b+SalE2+SalE7 at an application rate of 3+1+1+1 mg/kg meat (p-values for comparison at 1 h, 24 h, 48 h and 72 h post treatment were <0.0001, 0.0012, 0.0136 and 0.0035, respectively). In Figure 2-9, statistically significant reductions in bacterial contamination were found by application of a **low-dose** salmocin blend consisting of SalE1a+SalE1b+SalE2+SalE7 at an application rate of 0.3+0.1+0.1+0.1 mg/kg meat (last column on right in series). Statistically significant differences were seen even at $1/0^{th}$ the original application rate of salmocin mixtures (p-values for comparison at 1 h, 24 h, 48 h and 72 h post treatment were 0.0002, 0.004, 0.0139 and 0.005, respectively). In both figures, statistical differences were assessed in relation to salmocin-free carrier-treated meat samples.

E. Antibacterial efficacy of salmocin mixtures on poultry meat, skin-on matrix

Chicken breast fillet was purchased from a local supermarket (Halle, Germany). Nalidixic acid resistant mutants of strains of *S. enterica* ssp. *enterica* serovars Enteritidis (strain ATCC®13076™*), Typhimurium (strain ATCC®14028™*), Newport (strain ATCC®6962™*), Javiana (strain ATCC®10721™*), Heidelberg (strain ATCC®8326™*), Infantis (strain ATCC®BAA-1675™*) and München (strain ATCC®8388™*) were individually grown in LB medium supplemented with 25 µg/ml nalidixic acid to stationary phase, diluted with fresh LB and grown to exponential phase.

For contamination of poultry, bacterial cultures were diluted with LB medium to OD_{600}=0.001 (1.5 x 10^5 CFU/ml) and mixed 1:1:1:1:1:1:1. A pool of raw skin-on chicken breast fillets cut into pieces of about 20 g weight was inoculated at 10 ml/kg of a mixture of the seven (7) *S. enterica* strains to an initial contamination level of ~1 x 10^3 CFU/ml density and held at room temperature for 30 minutes with hand massaging to allow bacterial attachment to the meat muscle and skin matrices. Procedures were replicated in quadruplicate (N=4).

The pieces of contaminated skin-on chicken breast were treated at a rate of 20 ml/kg with either solutions of purified salmocin protein powders dissolved in buffer (20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl), or buffer alone. In this series of experiments, salmocin SalE1b was used alone for antibacterial efficacy determination, at application rates of 5 mg/kg, 0.5 mg/kg, 1.0 mg/kg or 0.1 mg/kg to assess the effects of high-, intermediate- and low-dose application rates.

Salmocin-treated meat samples were further incubated at room temperature for 30 min with hand massaging at application and at 15 and 30 min post application. Aliquots of meat trim corresponding to ~40 g each were packed into BagFilter®400P sterile bags (Interscience) and stored for 1 h, 24 h or 72 h at 10°C. That temperature was selected because it represents realistic industrial meat processing conditions that are suboptimal yet permissive for bacterial growth.

In total, meat samples were incubated at room temperature for 1.5 h during salmocin treatment before they were sealed and stored at 10°C. For analysis of bacterial populations, skin-on poultry aliquots were homogenized with 4 vol. peptone water using Bag Mixer®400CC® homogenizer and colony forming units (CFU) of *S. enterica* were enumerated on XLD medium (Sifin Diagnostics) supplemented with 25 µg/ml nalidixic acid upon plating of serial dilutions of microbial suspensions. Samples were analyzed in quadruplicate. Statistical significance was determined by two-tailed unpaired parametric t-test.

The results of this model skin-on poultry exposure are shown in Figure 2-10 and Figure 2-11. Figure 2-10 shows the relative inhibition of bacterial growth after a single application of broad-spectrum SalE1b at rates of 0.1, 0.5 and 5.0 mg/kg when the meat is incubated at 10°C and sampled at 1 h, 24 h and 72 h post application. Relative to vehicle control, SalE1b inhibits mixed-serovar *Salmonella* growth from about 1 to nearly 4 $\log_{10}$ CFU/g (>99.99 mean percent reduction) depending on concentration and incubation time.

In a repeat series under the same conditions, one other intermediate application rate of SalE1b was included, namely, 1.0 mg/kg, in addition to the rates shown in Figure 2-10. The results of these studies with readouts from N=4 replicates each are shown in the two panels of Figure 2-11. This figure shows the relative inhibition of bacterial growth after a single application of broad-spectrum SalE1b at rates of 0.1, 0.5, 1.0 and 5.0 mg/kg when the meat is incubated at 10°C and sampled at 1 h, 24 h and 72 h post application. Relative to vehicle solution control, SalE1b inhibits mixed-serovar *Salmonella* growth from <1 to >2.3 $\log_{10}$ CFU/g (>99.54 mean percent reduction) depending on salmocin concentration and incubation time.

Figure 2-10. Activity of salmocin SalE1b at various application rates against 7-serovar mix of *Salmonella enterica* on skin-on chicken matrix

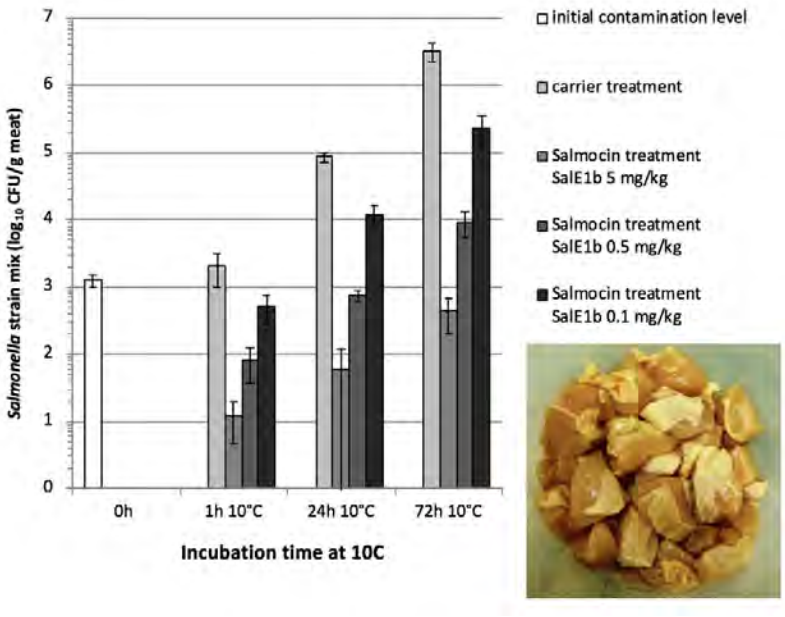


Figure 2-10. Inhibition of 7 mixed-serotype *Salmonella* strains treated with a single application of SalE1b at the rates shown and incubated for up to 72 h before sampling. Reductions in CFU/g meat relative to control vehicle solution were all statistically significant at all time points sampled (p<0.01 to <0.0001).

Figure 2-11. Confirmation of SalE1b activity at various application rates against 7-serovar mix of *Salmonella enterica* on skin-on chicken matrix

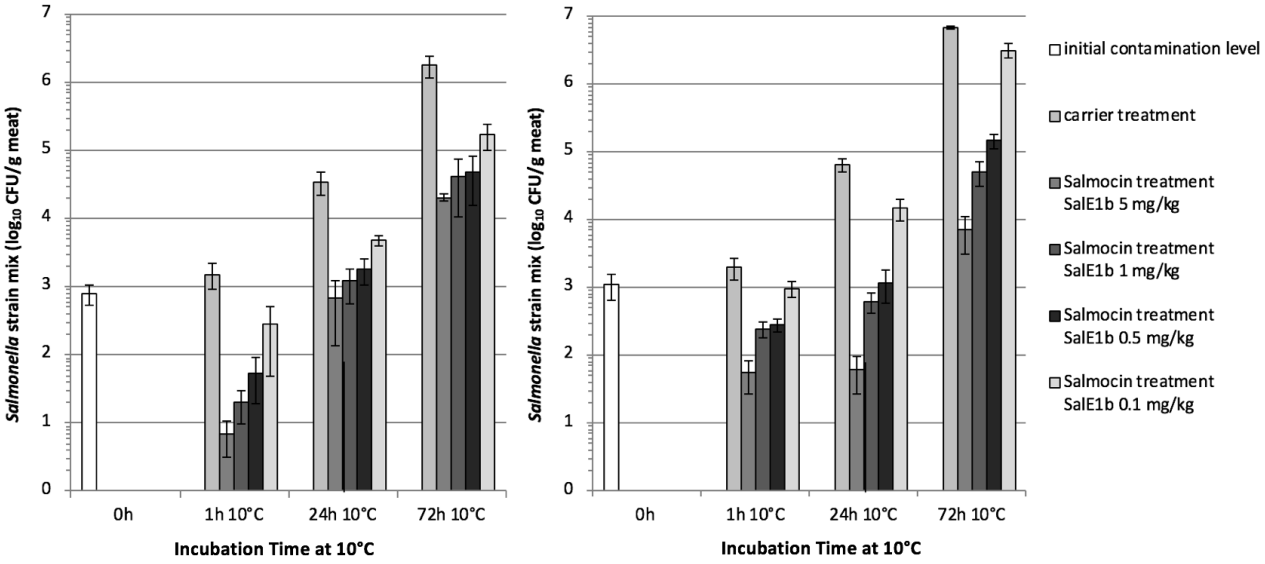


Figure 2-11. Inhibition of 7 mixed-serotype *Salmonella* strains treated with a single application of SalE1b at the rates shown and incubated for up to 72 h before sampling. Reductions in CFU relative to control vehicle solution were all statistically significant at all time points sampled (p<0.03 to <0.0001).

Conclusions from raw poultry skin-off and skin-on exposure studies

The conclusion that can be drawn from this series of studies is that broad-spectrum SalE1b or a mixed formulation of several salmocins can effectively reduce the contamination level of *Salmonella* (7-serovar mix) on poultry cuts whether the skin is left on or removed from the meat prior to contamination (i.e. skin-on and skin-off matrices).

The antibacterial effect is strong (>1 to >2.5 $\log_{10}$ CFU/g meat reductions relative to vehicle solution) for salmocin application rates of 1 and 5 mg/kg. The antibacterial effect decreases with longer incubation time due in part to degradation or inactivation of the salmocins on the meat matrix as well as unimpeded growth of surviving bacteria. Regardless, 99.95% to 99.99% mean CFU reductions in contamination can be achieved with a single application of salmocins.

F. Antibacterial efficacy of salmocin mixtures on raw beef matrix

Salmocins were also evaluated on beef cuts to assess their suitability in controlling *Salmonella* on red meat. In this series of studies, raw beef (round roast) obtained from a local supermarket (Halle, Germany) was cut into 20-gram pieces and the pieces were inoculated with nalidixic acid resistant mutants of two sample serovars of *S. enterica*, namely, *S. enterica* ssp. *enterica*, serotype Enteritidis (ATCC®13076™*nalR#6) and *S. enterica* ssp. *enterica*, serotype Typhimurium (ATCC®14028™*nalR#3).

Following procedures described in APPENDIX D, the meat matrices were inoculated with 10 mL/kg of the mixed serotype bacterial solution (at OD_{600}=0.001 =~$1.5x10^5$ CFU/ml or at OD_{600}=0.005 = ~$7.5x10^5$ CFU/ml) in LB medium. The inoculated cuts were hand mixed to assure uniformity and incubated at RT for 30 minutes to enable bacterial attachment to the matrix, with hand-mixing at 15 and 30 min post inoculation.

Purified powder of broad-spectrum salmocin SalE1b was dissolved in buffer consisting of 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, and 100 mM NaCl. The carrier/vehicle solution consisted of the same buffer minus salmocin. SalE1b was evaluated at two application rates, namely 5 mg/kg and 0.5 mg/kg meat. The contaminated meat samples were treated with SalE1b or control solution applied at a rate of 20 ml/kg, followed by hand mixing for uniformity and further incubation for 30 min at RT, with hand mixing at 15 and 30 min post treatment. The total incubation time during these procedures was 2 h.

The samples were placed in sterile bags, incubated at 10°C and aliquots were sampled at 1 h and 48 h post treatment to determine bacterial viability. Samples were mixed with 4 vol peptone water and homogenized in a bag mixer. Serial dilutions of recovered bacterial solutions were plated on XLD Agar supplemented with 25 µg/ml nalidixic acid and plated. The plates were incubated at 37°C for 16-20h, after which the CFU were enumerated.

Results of SalE1b activity on *Salmonella*-contaminated beef cuts are summarized in Figure 2-12. Highly potent bactericidal activity was found even at the lower application rate of 0.5 mg/kg (Panels **B** and **C**).

Conclusions from raw beef exposure studies

These results indicate the suitability of broad-spectrum salmocin SalE1b in controlling *S. enterica* serotypes Enteritidis and Typhimurium on contaminated raw beef. Nearly sterilizing effects were achieved using a single salmocin even at the lowest application rate of 0.5 mg/kg (0.5 ppm).

Figure 2-12. Activity of SalE1b against 2-serovar mix of *Salmonella enterica* on raw beef matrix

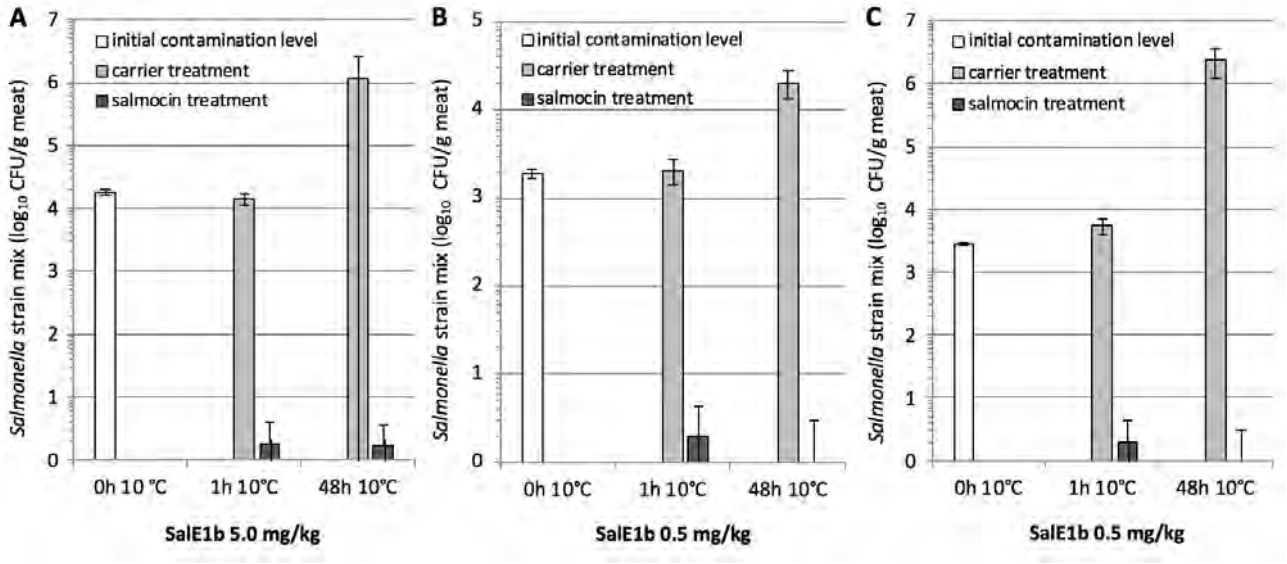


Figure 2-12. Inhibition of 2-serotype mix of *Salmonella enterica* (Enteritidis and Typhimurium) applied to raw beef cuts and treated with a single application of broad-spectrum salmocin SalE1b. Samples were incubated for up to 48 h at 10°C and sampled for enumeration. Panels A, B and C above show average values from 3 independent studies each conducted in quadruplicate (N=4). Panel **A** (left) shows results with a high bacterial inoculum of 1.81 x 10^4 CFU/g beef (initial contamination level) and SalE1b applied at a rate of 5 mg/kg (20 mL/kg). Panel **B** (center) shows results (N=4) with a moderate bacterial inoculum of 1.93 x 10^3 CFU/g and SalE1b applied at a rate of 0.5 mg/kg (20 mL/kg). Panel **C** (right) shows results (N=4) with moderate bacterial inoculum of 2.85 x 10^3 CFU/g and SalE1b applied at a rate of 0.5 mg/kg (20 mL/kg). Dramatic reductions in bacterial CFU/g of contaminated beef were obtained, all of which were statistically significant relative to vehicle control treatment. Mean CFU reductions of 99.96% to 100% (3.0 to 6.4 $\Delta\log_{10}$ CFU/g) were obtained. Statistical values for the high-dose SalE1b study (Panel A) ranged from a low of p=0.1575 due to high variability at 48 h, to p<0.0001 at 1 h. For the lower application rates of SalE1b (Panels B and C), p values ranged from 0.0075 to 0.0009.

G. Antibacterial efficacy of salmocins on sample seafood (raw tuna) matrix

The antibacterial efficacy of salmocins was also determined on a sample seafood matrix consisting of raw tuna fillet. For these studies, raw frozen tuna fillets obtained from a local supermarket (Halle, Germany) were thawed for overnight at 4°C and trimmed to ~20 g pieces.

The *Salmonella enterica* strains used (*S. enterica* ssp. *enterica*, serotype Enteritidis (ATCC®13076™*nalR#6) and *S. enterica* ssp. *enterica*, serotype Typhimurium (ATCC®14028™*nalR#3)) were nalidixic acid resistant mutants grown in LB medium supplemented with 25 µg/ml nalidixic acid to OD_{600}=~0.3, for a targeted contamination level of between ~1x10^3-1x10^4 CFU/g food product. For meat contamination, a 1:1 mixture of the two serotypes in LB medium (at OD_{600}=0.001 =~1.5x10^5 CFU/ml or at OD_{600}=0.005 = ~7.5x10^5 CFU/ml) were applied at 10 ml of bacterial suspension per kg tuna fillet. The bacterial inoculum was added to meat cubes and equally distributed on meat pieces by hand-mixing. The contaminated meat was incubated for 30 min at RT for attachment of bacteria and hand-massaged after 15 and 30 min of incubation.

The salmocin solutions were made by dissolving purified SalE1b protein powder in 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl. The carrier or vehicle control solution was the same buffer minus the salmocin protein. The salmocin solutions consisted of SalE1b applied at 5.0 mg/kg or 0.5 mg/kg tuna. Salmocin solutions or carrier solution were applied at a rate of 20 ml/kg on contaminated meat cubes and

equally distributed by hand-massage. The treated meat was incubated for 30 min at RT and hand-massaged at 15 and 30 min post treatment. Aliquots of treated tuna approximately 40 g each were packed into sterile bags for storage. The total elapsed time post treatments at RT was 2 h. The bags were stored at 10°C and aliquots taken at various times to determine bacterial viability. The tuna samples were supplemented with 4 vol peptone water and homogenized in Bag Mixer. Serial dilutions of recovered bacterial solution were plated on XLD Agar supplemented with 25 µg/ml nalidixic acid. The plates were incubated at 37°C for 16-20h to allow growth, and the CFU were subsequently enumerated.

The results of these studies are summarized in Figure 2-13. In Panel **A** (left) in the figure, the initial contamination level of mixed-serovar *S. enterica* was 1.44 x 10^4 CFU/g of raw tuna meat. The application rate of SalE1b solution was 5 mg/kg (20 mL/kg spray). Based on the average of 4 replicate samples (N=4), at 1 h of incubation at 10°C salmocin treatment reduced viable bacteria by 2.5 $\log_{10}$ CFU/g (99.66% mean CFU reduction) relative to vehicle control (statistically significant with p<0.0001). At 48 h incubation, the differential was 2.1 $\log_{10}$ CFU/g (99.21% mean CFU reduction) relative to control (statistically significant with p=0.0381). In Figure 2-12 Panel **B** (right) the initial bacterial contamination level was 1.6 x 10^3 CFU/g meat. This panel shows results of studies using a lower dose of salmocin SalE1b applied at 0.5 mg/kg of raw tuna meat (20 mL/kg). Based on averages of four replicates (N=4), the differential reduction of bacterial viability was 1.7 $\log_{10}$ CFU/g (98.06% mean CFU reduction) relative to vehicle control at 1 h of incubation (statistically significant at p<0.0001); at 48 h, the differential reduction was 3.0 $\log_{10}$ CFU/g (99.90% mean CFU reduction) relative to vehicle control (statistically significant at p=0.0189).

Figure 2-13. Activity of SalE1b against 2-serovar mix of *Salmonella enterica* on raw tuna matrix

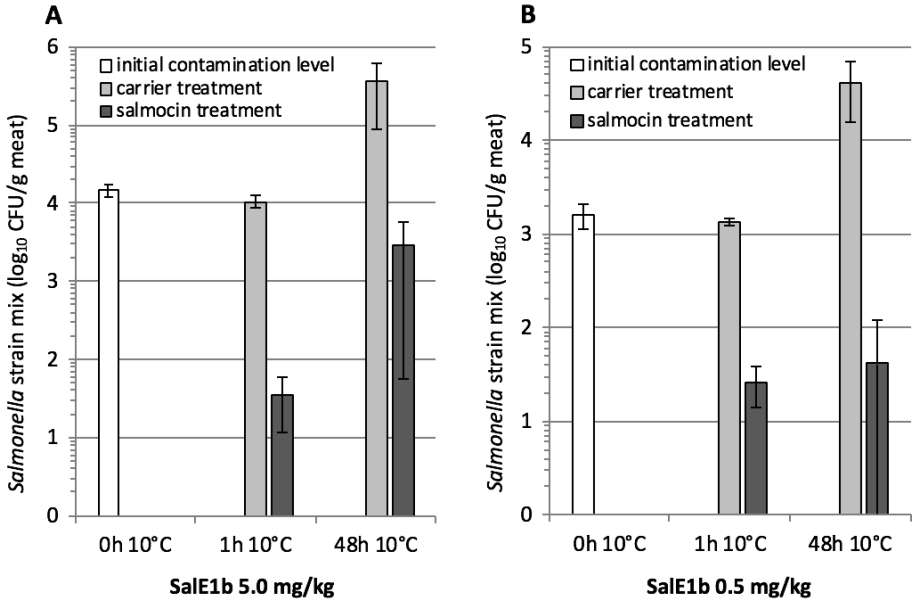


Figure 2-13. Antibacterial activity of broad-spectrum salmocin SalE1b against mixed serovars of *Salmonella enterica* on contaminated raw tuna meat. Solutions of SalE1b were applied (20 mL/kg) to a final rate of 5 mg/kg food (Panel **A**, left) or 0.5 mg/kg food (Panel **B**, right). Initial bacterial contamination levels were 1.44 x 10^4 CFU/g for experiments in Panel A and 1.6 x 10^3 CFU/g for experiments in Panel B. Details of experimental conditions and statistical significance are provided in text preceding the figure.

Clearly, broad-spectrum salmocin SalE1b can reduce *Salmonella* viable counts by 1.7 to 3 $\log_{10}$ CFU/g on raw tuna meat when applied a single time at rates ranging from 0.5 to 5 mg/kg food (0.5 to 5 ppm) with incubation of 1-48 h at 10°C. Results obtained with the two major and relevant *S. enterica* serovars

evaluated in these studies are exemplary rather than comprehensive and data are provided to support the breadth of activity of salmocins on various food matrices.

Conclusion from raw tuna exposure studies

These studies confirm that broad-spectrum salmocin SalE1b can control *Salmonella* contamination in raw tuna fillet at application rates of 0.5 to 5.0 mg/kg (0.5 to 5.0 ppm) for a minimum of 48 h at a suboptimal but permissive bacterial growth temperature of 10°C, modeling industrial food processing environments with low-level temperature abuse. Results of these studies also confirm that salmocins have utility beyond avian (poultry) and mammalian (beef) meat products and can also be used to control *Salmonella* in seafood, as exemplified in studies with raw tuna meat.

H. Antibacterial efficacy of salmocins in raw whole eggs

In addition to assessing salmocins' antibacterial activity against *Salmonella* in solid meat matrices (i.e. poultry with and without skin, beef, tuna) as described in the preceding sections, studies were conducted to assess the utility of salmocins in raw eggs.

In this series of studies, raw egg contents (eggwhite and eggyolk) were homogenized at RT using a laboratory blender for 2 min at 1000 rpm. *Salmonella enterica* strains used were nalidixic acid resistant mutants grown in LB medium supplemented with 25 µg/ml nalidixic acid to OD_{600}~0.3. Tester strains were *S. enterica* ssp. *enterica*, serotype Enteritidis (ATCC®13076™*nalR#6) and *S. enterica* ssp. *enterica*, serotype Typhimurium (ATCC®14028™*nalR#3). The target initial contamination level was set at ~$1x10^3$ to $1x10^4$ CFU/g food (intended).

The test food matrix (homogenized raw egg mixture) was contaminated by addition of a 1:1 mixture of the two *Salmonella* strains representing 2 different serotypes (at OD_{600}=0.001 =~$1.5x10^5$ CFU/ml or at OD_{600}=0.005 = ~$7.5x10^5$ CFU/ml) in LB medium, at an application rate of 10 ml of bacterial suspension per kg food. After addition of the bacterial inoculum, the mixture was homogenized gently using an immersion stirrer at 620 rpm for 1 min. The contaminated whole egg mixture was incubated for 30 min at RT and mixed again gently after 15 and 30 min post inoculation using a stirrer at 750 rpm for 1 min.

Salmocin solutions were made by dissolving purified powder of the broad-spectrum salmocin protein SalE1b in buffer consisting of 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, and 100 mM NaCl. The control or vehicle solution consisted of the same buffer minus the salmocin protein. The application rate for salmocin was 5.0 and 0.5 mg/kg food, added at a rate of 20 ml/kg to contaminated whole egg and mixed using a stirrer at 750 rpm for 1 min. The treated whole egg mixture was incubated for 30 min at RT and mixed again after 15 min and 30 min using a stirrer at 750 rpm for 1 min.

Contaminated aliquots (40 ml each) of the contaminated egg mixture were filled into bags and stored at 10°C to simulate suboptimal but growth-permissive temperature abuse conditions. The total incubation time of the raw egg mixture at RT during salmocin treatment was 2 h.

Aliquots of the egg mixture were collected and supplemented with 4 vol peptone water and homogenized in a Bag Mixer. Serial dilutions of recovered bacterial suspensions were plated on XLD Agar supplemented with 25 µg/ml nalidixic acid and plated. Plates were incubated at 37°C for 16-20h with subsequent enumeration of viable bacteria.

Results of three independent studies of salmocins' antibacterial activity in a raw whole egg substrate are summarized in Figure 2-14. Each study in the series consisted of N=4 replicates; the graphs show average CFU/g of egg mixture in each series.

Figure 2-14. Activity of SalE1b against 2-serovar mix of *Salmonella enterica* in raw whole eggs

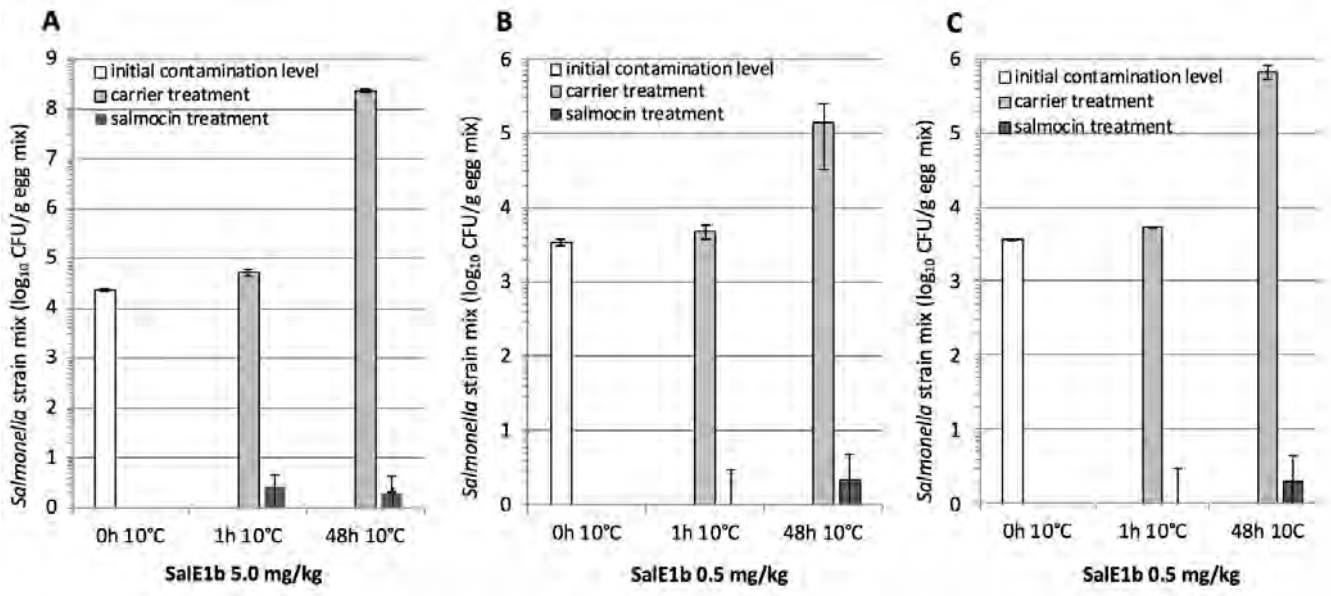


Figure 2-14. Panel **A** (left) shows antibacterial results for SalE1b applied at high dose of 5 mg/kg and Panels **B** and **C** (middle, right) show results of SalE1b applied at lower dose of 0.5 mg/kg to *Salmonella*-contaminated raw whole egg mixture.

As can be seen in the results of three (3) independent studies derived from averages of N=4 replicates each, broad-spectrum salmocin SalE1b can exhibit nearly sterilizing effects on the mixed-serovar *Salmonella*-contaminated whole egg (broken egg) mix.

In Figure 2-14 Panel **A**, the initial contamination level was 2.43 x 10^4 CFU/g mix and a high level of SalE1b was used, namely 5 mg/kg. In the series summarized in Panel **A**, the 1 h sampling point showed a differential of 4.3 $\log_{10}$ CFU/g egg mix relative to control (99.99% mean reduction) and the 48 h time point showed a reduction of 8.1 $\log_{10}$ CFU/g (100% reduction). Statistically, these values were significant at $p<0.0001$ for both sample times.

In Panel **B** and **C**, initial contamination levels were 3.36 x 10^3 and 3.59 x 10^3 CFU/g mix, respectively. In Panel **B**, when the egg mixture was treated with a lower dose of salmocin (0.5 ppm) the reduction in viable bacteria at 1 h was 3.7 $\log_{10}$ CFU/g, (99.98% reduction) with a 48-h reduction of 4.8 $\log_{10}$ CFU/g (100% reduction), relative to control treatment. Statistically, these values were significant at $p<0.0001$ at 1 h and $p=0.0401$ at 48 h. Similarly, for low-dose (0.5 ppm) experimental series shown in Panel **C**, the 1-h and 48-h CFU reduction values relative to control treatment were 3.7 $\log_{10}$ CFU/g (99.98% reduction) and 5.5 $\log_{10}$ CFU/g (100% reduction), respectively. Both sets of values significantly differed from controls at $p<0.0001$.

Concluson from raw whole egg product exposure studies

These results show that salmocin SalE1b can effectively control representative *Salmonella enterica* serovars Enteritidis and Typhimurium in raw whole (broken) egg product with nearly sterilizing effects even at the lower application rate of 0.5 ppm.

Summary of results of bactericidal efficacy and suitability of individual salmocins and salmocin mixtures on *S. enterica* spp. *enterica* serovars

The results of multiple studies summarized in the prior sections show that salmocins can control a very wide range of *Salmonella* serotypes that are responsible for world-wide foodborne outbreaks. We have also shown that salmocins can be used to control *Salmonella* contamination on a wide range of food products representing diverse physicochemical matrices, including raw poultry without and with skin, red meat, seafood such as tuna, and non-solid foods such as raw whole egg.

Efficacy and spectrum of activity *in vitro*

The results presented herein illustrate that plant-produced salmocins show bactericidal activity against food contamination-relevant serovars of *S. enterica*. Efficacy is demonstrated for salmocins evaluated singly and as mixtures, both *in vitro*, in susceptible model meat matrices, and in raw whole egg, under relevant food processing and storage conditions.

All plant-expressed salmocins were recovered in good yield and were active against some or most of the *S. enterica* serovars evaluated in an initial screen (Figure 2-1). The range of activities of individual salmocins was subsequently re-evaluated against 36 *S. enterica* spp. *enterica* pathovars that were verified to have caused human disease per CDC databases.

The broadest antimicrobial activity spectrum was identified for salmocins SalE1a and SalE1b, which showed positive antibacterial activity against 100% and 99% of all strains evaluated, respectively.

Significant breadth of activity was also observed for salmocins SalE7 (positive activity against 95% of serotypes), SalE2 (94%) and SalE3 (70%) as reflected by their activity on the subset of 36 strains (shown in Figure 2-2 through Figure 2-6). These findings support the use of salmocins as formulated mixtures to enhance the breadth of coverage as well as the potency of the product. Also, using mixtures of complementary acting salmocins allows for reduction in the amount of total salmocin protein applied to food.

The five salmocins analyzed were divided into four groups based on their ability to control major pathogenic *Salmonella* strains. Salmocins SalE1a and SalE1b were universally active, each being able to kill all tested pathovars and showing the highest average activity of higher than 10^5 AU/µg recombinant protein on all tested strains, and in most cases higher than 10^3 AU/µg protein against individual strains. The remaining salmocins fell into two groups, with salmocins SalE2 and SalE7 in one group having a 100-fold lower average activity (<10^5 AU/µg protein) and SalE3 in another group showing substantially lower average activity (10^2 AU/µg protein).

Our finding that two salmocins, SalE1a and SalE1b, each possessed broad antimicrobial activity against all 105 major pathogenic *Salmonella* serovars tested as well as remarkably high potency (average >10^6 AU/µg protein), was unexpected, and suggests that the SALMOCIN product can leverage its range of bactericidal activity as well as its potency by building on one or both of these salmocins as its main active ingredients.

Efficacy and suitability on contaminated meat matrices

On raw **skinless poultry meat** (chicken trim) matrices, individual salmocins and salmocin mixtures also proved effective in reducing the level of contamination of the meat with a 7-serovar mixture of *S. enterica* spp. *enterica*. The broad-spectrum salmocin SalE1a used singly at a higher application rate of 3 mg/kg meat

(3 ppm) reduced meat contamination by approximately 2 to 2.5 log CFU/g meat during incubations of 1 h to 72 h at 10°C, relative to carrier control (Figure 2-8 and Figure 2-9). Salmocin blends applied to contaminated meat by spraying at a lower rate of 0.3 mg/kg SalE1a plus 0.1 mg/kg each of SalE1b, SalE2 and SalE7 (0.6 ppm total salmocin) reduced meat contamination by 1-2 log CFU/g meat when incubated for 1-72 h at 10°C, relative to carrier control.

Using a spray application rate of 3 mg/kg meat for SalE1a plus 1 mg/kg each for SalE1b, SalE2 and SalE7 (6 ppm total salmocin) reduced on-matrix contamination by 3 to 3.5 log CFU/g meat, relative to control, when incubated for 1-72 h at 10°C (Figure 2-9). These differences were statistically significant and were derived from triplicate experiments analyzing quadruplicate samples in each study (p values discussed in caption following Figure 2-9).

Similarly, SalE1b was evaluated on contaminated **raw skin-on chicken cuts** under similar conditions. As shown in Figure 2-10 and Figure 2-11, the antibacterial effect was >1 to >2.5 $\log_{10}$ CFU/g meat reductions relative to vehicle solution for salmocin application rates of 1 and 5 mg/kg. The antibacterial effect decreased with longer incubation time due in part to degradation or inactivation of the salmocins on the meat matrix as well as unimpeded growth of surviving bacteria. Regardless, 99.95% to 99.99% mean CFU reductions in contamination can be achieved with a single application of salmocins to poultry cuts with retained skin.

Collectively, these studies showed that broad-spectrum SalE1b or a mixed formulation of several salmocins can effectively reduce the contamination level of *Salmonella* (7-serovar mix) on poultry cuts whether the skin is left on or removed from the meat prior to contamination (i.e. skin-on and skin-off matrices).

Contaminated raw **beef cuts** treated with salmocins or control solutions yielded similar results. Studies with raw beef contaminated with *S. enterica ssp. enterica* serovars Enteritidis and Typhimurium confirmed the suitability of broad-spectrum salmocin SalE1b in controlling these strains (Figure 2-12). Nearly sterilizing effects were achieved using a single salmocin even at the lowest application rate of 0.5 mg/kg (0.5 ppm).

Contamination of a model seafood, namely raw **tuna steak meat**, followed by salmocin treatment showed that the antibacterial could effectively control *S. enterica*. As shown in Figure 2-13, broad-spectrum salmocin SalE1b controlled *Salmonella* contamination in raw tuna fillet by achieving up to a 3-log CFU reduction in viable bacteria relative to control at application rates of 0.5 to 5.0 mg/kg (0.5 to 5.0 ppm) for a minimum of 48 h at a suboptimal but permissive bacterial growth temperature of 10°C. Results of these studies also confirm that salmocins have utility beyond avian (poultry) and mammalian (beef) meat products and can also be used to control *Salmonella* in seafood.

Lastly, contamination of **raw whole egg** followed by salmocin treatment showed that the antibacterial could effectively control *S. enterica*. As shown in Figure 2-14, salmocin SalE1b effectively controlled representative *Salmonella enterica* serovars Enteritidis and Typhimurium in raw whole egg product with nearly sterilizing effects even at the lower application rate of 0.5 mg/kg (0.5 ppm).

Suitability conclusion

Taken together, these results support our conclusion that salmocins can effectively reduce the viability of pathogenic strains of *Salmonella* and especially *S. enterica* spp. *enterica* pathovars in multiple foods, at concentrations (application rates) and application modalities (spray equipment; additive solution) that are commercially viable, and at processing times and temperatures that are relevant in food contamination, food processing and intervention scenarios.

Projected application rate of salmocins to food products

The results of these *in vitro* and on-matrix studies with foods that are susceptible to contamination with *S. enterica* pathovars, have shown that SALMOCIN product formulations consisting of single plant-made salmocins or blends thereof are effective food antimicrobials at application rates ranging from 0.01 to 6 mg total salmocin per kg treated food. However, based on the higher potency of salmocins relative to other antimicrobials including colicins, and their antibacterial activity on and in a wide range of foods having different physicochemical properties, textures and surfaces, we anticipate that a preferred application rate for individual salmocins and salmocin mixtures will be **≤3 mg SALMOCIN/kg treated food** (i.e. ≤3 ppm).

2.4.2 Duration of SALMOCIN's technical effect

The duration of SALMOCIN's technical (bactericidal) effect after application to meat cuts was derived from the studies described in Section 2.4.1.

Definition of technical effect and duration of technical effect

In this Notice, the **technical effect** is defined as the bactericidal effect of SALMOCIN and is quantified by determining the population of target pathogens remaining viable in an experimentally contaminated food matrix (CFU/g) after exposure to either SALMOCIN or a control (vehicle) solution. The **duration of technical effect** is the time after SALMOCIN treatment at which bacteria that have survived the effects of SALMOCIN are quantified to begin normal growth relative to the control treatment.

Summary of methods

Methods used to assess bactericidal effects and duration of technical effect on-matrix are summarized in APPENDIX C, Methods, Section C.7 and more extensively discussed in APPENDIX D. Briefly, samples of raw chicken breast meat were contaminated with measured concentrations of a mixture of 7-pathogenic serovars of *S. enterica* spp. enterica, incubated at RT to allow bacteria to adhere to the matrix, exposed to salmocins or a non-salmocin containing control vehicle by spraying at a defined rate of application, and incubated at 10°C to simulate temperature abuse. After treatment and storage, the suspensions/meat aliquots were sampled at 1 h, 24 h, 48 h and 72 h for enumeration. Viability of bacteria over time was determined as a function of treatment condition.

Results

The well-replicated results in Figure 2-8 and Figure 2-9 show that although multi-log reductions in CFU/g of contaminated meat are achieved when spraying salmocin solutions at application rates ranging from 0.6 to 6 ppm total salmocin, remaining viable bacteria post-treatment and incubation are able to grow. Hence, the bactericidal effect of salmocins and salmocin mixtures is transient and does not last much longer than 72 h of incubation at 10°C, as can be seen by the decreasing progression of growth control over time in a concentration-related manner (Figure 2-8). In fact, in these experiments there was no improvement in bacterial control beyond 48 h of incubation.

Conclusion

The duration of technical effect of single salmocins or mixtures of salmocins against enteropathogenic serovars of *S. enterica* ranges from 1 to 72 hours, with the strongest antibacterial effects observed from 1h to 48 h post application.

2.4.3 Digestibility: Susceptibility of salmocins to gastrointestinal degradation

An important factor influencing high potency/short duration kinetics of salmocins is their natural susceptibility to proteolytic digestion. In GRN 593, we described COLICIN as a food processing aid for controlling *E. coli* STEC strains. We demonstrated empirically the susceptibility of colicin proteins to gastroduodenal digestive enzymes by subjecting various colicins to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestion *in vitro*, followed by molecular size analysis. None of the proteins survived for more than a few minutes when exposed to conditions modeling the gastrointestinal tract.

Salmocins and colicins are bacteriocins that share a high degree of similarity, including high amino acid sequence homology. Repeating the studies with colicins described in GRN 593 with salmocins in the present Notice revealed similar results.

As before, we subjected the plant-made antimicrobial proteins to a two-stage digestion process to mimic ingestion of active salmocins. Gastric (phase I) and duodenal (phase II) digestions *in vitro* were performed with plant-produced lyophilized model salmocins SalE7, SalE1a or SalE1b dissolved in purified water by the method first described for colicins (Schulz 2015) and more recently described for salmocins (Schneider 2018).

Figure 2-15 summarizes the results of salmocin digestibility studies. The results shown in Figure 2-15 Panel **A** and Panel **B** were generated by incubating individual salmocins in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) containing pepsin or trypsin and chymotrypsin at physiological concentrations, respectively.

Incubation with pepsin in SGF for up to 60 min was followed by incubation with trypsin and chymotrypsin in SIF for up to 3 h. Aliquots of the reactions were evaluated for antimicrobial activity and protein degradation pattern by SDS-PAGE and Coomassie-staining upon different intervals of incubation.

It is clear from the results of digestion experiments in simulated gastrointestinal environment that pore-forming salmocins such as SalE1a and SalE1b and a DNase-active salmocin such as SalE7 would be degraded in the stomach or upper intestinal tract upon ingestion, and that their activity is almost totally lost after 3 h of incubation in simulated digestive passage (residual bactericidal activity of 0-001% of non-digested native proteins.

Because of their similarities to colicins, we expect that other salmocins not tested in this series or candidates for future inclusion in the SALMOCIN product would also be susceptible to digestive action.

Further, we have found no literature reports of salmocins (a.k.a. *Salmonella* colicins) being resistant to proteases. Yet another observation added in proof of digestibility is the fact that we analyzed salmocin amino acid sequences in part by tryptic digest peptide mapping. The salmocins were readily cleaved by enzymatic digestion prior to MS analysis (see APPENDIX C for a summary of methods).

Conclusion regarding salmocin digestibility

In sum, all evidence suggests that the candidate salmocins for inclusion in the SALMOCIN product are susceptible to digestion as are other bacteriocins reported in the public literature. This conclusion applies to salmocins that might be ingested from treated food that is uncooked or improperly cooked, especially meat, as cooking to a temperature of >60°C will inactivate bacteriocins (reviewed by Schneider (2018)).

Figure 2-15. Digestibility of salmocins in simulated gastroduodenal environment

A

Salmocin	Mean percent residual antimicrobial activity						
		Phase I – gastric digestion			Phase II – duodenal digestion		
	Not digested	0 min -/- SGF	60 min -/- SGF	60 min P/- SGF	0 min P/- SIF	180 min P/- SIF	180 min P/T, C SIF
SalE1a	100%	60%	60%	0.26%	0.06%	0.06%	0.001%
SalE1b	100%	100%	100%	75%	5%	5%	0%
SalE7	100%	55%	55%	0.1%	6%	6%	0%

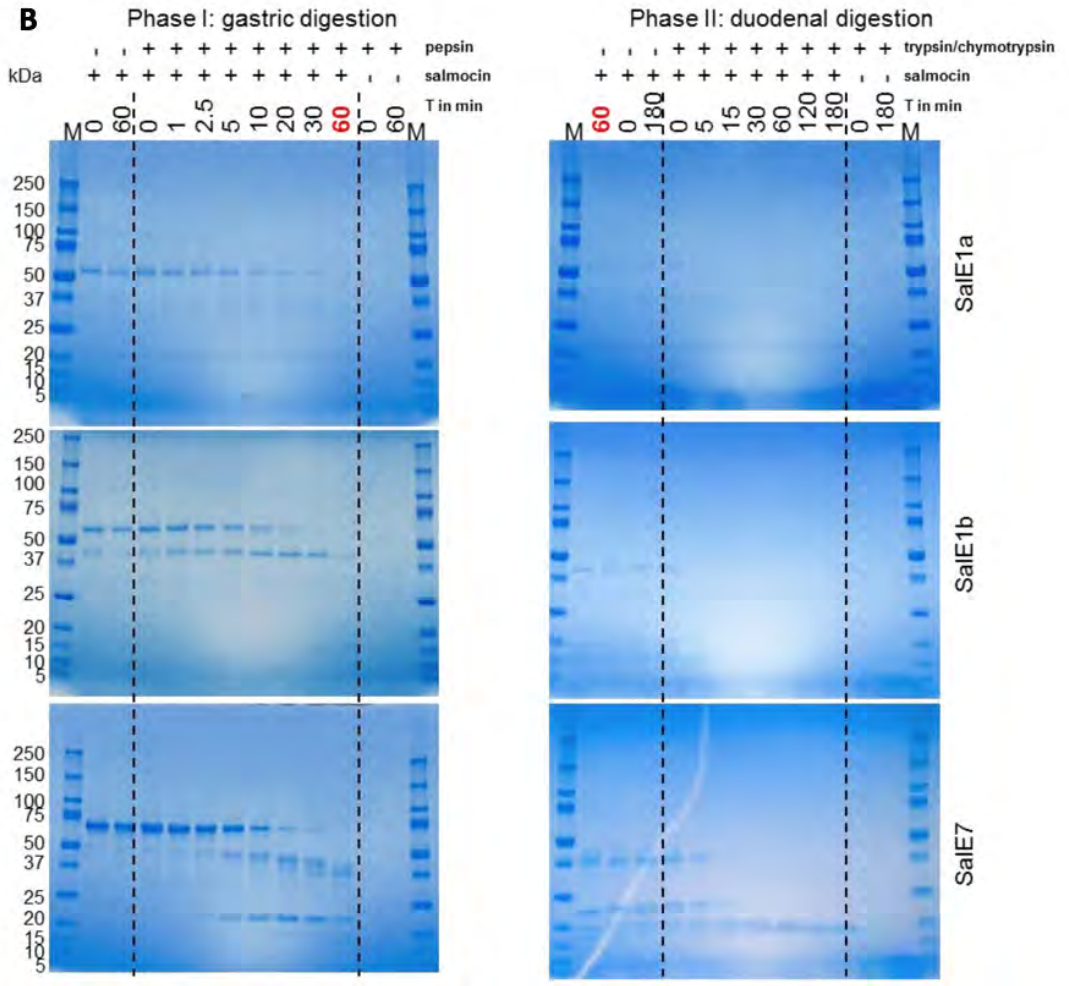


Figure 2-15. *In vitro* simulated gastro-duodenal digestion of salmocins. In phase I, gastric digestion, salmocins are incubated with pepsin (P) at 1:20 w:w; pepsin:salmocin for up to 60 min in simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5). In phase II, duodenal digestion, salmocin peptides generated within 60 min gastric digestion are incubated for up to 180 min with trypsin and chymotrypsin (T, C) at 1:4:400 w:w:w; trypsin:chymotrypsin:salmocin in simulated duodenal fluid (SIF, 4 mM sodium taurocholate, 4 mM glydeoxycholic acid, 10 mM $CaCl_2$, 25 mM Bis-Tris, pH 6.5. **Panel A** (top) shows antimicrobial activities of salmocin samples during gastro-duodenal digestion relative to non-digested protein measured by analysis of serial dilutions in soft agar overlay assay (*S. enterica* strain ATCC® 13076™*). **Panel B** (bottom) SDS-PAGE analysis of salmocin samples during gastro-duodenal digestion (left and right images, respectively). Loading corresponds to 1.5 µg or 1 µg protein for SalE7 and SalE1b or SalE1a, respectively.

2.4.4 SALMOCIN is a food processing aid

The FDA defines processing aids in 21 CFR 101.100(a)(3) as "substances that are added to a food for their technical or functional effect in the processing but are present in the finished food at insignificant levels and do not have any technical or functional effect in that food."

SALMOCIN meets this definition based on the following criteria:

a. SALMOCIN provides temporary antibacterial effect typically lasting 1-48 h (1-72 h maximally) post application upon incubation of treated, contaminated food at 10°C;

b. SALMOCIN residual proteins are present in the finished food product initially at insignificant levels of less than 3 ppm (typically 0.1 to 3 ppm). Those initial levels would be expected to decrease rapidly over time as the salmocin proteins would dissolve and degrade through enzymatic activity on the meat matrix (or other food matrices) and become indistinguishable from the matrix itself; and

c. SALMOCIN provides no continued technical or functional effect on the food.

As such, SALMOCIN-treated meats and whole (broken) egg products should be exempt from the FSIS labeling requirement.

2.5 Overall Conclusion

Results of the studies reported herein indicate that the plant-produced food antimicrobial product SALMOCIN, consisting of selected single broad-spectrum salmocins or mixtures of salmocins with complementary activity, is antibacterial against pathogenic strains of *S. enterica*, even at bacterial contamination levels >10^5 CFU/100g meat. The technical effect provided by SALMOCIN treatment was seen when contaminated meat and raw egg samples were incubated at 10°C, which simulates industrial perishable food (especially meat) processing conditions and elapsed processing times.

For the five salmocins evaluated at application rates ranging from 0.1 to 6 mg salmocin/kg of treated food, the antibacterial technical effect was observable from the first post-application sampling point of T = 1 h until T = 72 h. These experiments demonstrate that application of the plant-made salmocins over a wide range of 0.1 to 6 mg salmocin/kg food, results in rapid bactericidal control of pathogenic *S. enterica* applied to samples of raw food products, and that the technical effect observed is transient, lasting for approximately 1 to 48 h, maximally up to 72 h, depending on conditions. The growth of salmocin-surviving bacteria is clearly evident over time in a dose-dependent manner.

SALMOCIN application should not impact the organoleptic properties of the meat product. The SALMOCIN formulation is applied at a very low application rate of ~0.1 ppm to a maximum of 3 ppm initially and dissolves or diffuses in the meat (or other food) matrix post application. There is no color-masking of meat after application, and solutions of SALMOCIN are generally clear and have no objectionable odor. No organoleptic evaluation was conducted for this Notice because the meat and egg matrices were raw and/or were inoculated with strains of the pathogen *S. enterica*. Additional detail on lack of organoleptic impact is discussed in Section 6.7.

The observation that the small fraction of bacteria surviving SALMOCIN treatment can grow normally suggests that the product will not interfere with pathogen detection methods on food. Non-interference of salmocin application to food with procedures used to detect pathogens is discussed in Section 6.8.

Preliminary studies with plant-made salmocin proteins comprising SALMOCIN in aqueous solution were found compatible with a range of application devices including pressurized spraying equipment, showing no signs of denaturation or loss of potency after application (data not shown). While in-plant evaluation using industrial equipment will be conducted later in development, our initial tests suggest that application of SALMOCIN can be accomplished using standard equipment. On-going equipment compatibility studies with salmocins are equivalent to those described for colicins in GRN 676 Section 2.4.4, pp. 33-37.

Although the product has not yet been evaluated at-scale, no special handling procedures or protective measures are anticipated when using the product in industrial settings, and none are indicated from the public literature for these or other bacteriocins. The proteins and excipients used in SALMOCIN formulations are either GRAS or food-grade. As a precaution, eye (goggles), respiratory (mask) and skin (gloves) protection could be implemented during preparation of solutions of SALMOCIN if starting with a granular or powdered stock, and during mixing, transfer or disposal of the SALMOCIN solution.

A draft SDS for SALMOCIN is shown in APPENDIX A. Safety to USDA plant inspection personnel is discussed in Section 6.9.

3 Dietary Exposure

Application rates of 0.1 to 6 mg (mixed) salmocins/kg food have been shown effective in controlling enteropathogenic serotypes of *S. enterica* on artificially contaminated food matrices (Section 2.4). In practice, because of the higher potency of salmocins and their broader target coverage relative to other antimicrobial proteins including colicins and endolysins, the application rate of SALMOCIN is not expected to exceed 3 mg SALMOCIN per kg of treated food for controlling *S. enterica* in multiple food products.

3.1 Estimated dietary intake of selected foods susceptible to *Salmonella* contamination

Salmonella can contaminate a wide range of foods, but because *Salmonella* is an enteric pathogen, animal products can be a main source of contamination. To assess consumption of foods susceptible to *Salmonella* contamination and to subsequently estimate the amount of potential salmocin exposure of consumers from foods treated with SALMOCIN, public databases were consulted for the latest available figures.

Mammalian (red) meat. The estimated intake of red meats (e.g. beef, pork, lamb, mutton, veal) by the U.S. population varies depending on source of the survey, year of survey, method of estimation, whether total or only federally inspected facilities are counted, and how consumed weight is computed (e.g. carcass weight equivalents; total carcass vs. ready-to-cook carcass weight; retail weight; boneless net weight; served vs. consumed). These different methods can yield significantly different results. For example, for beef, the carcass weight of a steer may be 60% of its live weight, whereas the retail weight is only 42% as it may discount bones, ligaments, or tendons depending on the cut. Similarly, for pork, the carcass weight of a hog may be 70% of its live weight, in contrast to 56% for its retail weight (DeBruicker 2011). Also, carcass weights may vary from year to year depending on environmental and production conditions.

Table 3-1 summarizes results from recent consumption surveys. WASDE statistics published by USDA for 2014 domestic mammalian (red) meat production and disappearance indicate annual per capita consumption of 51.7 lbs of beef, 43.6 lbs of pork, 0.7 lbs of lamb and mutton, and 0.2 lbs of veal, for a red meat consumption total of 96.2 lbs/person, retail weight (USDA ERS 2014b). The 2015 WASDE estimates from the same USDA database suggest per capita consumption of all red meat of 142.4 lbs carcass weight, 104.8 lbs retail weight, and 99.1 lbs boneless retail weight (USDA ERS 2015).

The National Health and Nutrition Examination Survey (NHANES) by the Centers for Disease Control and Prevention (CDC) reports total yearly red meat consumption as 99.7 lbs/person retail weight for survey years 2003-2004 (analyzed by Daniel (2011)). The NHANES is based on interviews of >18,000 individuals and explores what people consume over a 24-hr period, from which yearly figures are projected. Calculations from the World Agricultural Supply and Demand Estimates (WASDE) database suggest a total U.S. red meat per capita consumption of 99.3 lbs/person retail weight for year ending 2015 and approximately 106 lbs/person retail weight for CY2016 (USDA WASDE 2016).

The per capita red meat consumption estimates may be slightly under-represented in many of these surveys because they do not take into account persons who do not consume meats. Results of a 2012 Gallup Poll showed that a consistent 5% of the U.S. population is vegetarian. This percentage remains largely unchanged from results of 1999 and 2001 surveys, which reported a value of 6% (Newport 2012). Hence, assuming that the projected domestic U.S. population in CY2016 is 325,032,763 (projected from US Census Bureau (2016)) this means that 16,251,638 people will not be consuming meat at all and should not be included in per capita consumption estimates in the above-referenced statistics. Therefore, exposure estimates may be more accurately calculated based on a U.S. population of 308,781,125 potential meat consumers (325,032,763 – 16,251,638). Using USDA ERS total red meat statistics for 2014 (USDA ERS 2014a)

and the adjusted population for the same year, yields an estimated red meat annual consumption of 104.3 lbs/person retail weight or 98.6 lbs/person based on boneless weight. These figures translate to 4.6 oz/day (130.4 g/day) retail weight, and 4.3 oz/day (121.9 g/day) boneless weight.

Avian (poultry) meat. Consumption figures for poultry included only chicken and turkey, which comprise the vast majority of retail poultry meat. USDA/ERS database entries published on July 26, 2017 (current to CY2015; USDA ERS (2017)) were used to derive per capita consumption of chicken ("broiler" and "other chicken") and turkey meat; only figures for boneless meat were used in our estimates.

The databases consulted and the variations in reported meat intake are shown in Table 3-1; all figures are based on retail weights and boneless products and include all subpopulations.

Table 3-1. Per capita US consumption of red meat and poultry based on various surveys

Survey Source, Database, Year	Consumption (retail wt)			
	Annual		Daily	
Red (mammalian) meat	**lbs**	**kg**	**oz**	**g**
USDA, ERS Livestock meat domestic data, 2014[1]	96.2	43.6	4.2	119.5
USDA, ERS Livestock meat domestic data, 2015[1]	104.8	47.5	4.6	130.2
USDA, ERS food availability per-capita data system, Jul 26, 2017[2]	98.6	44.72	4.32	122.5
CDC, NHANES, 2003-04 (analyzed by Daniel (2011))[3]	99.7	45.22	4.37	123.9
CDC, NHANES (DeBruicker 2011; Wang 2009)[3]	120.9	54.8	5.3	150.2
NCI analysis of NHANES (DeBruicker 2011)[4]	59.3	26.9	2.6	73.7
USDA, WASDE, 2015[1]	99.3	45.0	4.35	123.4
USDA, WASDE, March 2016 (projection)[1]	105.9	48.0	4.64	131.6
USDA Dietary Guidelines 8th Edition, 2015-2020[4]	57.0	25.9	2.5	70.8
Poultry (all chicken meat and turkey meat)	**lbs**	**kg**	**oz**	**g**
USDA, ERS food availability per-capita data system, Jul 26, 2017[2]	75.1	34.1	3.29	93.4

[1] USDA consumption estimates are based on annual regional animal meat production and disappearance data and the US population based on census results

[2] Current to end of CY2015; excludes mutton

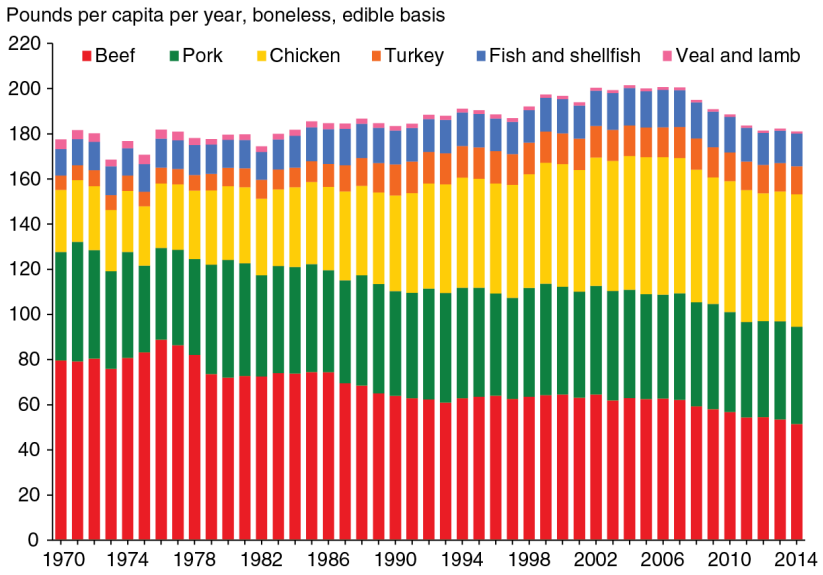
[3] NHANES extrapolates consumption from a single day survey of typical dietary intake

[4] NCI "usual intake" method of analysis of NHANES data distinguishes foods that tend to be consumed daily from foods that are consumed infrequently ("ubiquitous" compared to "episodic" foods). Going beyond what people happen to report from a given 24 hours (NHANES), the procedure goes for a long-term average, aiming to determine what people "usually" eat. The most recent US dietary guidelines use the same "usual intake" method, and hence results are similar to the NCI figures.

Seafood. Seafood consumption in the USA is much lower than that of other types of meat. Further, seafood susceptibility to *Salmonella* contamination could vary from species to species and how the foods are prepared prior to consumption. Our consumption estimate came from trends in consumption of all meats including seafood as recently published by USDA/ERS (Bentley 2017).

Although meat consumption trends may vary, with reported reduced domestic consumption in recent years, the totality of data published for the last 3-5 years in the above-referenced surveys suggest that U.S. per capita consumption of all red meat ranges from about 57-120 lbs per year (~26 to 55 kg/yr), or 2.5-5.3 oz/day (~71 to 150 g/day). For poultry (all chicken and turkey products, bone-free weights), per capita consumption is estimated at 75.1 lbs or 34.1 kg per year, or 3.29 oz/day or 93.4 g/day. For all seafood, estimated consumption is 14.5 lbs or 6.6 kg per year, or 0.64 oz/day or 18 g/day. The graphic reproduced in Figure 3-1 from USDA/ERS summarizes these consumption trends (from Bentley (2017)).

Figure 3-1. Trends in US per capita consumption of various meat products 1970-2014



Therefore, to err on the side of safety for SALMOCIN exposure calculations, we assumed that the product would be applied to all meats, poultry and seafood, for a total consumption of 180 lbs/person-year or ~82 kg/person-year. These figures translate to ~8 oz/person-day or ~225 g/person-day.

Estimating that the product could also be applied to foods during preparation, for example to meat sauces or meat-based gravies at 15% (w/w) of the consumption of meats, the calculated estimate for all subpopulations was rounded upwards to 208 lbs/person-year, which translates to **260 g/person-day**. Obviously, not every person (or age group) will consume the same quantities or ratios of red meat, poultry or seafood plus gravy or sauces on a given day, but the estimated consumption values allow for a calculation of average consumer exposure to salmocins applied to food.

Eggs and egg products. Americans consume an average of 276 eggs per capita (USDA ERS 2017). Of the 7,507 million dozen shell eggs shipped/consumed in 2017 including US territories (total disappearance; USDA ERS (2017)), approximately 30% (2,307 million dozen) were processed into egg products (broken) at USDA-inspected facilities. Those egg products include categories such as whole egg, egg white, egg yolk, whole plus 10% salt, whole plus 10% sugar, yolk plus 10% salt and yolk plus 10% sugar.

Salmonella contaminates eggs and egg products and in fact eggs and egg products are a significant cause of foodborne outbreaks. The CDC estimates that 1 million cases of salmonellosis occur per year in the US leading to 19,000 hospitalizations and 380 deaths (https://www.cdc.gov/salmonella/index.html). FDA estimates that 79,000 cases each year are the result of consuming eggs contaminated with *Salmonella*, of which 30 result in death (FDA 2016). This is still a substantial health problem because nearly 4 of 5 cases of salmonellosis derive from contaminated raw or undercooked shell eggs.

In eggs the major risk factor is *S. enterica* ssp. *enterica* serotype Enteritidis, which can be transmitted via trans-ovarian and trans-shell infection routes in the hen (USDA FSIS 2005). Other *Salmonella enterica* serotypes can also contaminate egg products and remain on-shell or reach the egg's interior through migration. Pasteurization of egg products has reduced the incidence of salmonellosis, but pasteurization is not 100% effective and egg products can be contaminated after pasteurization (USDA FSIS 2005).

It is difficult to estimate product usage and consumer exposure from SALMOCIN-treated egg/egg products because of the diversity of uses for egg and egg fractions in a wide range of foods. For purposes of this discussion, and using USDA ERS 2017 figures, we assumed a volume of 2,307 million dozen broken eggs consumed as egg products by 326 million US consumers to yield 85 shell egg-equivalents per year, which is 30% of 276 shell eggs consumed per capita. Although nearly all broken egg products are pasteurized, we assumed 85 egg-equivalents and 100% market coverage for a "worst case" exposure calculation.

Assuming an average size hen egg weighs 57 g and 11% of the mass is the shell, the contents of the whole egg would weigh about 50 g. The per capita consumption of SALMOCIN-treated egg products would be 85 eggs x 50 g/egg = 4.25 kg/person-year, or about 9.35 lb/year. This amount was added to the treated meat consumption estimate (208 lbs/yr + 9.35 lb/yr) to arrive at a sum of ~217 lbs/yr, or **270 g/person-day** (see calculation below).

3.2 Dietary intake (exposure) of salmocins from SALMOCIN-treated food products

The projected exposure to SALMOCIN from ingestion of treated meat, poultry, seafood and egg products was calculated as follows using the per capita statistics summarized in Section 3.1 and the maximum (highest envisioned) application rate of SALMOCIN (i.e. 3 mg/kg food; Section 3.3) to various meat, meat-containing and egg products during processing.

Weight of total treated food consumed per day per person (rounded off to nearest whole unit):

$$\frac{217\ \text{lbs}}{\text{person-year}} \times \frac{1\ \text{year}}{365\ \text{days}} \times \frac{1000\ \text{g}}{2.2\ \text{lbs}} = \frac{270\ \text{g treated food}}{\text{person-day}}$$

At a projected SALMOCIN application rate of 3 mg/kg treated food, the highest amount of SALMOCIN active ingredient consumed would be:

$$\frac{3\ \text{mg SALMOCIN (max)}}{1000\ \text{g food}} \times \frac{270\ \text{g treated food}}{\text{person-day}} = \frac{0.8\ \text{mg SALMOCIN consumed}}{\text{person-day}}$$

The total projected per capita maximum intake of SALMOCIN active ingredients from consumption of treated meat, poultry, seafood and egg products would be **0.8 mg per day** or **<0.3 g/year**. It bears mentioning that this calculated maximum exposure would be from **uncooked foods**, as cooking the food products to recommended temperatures would destroy all salmocins (see Table 3-2 and Section 6 for additional detail).

3.3 Additional, natural exposure to salmocins (intake not related to SALMOCIN product)

Exposure of consumers to various salmocins would be expected from a variety of sources, including ingestion of foods as well as from commensal microbiota. However, we found no reports in the literature that directly quantify human exposure to *Salmonella* bacteriocins. Small amounts of salmocins could be present in foods if they contain low levels of *Salmonella* strains with low pathogenicity. Also, ~2.2% to 5% of the US population (7.2 to 16.5 million people) reportedly carries non-typhoidal *Salmonella* (NTS) species asymptomatically (Marzel 2016; Monack 2012, 2013; Ruby 2012). It can be expected that if the commensal or environmental Salmonellae are producing salmocins in the gut in much the same way commensal *E. coli* produce colicins, humans may be routinely and chronically exposed to salmocins naturally. As reviewed by Gillor (2008), up to 26% of environmental samples of Enterobacteriaceae, including *Salmonella enterica* isolates, can produce various bacteriocins.

Table 3-2 summarizes exposure to salmocins and estimates the intake by food source. A maximum application rate of 3 mg total salmocins per kg food products (3 ppm; worst case) was used in the calculations. A worst-case intake estimate for all treated food is provided and assumes exposure to SALMOCIN from consumption of treated uncooked meats (a rarity in the USA) and raw egg products, as salmocins are destroyed by heating (cooking) as well as by proteases of the type found in the gastric environment and upper-intestinal tract. Cooking treated foods to recommended temperatures would essentially reduce salmocins levels to zero, regardless of their source, as is also shown in the table.

For the purpose of estimating risk, the exposure to salmocins not derived from treatment of food with Notifier's product was assumed to be 10% of the level applied via SALMOCIN treatment, or less than **~0.1 mg/person-day**. This level is likely to be much lower for the reasons stated above.

Table 3-2. Estimated human daily exposure to salmocins

Source of exposure	Estimated daily per capita salmocin exposure	
	If food is not cooked	**If food is cooked**
SALMOCIN treatment, total of all meat, poultry, seafood and egg products consumed (detailed in this GRN; derived from Table 3-1)	**0.8 mg**	**nil**
Commensal microflora and incidental natural exposure from food and environmental sources	**0.1 mg**	**nil**
Total (estimated maximum, all food sources)	**0.9 mg**	**nil**

Perspective on the significance of these intake levels from all sources vis-à-vis consumer safety is provided in Section 6 of this Notice.

4 Information on Any Self-Limiting Levels of Use

There are no known self-limiting levels of use for salmocins.

5 Experience Based on Common Use in Food Before 1958

SALMOCIN (individual salmocins or mixtures of salmocin proteins) has not previously been used in food.

6 Basis for Conclusion of SALMOCIN's GRAS Status

6.1 Overall Safety of Salmocin Proteins

Notifier has used scientific procedures to conclude that its SALMOCIN food antimicrobial product is GRAS under the conditions of intended use. Information supporting our determination of SALMOCIN as GRAS for use as an antimicrobial processing aid on meat, poultry, seafood and egg products is summarized in this current Notice. Methods specifically used to assess (1) efficacy, suitability, residual technical effect after application to meat products, and (2) safety upon ingestion of treated foods, are described separately in this Notice, specifically in Section 2 and Section 3, respectively.

In Schneider et al. (2018) we described the identification of *Salmonella*-specific, colicin-like antibacterial proteins we termed salmocins. These proteins were found by searching *Salmonella* genomic sequences for patterns that showed similarity to colicins. Salmocins, colicins and several other families of bacteria-derived antimicrobial proteins belong to a class of proteins known as bacteriocins and share many common targeting, transporter and antibacterial features, as extensively reviewed by Cascales (2007). The *Salmonella* bacteriocin sequences identified bear strong similarity to *E. coli* colicins that Notifier has determined to be GRAS when used as food antimicrobials in fruits and vegetables (GRN 593) and meat products (GRN 676; FSIS 7120.1 Rev 42 Aug 2017 (USDA FSIS 2017)).

Consequently, the *Salmonella* bacteriocin gene sequences subsequently cloned and expressed in Notifier's plant-based production system yield salmocin proteins with high structural homology to *E. coli* colicins yet with high specificity against *Salmonella*. In fact, the features of colicins and salmocins are so similar that the main differences appear to be the genus-specific transporters used to gain access to cellular compartments and the serovar target site specificity. The mode of action of colicins and salmocins is essentially the same; namely, bactericidal activity is via membrane pore formation (porins) or by enzymatic degradation of macromolecular structures in the target cell (Cascales 2007; Schneider 2018). As we showed, some colicins can in fact inhibit *Salmonella* serovars and some salmocins can inhibit *E. coli* serovars; that is, there is cross-genera antibacterial activity, corroborating the close evolutionary and structural relatedness between the genera *Salmonella* and *Escherichia,* which co-evolved in the same hosts (Cascales 2007; Schneider 2018).

We can conclude that *Salmonella*-derived/*Salmonella*-specific bacteriocins, salmocins, are safe from the following observations:

- Salmocins and colicins are highly similar in structure and function, including susceptibility to degradation and gastroduodenal digestion, and colicins are GRAS (GRN 593; GRN 676; GRN 775);
- Salmocins have high antibacterial specificity towards *Salmonella* pathovars, low to moderate activity against *E. coli* strains (not a beneficial organism), and no known activity against beneficial intestinal genera;
- Like colicins, salmocins attack bacterial structures and cellular targets that do not exist in mammalian tissues.

The evidence supporting these observations derive from published studies and Notifier's own work.

Salmonella strains have been known to harbor colicin-like gene sequences for nearly forty years (Barker 1980; Campos 1988; Nedialkova 2014; Vicente 1984). These prior studies focused on evaluating the presence and type of colicins as potential taxonomic differentiators for *Salmonella* serovars. These studies identified colicin-encoding plasmids and only a few studies isolated colicins in crude (Guterman 1975) and purer (his-tagged) forms (Nedialkova 2014) to determine strain susceptibility and/or ecological competitiveness. These latter studies focused on the activity of *Salmonella* colicins against *E. coli.* In Notifier's studies with plant-produced bacteriocins, activity against *Salmonella* was confirmed for colicins M, Ia, Ib, 5, 10 and S4, in agreement with literature reports. However, *Salmonella enterica* serovar Typhimurium was reported to be insensitive to Group A colicins E1, E2 and E3 (Graham 1977; Guterman 1975), yet some *Salmonella* strains are sensitive to colM (Graham 1977). NCBI database searches showed that analogues of *E. coli* colicins Ia, Ib, M, and B seem to be widely distributed in *Salmonella* with 99-100% identity in amino acid sequence between the bacteriocins of each genus. Thus, even though *Salmonella* bacteriocins were only recently expressed, purified and characterized, we can state with some confidence that the similarities in structure, mode of action, molecular features, environmental instability and gastroduodenal digestibility between colicins and salmocins are high (Section 2.4.3; GRN 593; GRN 676). Hence, the overall high safety profile of the two types of bacteriocins is also expected to be comparable.

Humans have a very long history of exposure to bacteriocins from various natural sources, including exposure from human commensal and domestic animal microflora. Original studies that isolated and characterized colicins (Gratia 1945, 1946 as reviewed in Cascales (2007)), colicin-producing *E. coli* have been isolated from fecal samples of healthy humans, animals and multiple environmental samples (Cascales 2007; Hossneara 2007; Obi 1978; Riley 1992; Riley 1994; Schamberger 2002; Smarda 2001; Smarda 2007). Estimates for the number of colicin-producing *E. coli* in the colon of healthy humans have ranged from as low as 9% of the total number of *E. coli* isolated (Lorkiewicz 1964 as reviewed in Smarda (2001)) to as high as 83% of recovered isolates (Hossneara 2007).

Similar commensalism has been reported for *Salmonella* strains; while some appear to be normal non-pathogenic inhabitants of the colon (Todar 2012), other strains can cause notable gastroenteric pathology. Campos (1988) described the colicinogeny of *Salmonella* strains isolated from various sources. They reported that 3% of *Salmonella* strains isolated from human feces expressed *Salmonella* colicins; in contrast, 10% and 30% of strains isolated from the environment and from various foods, respectively, were colicinogenic. Gillor (2008) reported that 3% to 26% of enteric bacteria including *S. enterica* expressed bacteriocins, compared to 9-83% of *E. coli* strains. It is not unexpected to find lower level of colicin-positive *Salmonella* strains in humans relative to colicin-positive *E. coli* due to the higher colonization of the gut by *E. coli* relative to *Salmonella*. A small percentage of the population (~2.2%–5%) reportedly carries non-typhoidal *Salmonella* (NTS) species asymptomatically (Marzel 2016; Monack 2012, 2013; Ruby 2012). Therefore, it can be expected that if the commensal, food-borne and environmental Salmonellae are producing salmocins in the gut in much the same way commensal *E. coli* produce colicins, humans may be routinely and chronically exposed to salmocins naturally and without consequential pathology.

Therefore, it is likely that humans have been exposed to *Salmonella* bacteriocins (salmocins) as we have been exposed to *E. coli* colicins endogenously for as long as the human gut has carried these bacteria as commensal organisms, and to low levels of both bacteriocins from foods and the environment. It is also possible that humans are exposed to salmocins during pathogenic *Salmonella* exposures during the bacterium's virulent stage of infection. However, the presence of salmocins during pathogenesis has not been reported and, like colicins, they are unlikely to cause or exacerbate disease as the common mechanisms of action of salmocins and colicins are highly specific and act preferentially against related

strains. Further, molecular target moieties attached by bacteriocins are not present in mammalian cells (Cascales 2007).

The high degree of species- and strain-specificity of salmocins, like colicins, ameliorates concerns of the impact of SALMOCIN on beneficial intestinal microbiota. Nedialkova (2014) suggested that *Salmonella* serovar Typhimurium may depend on gut inflammation to synthesize *E. coli*-active colicins to gain a competitive advantage. However, when examining the bactericidal activity range of colicins against *Salmonella* serovars and conversely of salmocins against *E. coli* serovars, positive yet weak cross-genera activity was found, even though the two genera share many physiological and structural features (Cascales 2007; Schneider 2018). This finding suggests that even if ppm levels of salmocins applied to food were to reach the human colon intact (a fact not substantiated by digestibility results; Section 2.4.3), these proteins are unlikely to affect the gut microbiome given their specificity and short duration of technical effect. In addition, *E. coli* is not necessarily a beneficial organism in humans and niche competition via bacteriocin secretion is already a well-known natural process.

An additional observation supporting safety of salmocins is the finding that *Salmonella enterica* serovar Typhimurium utilizes a T6SS-mediated antibacterial weapon to establish in the host gut (Sana 2016). The mammalian gastrointestinal tract is colonized by a high-density polymicrobial community where bacteria compete for niches and resources. One key competition strategy includes cell contact-dependent mechanisms of interbacterial antagonism, such as the type VI secretion system (T6SS), a multiprotein needle-like apparatus that injects effector proteins into prokaryotic and/or eukaryotic target cells. The two key results reported by Sana et al. are that (a) the T6SS antibacterial activity is essential for *Salmonella* to establish infection within the host gut and (b) by exclusion, *Salmonella*-produced colicins (salmocins) are not involved in host pathology.

The endogenous steady state level of *E. coli*-specific colicin biosynthesis was estimated at about 3 mg/day (GRN 593). Because of the lower incidence of commensalism for *Salmonella* in humans, even assuming equivalent rates of biosynthesis of each bacteriocin the endogenous level of salmocins is likely to be lower than that of colicins (i.e. <3 mg/day). Through traditional practices used in food cultivation, preparation and consumption, humans have likely been chronically exposed to colicins and salmocins from food and environmental sources for millenia. The published studies cited above suggest that the level of human exposure to bacteriocins from various foods, while small (parts per million levels ingested per day), may nevertheless be consistent due to dietary and cultural habits.

Hence, there exists the possibility that salmocins could be consumed from multiple sources, including from SALMOCIN-treated foods plus residues from natural sources in various foods. This would increase the total daily exposure to colicins from food consumption. Estimates of potential exposure from all sources are included in Table 3-2. Most meats will be consumed after cooking (i.e. thermal processing through baking, broiling, sautéing, boiling, etc.) while a minority might be consumed raw (e.g. seafood sashimi, steak tar tar, etc.). Most egg products are pasteurized or cooked but a minority are consumed raw (e.g., in salad dressings).

Therefore, the estimates in Table 3-2 include a worst-case situation where (a) SALMOCIN is applied to 100% of all meat, poultry, seafood and egg products in the USA, (b) none of the SALMOCIN-treated foods are cooked, and (c) there is additional albeit limited exposure to salmocins from natural (commensalism and environmental) sources. From this exposure scenario, the maximum daily intake of salmocins can be estimated to be on the order of 0.8 mg from foods treated with the SALMOCIN food processing and 0.1 mg from non-treated foods and the environment, for a total exposure of 0.9 mg/person-day.

No reports have appeared in the literature linking consumption of any bacteriocin (ingestion) with onset of disease, morbidity or mortality. In large part, this can be explained by the specificity of these molecules for bacterial target structures, plus the evolutionary adaptation by humans and animals to bacteriocin protein exposure, including immune tolerance.

Importantly, the physicochemical properties of salmocins shared with other bacteriocins including colicins, notably their instability to heat and their degradation in the gastric and upper intestinal environments (Cascales (2007); GRN 593; this Notice), contribute significantly to their safety profile and support the use of salmocins as food preservatives and food processing aids.

Meats and egg dishes are typically cooked domestically and in most other countries; therefore, the level of intake of salmocins from the use of the SALMOCIN product in meat processing is expected to be less than the intake from treated fresh and minimally processed foods such as some meat and fish dishes, albeit these raw foods represent a minority of meat preparation options.

Taken together, extensive published reports on bacteriocins including colicins, our determination that colicins are GRAS (GRN 593, GRN 676, GRN 775), the similarities between colicins and salmocins, and our verification of the high specificity, environmental instability and digestibility of salmocins (Cascales 2007; Schneider 2018) allow us to conclude that the proteins salmocins (*Salmonella*-specific bacteriocins) that comprise the SALMOCIN product can be Generally Recognized As Safe.

6.2 Low Safety Risk from Consumption of Plant Host Impurities in Salmocins

Multiple plant hosts can be used in the manufacture of salmocin proteins. Species such as spinach, red beet and lettuce are food crops and can be consumed in unrestricted quantities. Residual host-derived impurities from these plants pose no safety risks, as discussed in detail in Notifier's prior GRAS notices, including GRN 593, GRN 676 and GRN 738.

Plants of the genus *Nicotiana* can also be used in the manufacture of salmocins, often with higher efficiencies relative to other crops. *Nicotiana* species, including *N. benthamiana,* share the main structural, physiological and biochemical constituents with all other land plants. As extensively reviewed by (Leffingwell 1999), such constituents include (a) carbohydrates, including starch, sugars, sugar esters, cellulose and pectin; (b) nitrogenous constituents, including protein, soluble amino acids, nitrate, and certain alkaloids; (c) plastid pigments, including chlorophyll and carotenoids; (d) and isoprenoids and diterpenoids (both carotenoid-derived and non-carotenoid-derived), cembranoids and labdanoids; (e) phenolics, including polyphenols, lignin and various other phenolics; (f) sterols such as cholesterol and stigmasterol, and (g) various inorganics, including calcium, potassium, magnesium, sodium, chloride, and various other minerals that are absorbed from the soil (Leffingwell 1999). These major structural, proteinaceous and biochemical components of *Nicotiana* species, including *N. benthamiana*, are shared with other plant species, including edible species, and are not considered inherently toxic. In fact, several studies have appeared in the peer-reviewed literature where members of the genus were assessed as a source of nutritional protein and other valuable biochemicals (discussed in GRN 775 Section 6.1, pp 28-39).

Of the minor constituents of *Nicotiana* species, several alkaloids could present a potential safety concern. These potential toxicants need to be removed or diluted to ensure that the final product is safe at the levels applied to food. *Nicotiana* species synthesize a number of bioactive substances, some of which are toxic in high doses. The major bioactive alkaloids in the genus include **nicotine**, **nornicotine**, **anabasine** and **anatabine**. Due in large part to tobacco variety improvement and tobacco safety research, the synthesis, accumulation and biological effects of these alkaloids have been extensively studied.

A full discussion of these alkaloids and the relative risk they present from their consumption in common foods that contain them (e.g. tomato, pepper, eggplant, cauliflower, etc.) and from foods treated with Notifier's antimicrobials was included in GRN 775 (Section 6.1, pp 32-39; Section C.3, pp 76-77). That document is publicly available and hence the same discussion of methods, results and risks is not repeated here; however, we include parts of that discussion here and in Section 3.3 for convenience.

Nicotine is the most abundant bioactive alkaloid in *N. benthamiana*, followed distantly by anabasine. Nicotine constitutes 80-90% of the total alkaloid content of *N. benthamiana* and anabasine 8-12% of the total; the typical ratio of nicotine to anabasine is ~10:1 (Sisson 1990). Nornicotine and anatabine may be present in trace amounts (<1% of total alkaloids each), and at the levels of salmocins applied to food these two alkaloids are not a risk. The high genetic homogeneity of *N. benthamiana* cultivars suggests that alkaloid levels and ratios will remain consistent (Goodin 2008).

Table 6-1 summarizes levels of nicotine and anabasine measured by Notifier in multiple analyses of three salmocins expressed in *N. benthamiana*. Because these salmocins could be blended in different ratios, the average content of nicotine and anabasine were used in subsequent calculations. To simulate a worst-case exposure scenario, plant extracts of salmocins were used to measure the levels of residual alkaloids.

Table 6-1. Residual alkaloid content in *N. benthamiana*-produced salmocins

	Residual Alkaloid Levels in Salmocin Protein Samples		
Salmocin	Nicotine (ng/mg protein; ave. ± s.d.)	Anabasine (ng/mg protein; ave. ± s.d.)	No. non-consecutive batches analyzed
SalE1a	477.83 ± 253.72	40.16 ± 33.69	3
SalE1b	353.33 ± 250.31	96.76 ± 104.61	3
SalE7	122.17 ± 20.71	22.31 ± 4.12	3
Average	317.78 ± 255.19	53.08 ± 31.75	9
Average total alkaloid content: 371 ng/mg protein (i.e. 318 ng nicotine/mg + 53 ng anabasine/mg salmocin)			

Table 6-1 shows the residual content of nicotine and anabasine in salmocins expressed in *N. benthamiana*. The alkaloids were analyzed by HPLC-MS/MS. The method's lower limits of quantitation (LLOQs) is 0.005 µg/ml for nicotine (5 parts per billion) and 0.001 µg/ml for anabasine (1 part per billion). Because salmocins can be blended in different ratios, the average content of nicotine and anabasine and total mixed alkaloids were calculated (bottom rows).

The average total level of mixed alkaloid impurity in salmocin proteins was used to calculate alkaloid intake levels on the basis of SALMOCIN application rates and per capita consumption of treated foods. Those estimates are presented in Section 3.3. In a "worst case" scenario where all applicable foods are treated with a maximum of 3 mg/kg *N. benthamiana*-produced SALMOCIN and the product achieves 100% market penetration, exposure to solanaceous alkaloids from SALMOCIN would be 0.8 mg salmocins/person-day. Only the salmocin ingested from the product would have residual alkaloids; hence, our estimates do not include salmocins from commensal or environmental exposure. The daily per capita dietary alkaloid exposure from SALMOCIN-treated foods can then be calculated as follows:

$$\frac{371 \text{ ng total alkaloid}}{\text{mg salmocin}} \times \frac{0.8 \text{ mg salmocin ingested}}{\text{person-day}} = \frac{297 \text{ ng alkaloid}}{\text{person-day}}$$

As discussed extensively in GRN 775 (Section 6.1.4, pp 32-38), the daily alkaloid intake from consumption of common vegetables such as **tomato, potato, eggplants, peppers, cauliflower**, etc., in a typical US diet is **900-1,300 ng/person-day (~1 μg/day)** depending on dietary habits. There are no health consequences from ingestion of alkaloids at these low levels. The exposure to these alkaloids from the SALMOCIN product would be <0.3 μg/person-day (0.9 mg/year), even if the most liberal assumptions are made about 100% product use in all foods.

Therefore, total use of the product in all treated food categories would raise the exposure to alkaloids from 1 μg per day to 1.3 μg per day. That difference is less than the amount in a second helping of vegetables, and 3-times lower than what a person would absorb by attending a meeting in a room with a minimal amount of residual tobacco smoke (Domino 1993). We conclude that residual alkaloids in SALMOCIN would pose no significant health risk to any subpopulation of consumers.

6.3 Low Safety Risk from Consumption of Process Impurities in Salmocins

Biotic and organic impurities

The process for manufacturing antimicrobial proteins from *N. benthamiana* is described in Stephan (2017) and in APPENDIX B of this Notice. The same gene expression options are available with *N. benthamiana* as with food species hosts to express other bacteriocins (Schulz (2015); GRN 593, GRN 676 and GRN 775). Biosynthesis of salmocin proteins can be initiated via agroinfiltration, agrospray, or via ethanol induction of transgenic hosts.

The agrobacterial vectors used are the same regardless of plant host, and consumables, buffers, salts, etc. used in the extraction and purification of salmocins from *N. benthamiana* are commonly used in food processing. No pesticides are used in the production process. Hence, there are no additional biologic or organic compound risks introduced in the manufacturing of the final product when using *N. benthamiana* as the host.

Inorganic impurities

Potentially harmful heavy metals, mercury (Hg), lead (Pb), cadmium (Cd) and arsenic (As), were quantified for individual salmocins and the average levels of these compounds are reported in Table 6-2. The methods used in elemental analyses of salmocins are summarized in APPENDIX C, Section C.12.

The levels of heavy metals were included in the Specification in GRN 775 out of an abundance of caution because it was the first time that *N. benthamiana* was used as a production host for antimicrobial proteins. The process used to produce colicins in GRN 775 is the same as the process used to produce salmocins in this Notice.

The table also provides, based on a worst-case assessment, the highest level of exposure to these heavy metals from SALMOCIN, expressed on a ng/person-day basis. Using the NHEXAS database, Ryan (2001) reports that the estimated daily intake of heavy metals in Maryland residents is 28 **mg** for arsenic, 10 **mg** for cadmium, and 8 **mg** for lead. ASTSDR (1999) states that the mercury intake through food is approximately 3.5 **mg**/day.

Compared to reported levels of intake of these heavy metals from food, the level of exposure to these elements from 0.8 mg/person-day residual SALMOCIN in foods is inconsequential; as such, we excluded heavy metal release limits from the target Specification.

Table 6-2. Heavy metal impurities in salmocins SalE1b and SalE7

Salmocin / Element	Elemental level (ng metal/mg salmocin)	Elemental level in 0.8 mg of ingested salmocin (ng)
SalE1b		
Hg	9.83	7.9
Cd	86.17	68.9
Pb	49.59	39.7
As	54.42	43.5
Sum		160.0 ng/person-day
SalE7		
Hg	13.32	10.6
Cd	85.36	68.3
Pb	41.93	33.5
As	60.94	48.7
Sum		161.1 ng/person-day

The conclusion that can be drawn from these analyses is that ingestion of the projected amount of 0.8 mg salmocins/person-day from SALMOCIN-treated foods, based on measured levels of heavy metals, would expose consumers to low amounts of heavy metals relative to ambient and food-borne levels of exposures.

6.4 Low Potential for Development of Bacterial Resistance

We have also considered the potential for development of resistance to salmocins and its implications on safety. Like other bacteriocins, the mechanism of action of Gram-negative active salmocins entails multiple coordinated steps including receptor-recognition, active translocation to the cellular target, and pore formation or macromolecular destruction depending on the molecule. Although resistance and tolerance of target bacteria to Gram-positive and Gram-negative bacteriocins is known (e.g. Alonso (2000); Bastos (2015); Martinez (2016)), including co-synthesis of immunity proteins by bacteriocinogenic strains of bacteria, resistance development appears to be a rare event in nature (Martínez 2016) and if it occurs, it typically involves resistance to a single bacteriocin rather than to multiple bacteriocins in a class (Schamberger 2005).

The reason for this infrequency is that the target cell would need to simultaneously mutate more than one cellular structure to avoid the action of the bactericide, making avoidance to attack improbable. When resistance has been documented, it appears to have resulted more from random mutational changes than from bactericide pressure (Bastos 2015).

Salmocins and colicins are highly similar in structure and action. Our own studies with colicins failed to select resistant strains of *E. coli* upon multi-generational exposure of surviving bacteria (GRN 593). In brief, we conducted a series of studies wherein a tester strain of *E. coli* (pathogenic strain O157:H7) was applied to apples, subsequently treated with COLICIN product or a control plant extract containing no colicins, and

any surviving colonies were re-grown and re-exposed to COLICIN product, repeating the experiment in series and testing susceptibility to the active ingredients. Under such realistic application conditions in produce, we found no development of resistance to COLICIN components, including a two-component mixture of colicins M + E7, and a five-component mixture of colicins M + E7 + K + B + 5. The methodology used in these assays and the results obtained were presented in detail in GRN 593.

Due to the very high similarity of salmocins and colicins, including their mode of action, we deduce that the use of salmocins on food as bactericides and/or consumption of salmocins through ingestion, regardless of level, are unlikely to select for salmocin-resistant bacteria in food processing scenarios or in the intestinal tract of humans. Even if resistance did develop, no significant health impact is expected for the following main reasons:

- Application of salmocins to foods to control contamination by *Salmonella* exerts a potent but short-lived technical effect not conducive to resistance development; a process that more typically entails prolonged exposure to low levels of antimicrobials (Cotter 2013);
- Cooking salmocin-containing foods prior to ingestion will lead to inactivation of the salmocins by thermal denaturation of the polypeptides;
- Even without cooking/heating, ingested salmocins will be denatured by the low pH of the stomach;
- Salmocin-class proteins potentially surviving stomach acid will be rapidly digested by proteases in the upper and mid gastrointestinal tract;
- Commensal *Salmonella* are opportunistic members of the microbiome of asymptomatic carriers that comprise a small segment of the population (~2.2%–5%). Hence, elimination of resident strains by oral consumption of salmocins (improbable due to their instability) should not have a negative health impact since *Salmonella* is not considered a beneficial or essential gut microorganism; and
- Even if *Salmonella* strains became resistant, their pathology is not known to be mediated by salmocins; hence, salmocin-resistant pathovars are not expected to be more virulent or pathogenic than salmocin-susceptible strains.

We also considered whether there might be an impact on commensal *E. coli* from ingestion of salmocins, since due to the close relatedness of the genera some salmocins may be toxic to non-pathogenic *E. coli* strains (Cascales 2007; Schneider 2018). Our conclusion is similar, in that (a) it would be highly improbable for salmocins to reach the colon via oral ingestion due to instability and (b) elimination of intestinal *E. coli* should not physiologically impact the health of the host. In situations where both *E. coli* and *Salmonella* are commensal, both colicins and salmocins would likely already be produced endogenously in this population of carriers.

In sum, results from our own studies and from literature reports on the structure and function of bacteriocins and the conditions for development of resistance to bacteriocins, suggest that resistance development to salmocins should be unlikely.

6.5 Low potential for Development of Allergenicity or Immunogenicity

Although recombinant salmocins have the same amino acid sequence as natively encoded *Salmonella* salmocins to which humans and other animals may have been exposed throughout evolution, allergenic or immunogenic reactions cannot be ruled out *a priori* because salmocins are, after all, foreign proteins.

As summarized in Section 6.2, it is highly unlikely that ingestion of significant amounts of salmocins from food treatment will be experienced due to the instability of these proteins. Nevertheless, for verification, the potential for induction of allergic responses was investigated at the molecular level by analyzing the amino acid sequences of plant-produced salmocins for known immunogenic/allergenic domains against databases of known allergenic epitopes (e.g. AllergenOnline (2018)). Proteins with greater than 50% identity to known allergens are considered potentially allergenic (Aalberse 2000). CODEX Alimertarius (2003), lists 35% as the minimum homology required for categorizing a protein as potentially allergenic.

More detailed sequence searches at the 80-mer and 8-mer level were also conducted because searching by different peptide sequence lengths reduces the probability of both false-positive and false-negative results.

Allergenicity/hypersensitivity protein domain searches *in silico*

The following amino acid sequences for the five salmocins that could comprise the SALMOCIN product formulation were analyzed using the AllergenOnline database v18B (released March 23 2018; accessed May 15-16 2018; http://allergenonline.org/databasefasta.shtml) to assess whether Notifier's salmocins shared amino acid sequences with known allergens, including newly identified and peer-reviewed allergens.

SalE2 583 aa (GenBank: KTM78572.1)

```
MSGGDGIGHN SGAHSTGGVN GSSSGRGGSS SGGGNNPNSG PGWGTTHTPD GHDIHNYNPG EFGGGGHKPG GNGGNHSGGT
GDGQPPGAAM AFGFPALVPA GAGGLAVTVS GDALAAAIAD VLAVLKGPFK FGAWGIALYG ILPTEIAKDD PRMMSKIVTS
LPADAVTESP VSSLPLDQAT VSVTKRVTDV VKDERQHIAV VAGVPASIPV VDAKPTTHPG VFSVSVPGLP DLQVSTVKNA
PAMTALPRGV TDEKDRTVHP AGFTFGGSSH EAVIRFPKES GQAPVYVSVT DVLTPEQVKQ RQDEENRRQQ EWDATHPVEV
AERNYRLASD ELNRANVDVA GKQERQIQAA QAVAARKGEL DAANKTFADA KEEIKKFERF AHDPMAGGHR MWQMAGLKAQ
RAQNEVNQKQ AEFNAAEKEK ADADAALNVA LESRKQKEQK AKDASDKLDK ENKRNHPGKA TGKGQPVGDK WLEDAGKEAG
APVPDRIADK LRDKEFKNFD DFRKKFWEEV SKDPELSKQF IPGNKKRMSQ GLAPRARNKD TVGGRRSFEL HHDKPISQDG
GVYDMDNIRV TTPKLHIDIH RGK
```

SalE3 587 aa (GenBank: GAS18013.1)

```
MSGGDGRGHN TGAHSTSGNI NGGPTGLGVS GGASDGSGWS SENNPWGGGS GSGIHWGGGS GRGNGGGNGN SGGGSGTGGN
LSAVAAPVAF GFPALSTPGA GGLAVSISAS ELSAAIAGII AKLKKVNLKF TPFGVVLSSL IPSEIAKDDP NMMSKIVTSL
PADDITESPV SSLPLDKATV NVNVRVVDDV KDERQNISVV SGVPMSVPVV DAKPTERPGV FTASIPGAPV LNISVNNSTP
AVQTLSPGVT NNTDKDVRPA GFTQGGNTRD AVIRFPKDSG HNAVYVSVSD VLSPDQVKQR QDEENRRQQE WDATHPVEVA
EREYENARAE LEAENKNVHS LQVALDGLKN TAEGLALSDA GRHPLTSSES RFVAVPGYSG GGVHFDATAT VDSRDRLNSL
LSLGGAAYVN NVLELGEVSA PTEDGLKVGN AIKNAMIEVY DKLRQRLITR QNEINHAQVS LNTAIESRNK KEEKKRSAEN
KLNEERNKPR KGTKDYGHDY HPAPETEEIK GLGDIKKGIP KTPKQNGGGK RKRWIGDKGR KIYEWDSQHG ELEGYRASDG
QHLGSFDPKT GKQLKGPDPK RNIKKYL
```

SalE7 585 aa (GenBank: KSU39545.1)

```
MSGGDGIGHN SGAHSTGGVN GSSSGSGGSS SGSGNNPNSG PGWGTTHTPN GDIHNYNPGE FGGGGNKPGG HGGNSGNHDG
SSGNGQPSAA PMAFGFPALA PAGAGSLAVT VSGEALSAAI ADIFAALKGP FKFGAWGIAL YGIMPTEIAK DDPNMMSKIM
TSLPADTVTD TPVSSLPLDQ ATVSVTKRVA DVVKDERQHI AVVAGVPMSV PVVDAKPTTR PGIFSATVPG LPALEVSTGK
SIPASTALPR GITEDKDRTE HPAGFTFGGS SHDAVIRFPK ESGQAPVYVS VTDVLTPEQV KQRQDEESRR QQEWDATHPV
EVAERNYRLA SDELNRVNAD VAGKQERQAQ AGQAVAARKG ELDAANKTFA DAKEEIKKFE HFARDPMAGG HRMWQMAGLK
AQRAQNEVNQ KQAEFDAAEK EKADADAALN AALESRKQKE QKAKDTKERL DKENKRNQPG KATGKGQPVS DKWLEDAGKE
SGSPIPDSIA DKLRDKEFRN FDDFRKKFWE EVSKDPELSK QFIKGNRDRM QVGKAPKSRK KDAAGKRTSF ELHHDKPVSQ
DGGVYDMDNL RITTPKRHID IHRGQ
```

SalE1a 483 aa (GenBank: OIN35410.1)

```
MADNTIAYYE DGVPHSADGK VVIVIDGKMP VDTGAGGTGG GGGGKVGGTS ESSAAIHATA KWSTAQLKKT LAEKAARERE
TAAAMAAAKA KRDALTQHLK DIVNDVLRHN ASRTPSATDL AHANNMAMQA EAQRLGRAKA EEKARKEAEA AELAFQEAER
```

QREEAVRQLA ETERQLKQAE EEKRLAALSD EARAVENARK NLDTAKSELA NVDSDIERQR SQLSSLDADV KKAEENLRLT
MRIKGRIGRK MQAKSQAIVD DKKRIYSDAE NVLNTMTVNR NLKAQQVTDA ENELKVAIDN LNSSQMKNAV DATVSFYQTL
TEKYGEKYSL IAQELAEKSK GKKIGNVDEA LAAFEKYKDV LDKKFSKADR DAIVNALKSF NYDDWAKHLD QFAKYLKITG
HVSFGYDVVS DVLKASETGD WKPLFITLEQ KVLDTGMSYL VVLMFSLIAG TTLGIFGVAI ITAILCSFVD KYILNALNDA
LGI

SalE1b 527 aa (GenBank: OIN32443.1)

MSDNTIAYYE DGVPYSADGQ VVIVIDGKMP VDTGAGGTGG GGGGKVGGTS ESSAAIHATA KWSKAQLQKS LEEKAARERE
TAAAMAAAKA KRDALTQHLK DIVNDVLRYN ASRTPSATDL AHANNMAMQA EAQRLGRAKA EEKARKEAEA AEKSLQEAER
QREEAARQRA EAERQLKQAE AEEKRLAALS EEARAVEITQ KNLAAAQSEL SKMDGEIKSL NVRLSTSIHA RDAEMNSLSG
KRNELAQESA KYKELDELVK KLEPRANDPL QNRPFFDATS RRARAGDTLA EKQKEVTASE TRINELNTEI NQVRGAISQA
NNNRNLKVQQ VTETENALKV AIDNLNSSQM KNAVDATVSF YQTLTAKYGE KYSLIAQELA EQSKGKKISN VDEALAAFEK
YKDVLDKKFS KADRDAIVNA LKSVDYADWA KHLDQFSRYL KISGRVSTGY DIYSDIRKGM DTNDWRPLFL TLEKLAVDAG
VGYIVALGFS VIASTALGIW GVAIITGVIC SFVDKKDLEK LNEALGI

The results of informatic searches are summarized.

Results

Salmocins' amino acid full-length, sliding window 80-mer, and exact match 8-mer sequence searches *in silico*

The complete amino acid sequence of each plant-produced salmocin was scanned for potentially allergenic or hypersensitivity inducing sequences. The results are summarized in Table 6-3 and described in the accompanying text for each salmocin entry. Plant-produced salmocins having amino acid sequence similarities to known allergens of greater than 50% (Aalberse 2000) could be considered potentially allergenic. However, Table 6-3 also shows similarity at the more stringent threshold of >**35% identity** to known allergens (CODEX Alimentarius 2003). Using criteria from these algorithms all salmocins were found to have low potential for inducing an allergic response.

Table 6-3. Bioinformatic amino acid scan for potentially allergenic sequences in plant-made salmocins

Salmocin	Number of salmocin amino acid sequences with ≥50% similarity at the full-sequence level and ≥35% full-alignment identity to known allergen sequences at the 80-mer or 8-mer level			Allergenicity Potential
	Full seq	80-mer	8-mer	
SalE2	11	2	0	low
SalE3	14	6	0	low
SalE7	7	1	0	low
SalE1a	74	0	0	low
SalE1b	72	1	0	low

SalE2. A bioinformatic FASTA search of the full 583 amino acids of plant-produced SalE2 revealed only a distant relationship to 11 AllergenOnline database entries with similarities of at least 50%, including 4 for ragweed pollen (*Ambrosia* sp.) a respiratory allergen. However, none of these were higher than 34.7% identity. A more specific bioinformatic comparison at the 80-mer level (sliding window of 504 80-mers in 583 aa) revealed 2 hits, with 37% identity to ragweed pollen protein (a respiratory allergen) and 36.1% identity to bovine collagen. An even more precise search at the 8-aa level revealed no (zero) exact matches to any allergen. In sum, only two amino acid sequences in salmocin SalE2 were found to match known proteinaceous allergens at slightly above the threshold >35% identity level and hence the allergenic potential of SalE2 should be considered low.

SalE3. A bioinformatic FASTA search of the full 587 amino acids of salmocin SalE3 revealed 14 aa sequences that are 50-61.8% similar to known allergens. Of these, the top four were most similar to portions of ragweed protein (*Ambrosia* sp). More precise sliding window 80-mer scan (508 80-mers in 587 aa) revealed 6 hits with 35-39% identity mostly to ragweed protein (a respiratory allergen). However, an 8-mer exact-match search revealed no (zero) sequences with >35% identity to any known allergens. Hence, the allergenic potential of SalE3 should be considered low.

SalE7. A bioinformatic FASTA search of the full 585 amino acids of SalE7 revealed 7 aa sequences with >50% similarity to known allergens, including 4 sequences with $\geq$60% similarity to ragweed protein (*Ambrosia* sp.) a respiratory allergen, 2 sequences 58.2% similar to dust mite protein (*Dermatophagoides*), and 1 sequence 76.7% similar to an *Aspergillus* protein. A more precise sliding window 80-mer scan (506 80-mers in 585 aa) revealed 1 sequence with 36.2% identity to a known allergen (26.1% full-alignment identity to a protein from the herring worm *Anisakis simplex*). An 8-mer exact match search revealed no (zero) identities to any known allergen in the database. The allergenic potential of salmocin SalE7 should be considered low.

SalE1a. A bioinformatic FASTA search of the full 483 amino acids of salmocin SalE1a revealed 74 aa sequences with >50% similarity to known allergens, predominantly variants of tropomyosin or paramyosin (69/74). A more specific sequence search at the sliding 80-mer level (404 80-mers in 483 aa) revealed 54 sequences with 35-39.9% identity to known allergens but no (zero) hits at the full-alignment level with $\geq$35% identity to known allergens. Likewise, an exact match 8-mer search revealed no (zero) matches of $\geq$35% identity to any known allergens. Hence, the similarity of SalE1a to tropomyosin and paramyosin proteins from various natural sources can be considered coincidental and given the lack of exact sequence matches the allergenic potential of SalE1a should be considered low.

SalE1b. A bioinformatic FASTA search of the full 527 amino acids of salmocin SalE1b revealed 72 sequences in the protein having >50% similarity to allergen sequences in the database. All but 7 of those sequences were similar to tropomyosin (65/72) and less commonly similar to paramyosin (5/72) proteins from various sources. A more specific 80-mer level search (448 80-mers in 527 aa) revealed 21 sequences with 35-41.5% identity to known allergens but only 1 sequence at the full-alignment level with >35% identity (36.1%) to a mite allergen (*Dermatophagoides farinae*). Importantly, a precise 8-mer exact match search revealed no (zero) matches to any known allergens. The allergenic potential of salmocin SalE1b can be considered low.

Conclusion

Amino acid sequence scans at 80-mer granularity revealed only 10 sequences at the full-alignment level for all salmocins combined that could potentially cross react with IgE generated against the corresponding allergens. However, in the five salmocin proteins analyzed for specific sequence identity, comprising a total

of 2,765 amino acids, there were no (zero) specific peptide sequences matching the sequences in known proteinaceous allergens. Hence, the potential for allergenicity/hypersensitivity of plant-made salmocins is considered low. This low risk is corroborated by the lack of reports in the literature linking bacteriocins of the colicin class, which includes *Salmonella*-specific colicins (salmocins), with development of allergenicity or hypersensitivity.

Furthermore, intact salmocin proteins applied to food do not survive cooking, stomach acid or digestive enzymes once ingested, and low MW peptides generated during digestion of proteins are not known to interact with the immune system in deleterious ways (Dupont 2010), further lowering the concern over allergenicity.

In addition to the salmocin active ingredients, the formulated product SALMOCIN may contain small amounts of proteinaceous residues derived from the host plant and/or the process of genetic induction (i.e. vector). The salmocins are purified by chromatography in the downstream component of the manufacturing process, and hence proteinaceous impurities would be present in low amounts. For example, a salmocin mixture that is >70% salmocin protein would contain <30% non-salmocin proteins in the final formulation.

Considering that salmocins would be applied to food at $\leq$3 ppm, any residual protein impurity that might pose an allergenic concern would be co-applied with salmocins at <1 ppm, and also face denaturation or degradation through food preparation and/or on-matrix digestion. Hence, the allergenic potential of any proteinaceous impurity in the SALMOCIN product is expected to be low as well.

6.6 Safety in Relation to Dietary Intake of SALMOCIN

In Section 3 we summarized Notifier's estimated SALMOCIN application rate and projected potential intake based on consumption of red meats, poultry, sea food and egg products treated with SALMOCIN. The projected intake was 0.8 mg salmocins/person-day from SALMOCIN treatment of food, assuming 100% market utilization of Notifier's product. We rounded that figure up to account for additional salmocins that consumers may ingest from foods and the environment and/or from commensal salmocin synthesis *in situ*. We estimated that this additional non-product exposure would be very low and assumed an additional 10% of 0.8 mg from SALMOCIN for a total of ~0.9 mg/person-day total salmocin exposure.

If viewed as food (safety) enzymes, salmocins can be considered non-toxic at the levels applied. As reviewed by Sewalt (2016), microbial enzymes applied to foods have an excellent record of safety. Enzymes are proteinaceous molecules with a globular structure produced by all living cells in order to perform the biochemical reactions required to support life. Enzymes operate within a narrow set of conditions, such as temperature and pH, and are subject to inhibition by various means. Enzymes are found to be ubiquitous in fresh and processed foods and have not been associated with toxicity in the human diet (Federal Register 2010). Much like other proteins, once ingested, enzyme proteins are generally easily broken down into their constituent amino acids and cofactors that are indistinguishable from other food molecules.

From a safety point of view, the high specificity of salmocins can be considered one of their most desirable traits. The fact that these enzymes specifically destroy a target pathogen such as *S. enterica* without affecting the beneficial commensal microbiome gives them an advantage over many commonly used chemical preservatives.

With respect to nutritional content, at the projected application rates of <3 mg salmocin/kg food, even if all of the salmocins were to survive in cooked foods and be ingested at the levels applied, daily per capita

ingestion would be ≤1 mg salmocin/person-day. Such a minute amount of salmocins would represent <0.001% of the total adult daily protein intake and is nutritionally insignificant.

No reports have appeared in the literature linking consumption (ingestion) of any bacteriocin with onset of disease, morbidity or mortality. In large part, this can be explained by the specificity of these molecules for bacterial target structures, plus the evolutionary adaptation by humans and animals to bacteriocin protein exposure, including immune tolerance.

Importantly, the physicochemical properties of salmocins that are shared with colicins, notably their instability to heat and their susceptibility to enzymatic digestion (e.g. degradation in the gastric and upper intestinal environments; (Cascales 2007; Schneider 2018) contribute significantly to their safety profile and support the use of salmocins as antimicrobials for food safety interventions. Meats and egg products are typically cooked domestically and in most other countries; therefore, the level of intake of salmocins from use of the SALMOCIN product in meats, sea food and egg products, which are some of the most susceptible foods to *C. perfringens* contamination, is expected to be very low, and so is the estimated risk to consumers.

6.7 Effect of SALMOCIN Application on Organoleptic Properties of Food

No organoleptic assessment of foods treated with SALMOCIN were conducted in these studies because the treated substrates consisted of non-sterile raw meats and raw whole eggs. Solutions of single or mixed salmocin proteins are generally visually clear and have no objectionable odor. The solubles in the SALMOCIN product are typically applied at 0.1 – 3 mg/kg food (<3 ppm) initial concentration and become diluted by diffusion into the matrix after application.

There is no observed masking of the color of food after SALMOCIN application. No organoleptic changes are anticipated in raw foods after product application at the low rates specified. Importantly, no organoleptic impact is expected after cooking foods treated with SALMOCIN because cooking will destroy the proteins prior to consumption. Independent evaluation of organoleptic impact on treated foods will be conducted later in product development.

6.8 Non-Interference of SALMOCIN with Pathogen Detection Methods

The component salmocin proteins in the SALMOCIN product formulations are specific for *Salmonella* strains and exert antibacterial effects quickly even at low levels (0.1 – 3 ppm). In liquid suspension cultures, multi-log reductions in CFU are seen within 1 h of salmocin application (Section 2.4.1).

On-matrix studies with various foods susceptible to *Salmonella* contamination showed similar results. Raw chicken meat with or without skin inoculated at 3-4 log CFU/g contamination levels with a mixture of pathogenic *S. enterica* strains were sampled at 1 h, 24 h, 48 h and 72 h under low temperature yet growth-permissive conditions. Uniformly, rapid and significant reductions in CFU were seen at the earliest time of sampling, followed by normal growth of surviving bacteria at permissive temperature in SALMOCIN-treated samples relative to control treatments as the technical effect diminished.

Similarly, raw beef cuts inoculated with 3-4 log CFU/g of mixed serotype *S. enterica* strains and treated with 0.5-5 mg salmocin/kg at 10°C showed dramatic reductions in viable bacteria (99.96% to 100%, or 3.0 to 6.4 $\Delta\log_{10}$ CFU/g). Raw tuna fillet contaminated with mixed *S. enterica* strains at 3-4 log CFU/g and treated similarly with 0.5 or 5 mg/kg salmocin at 10°C showed 1.7 to 3.0 $\log_{10}$ CFU/g (98.06% to 99.90% mean CFU

reduction) relative to vehicle control, with visible regrowth of surviving bacteria at permissive temperature at 48 h.

Lastly, raw whole egg homogenate contaminated at 3-4 log CFU/g with mixed serotype *S. enterica* strains and treated with salmocin at 0.5 or 5 mg/kg at 10°C showed 3.7 to >8 $\log_{10}$ CFU/g reductions in bacterial viability (nearly 100% kill) yet surviving bacteria were viable at the 48-h time sample.

Inspection of meat and egg products for the presence of pathogens can be done by taking samples from meat surfaces or egg mixtures prior to and after SALMOCIN application. As shown in the studies reported in this Notice, any viable (surviving) bacteria will grow after SALMOCIN treatment if incubated under permissive growth conditions. Therefore, SALMOCIN application should not interfere with pathogen determination methods used in food safety analyses during food processing, including protein-protein interactions for ELISAs, or PCR-based amplification reactions.

6.9 Occupational and In-Plant Safety Related to Use of SALMOCIN Product

The safety of consumers and the occupational safety of in-plant inspectors and food processing industry personnel were prioritized during the earliest stages of SALMOCIN product development. The product is undergoing formulation optimization; as such, to date no in-plant testing in industrial food processing (including meat or egg processing) facilities has taken place.

The antibacterial proteins comprising SALMOCIN are very similar to colicins synthesized in the human commensal microbiome as well as ingested naturally in the diet. The salmocin proteins used in the SALMOCIN product have low allergenic potential. Salmocins made in non-food species, such as in *N. benthamiana*, are purified to reduce residual plant alkaloids to non-toxic levels at the application rates used on various foods. Therefore, any residual host or process impurities remaining in the SALMOCIN formulation should be safe and not pose undue risk to plant personnel. In addition, the excipients in the formulation are food grade and approved for food use.

Consequently, only minimal personnel protection should be required during product preparation for application, and during application and disposal. Protective devices such as a mask, goggles and gloves are suggested as a precaution to prevent inhalation, eye and skin exposure to particulates of the dry concentrated product. Aerosols may be generated when the solubilized product is applied as a spray, but spray cabinets should minimize exposure.

Specific use procedures, personnel protection practices, and additional safety, use, storage and disposal information will be included in the product label and safety data sheet (see APPENDIX A for draft SDS), as well as included in individual HACCT plans.

7 Supporting Data and Information

Multiple sources of information were used to support the conclusion that the SALMOCIN product is GRAS when used as intended. Table 7-1 lists the various data and other information discussed in this Notice and used in reaching this conclusion. Also listed in the table is whether the specific information cited was generated by Notifier and/or obtained from databases or references in the public domain.

Table 7-1. Information supporting SALMOCIN GRAS determination

Topic	Document	Location	Source	Availability
Salmonella enterica biology; role in food contamination	This Notice	Introduction, pg 1-2 Section 2.4, pp 18-21	(CDC 2016); (CDC 2017b) ; (CDC 2017a); (FDA 2016); (Barker 1980); (Todar 2012); (USDA FSIS 2005)	Public
Source, traits and selection of target *S. enterica* pathovars	This Notice	Section 2.4, pp 18-21 Table 2-3, pp 19-21	(CDC 2016); (Schneider 2018)	Public
Salmocins' mode of action; properties; specificity; spectrum of activity	This Notice	Section 2.1, pp 11-14 Table 2-1, pg 11	(Barker 1980); (Campos 1988); (Cascales 2007); (Graham 1977); (Guterman 1975); (Nedialkova 2014); (Schneider 2018); (Vicente 1984)	Public
History of human exposure to *Salmonella* and salmocins, including exposure from food	This Notice	Section 6.1, pp 47-50	(Barker 1980); (Campos 1988); (Cascales 2007); (Gillor 2008); (Hossneara 2007); (Marzel 2016); (Monack 2012, 2013); (Nedialkova 2014); (Obi 1978); (Ruby 2012); (Schamberger 2002); (Smarda 2001); (Todar 2012); (Vicente 1984)	Public
Lack of glycosylation; low risk from small modifications to proteins; safety of enzymes added to food	This Notice	Section 2.3, pg 14-15 Section 6.6, pp 58-59	(Kamionka 2011); (Sewalt 2016); (Federal Register 2010); (Schneider 2018)	Public
Safety of salmocin production organism	GRN 593	Section A.2.2, pp 25-26 Appendix B, pp 51-57	Notifier	Public
Safety of production host species	GRN 593	Section A.2.2, pp 26-28	Multiple references	Public
Safety of manufacturing process	GRN 593	APPENDIX B, pp 51-57	Notifier	Public
(Continued)				

Topic	Document	Location	Source	Availability
Safety of edible plant hosts and process impurities	GRN 593	Section A.2.2, pp 27-28	Notifier	Public
Safety of *Nicotiana benthamiana* plant host, host and process impurities	GRN 775 This Notice	Section 6.1, pp 28-39 Section 6.2, pp 50-52 Table 6-1, pg 51 Section 6.3, pp 52-53 Table 6-2, pg 53	Notifier (ASTSDR 1999); (Ryan 2001); Notifier	Public Public, and to be made public via this GRN
Salmocin digestibility and degradability	This Notice	Section 2.4.3, pp 38-39	(Schneider 2018)	Public
Low potential for development of bacterial resistance	GRN 593 This Notice	APPENDIX A, pg 34 APPENDIX C, pp 64-66 Section 6.4, pp 53-54	(Alonso 2000); (Bastos 2015); (Cotter 2013); (Martínez 2016); (Schamberger 2005)	Public
Low potential for development of allergenicity or immunogenicity	This Notice	Section 6.5, pp 54-58	(Aalberse 2000); (AllergenOnline 2018); (Schneider 2018); Calculated by Notifier	Public To be made public via this GRN
Safe ingestion estimates of salmocins applied to various foods from treatment with SALMOCIN product	This Notice	Section 3.2, pg 45 Table 3-2, pg 46 Section 6.6, pp 58-59	Calculations of salmocin intake based on application rates and food consumption data from: (USDA ERS 2014a); (USDA ERS 2014b), (2015); CDC NHANES (Daniel 2011); (USDA WASDE 2016); (USDA 2015 Dietary Guidelines); (USDA ERS 2017)	Public To be made public via this GRN
Safety from additive consumption of salmocins from applied SALMOCIN product and from other sources of salmocins	This Notice	Section 3.3, pg 46 Table 3-2, pg 46	Notifier	To be made public via this GRN
Non-interference with pathogen-detection methods	This Notice	Section 6.8, pp 59-60	Notifier	To be made public via this GRN
Occupational and in-plant inspector safety of SALMOCIN product usage	This Notice	Section 6.9, pg 60 APPENDIX A, pp 67-72	Notifier	To be made public via this GRN

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APPENDIX A. SALMOCIN Safety Data Sheet

NOMAD

SALMOCIN SAFETY DATA SHEET
Version 1.0
DRAFT – Date 1 October 2018
Print Date 5 October 2018

1. PRODUCT AND COMPANY IDENTIFICATION

1.1 Product identifiers

Product name : SALMOCIN, mixture of *Salmonella*-specific colicin-class bacteriocins produced in plants
: Contains one or more of the following proteins (Registry Number = GeneBank Entry No): SalE2 (KTM78572.1), SalE3 (GAS18013.1), SalE7 (KSU39545.1), SalE1a (OIN35410.1), SalE1b (OIN32443.1)

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

Identified uses : Antibacterial processing aid to control *Salmonella enterica* in food

1.3 Details of the supplier of the safety data sheet

Company : Nomad Bioscience GmbH
Weinbergweg 22
Halle 02160, Germany
Telephone : +49 345 555 9887
Fax : +49 345 1314 2601

1.4 Emergency telephone number

Emergency Phone # : +49 345 555 9887 in the EU (US emergency phone number to be provided)

2. HAZARDS IDENTIFICATION

2.1 Classification of the substance or mixture

Not a hazardous substance or mixture

2.2 GHS Label elements, including precautionary statements

Not a hazardous substance or mixture

2.3 Hazards not otherwise classified (HNOC) or not covered by GHS

None

3. COMPOSITION/INFORMATION ON INGREDIENTS

3.1 Substances

GeneBank Registry Numbers : See Section 1.1
No ingredients are hazardous according to OSHA criteria
No components need to be disclosed according to the applicable regulations

4. FIRST AID MEASURES

4.1 Description of first aid measures

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration

In case of skin contact
Wash off with soap and plenty of water

In case of eye contact
Flush eyes with water as a precaution

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water

Page 1

4.2 Most important symptoms and effects, both acute and delayed
None known. See section 2.2 and/or Section 11

4.3 Indication of any immediate medical attention and special treatment needed
No data available

5. FIREFIGHTING MEASURES

5.1 Extinguishing media - Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide

5.2 Special hazards arising from the substance or mixture
Nature of decomposition products not known

5.3 Advice for firefighters
Wear self-contained breathing apparatus for firefighting if necessary

5.4 Additional information
No additional information is available

6. ACCIDENTAL RELEASE MEASURES

6.1 Personal precautions, protective equipment and emergency procedures
As with any concentrated protein, avoid dust formation and inhalation of particulates or aerosols. For personal protection see Section 8

6.2 Environmental precautions
Product active ingredients are biodegradable. No special environmental precautions are necessary

6.3 Methods and materials for containment and cleaning up
Sweep up and shovel solid. Water-wash surfaces. Use closed containers for disposal of any unused product

6.4 Reference to other sections
For disposal see Section 13

7. HANDLING AND STORAGE

7.1 Precautions for safe handling
Provide appropriate exhaust ventilation during preparation and use. For precautions see Section 2.2

7.2 Conditions for safe storage, including any incompatibilities
Keep container tightly closed. Store dry at 2 - 8 °C

7.3 Specific end use(s)
Apart from the uses mentioned in Section 1.2 no other specific uses are stipulated

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

8.1 Control parameters

Components with workplace control parameters
Contains no substances with occupational exposure limit values

8.2 Exposure controls

Appropriate engineering controls
General industrial hygiene practice

Personal protective equipment

Eye/face protection
Use government tested and approved eye protection devices (e.g. NIOSH - US or EN 166 - EU)

Skin protection
Handle with gloves that are inspected prior to use. Use proper glove removal technique to avoid skin contact. Dispose of used gloves in accordance with applicable laws and good laboratory practices. Wash and dry hands

Body Protection
Wear lab coat or similar cover during preparation, application and disposal of product in keeping with specific practices in

the work environment. This recommendation is advisory only and must be evaluated by an industrial hygienist and safety officer familiar with the specific situation of anticipated use by the customers. It should not be construed as offering an approval for any specific use scenario.

Respiratory protection
Use type N95 (US) or type P1 (EN 143) dust masks, or respirators, depending on the product formulation and preparation and use environment. Use devices approved under appropriate government standards such as NIOSH (US) or CEN (EU)

Control of environmental exposure
Product components are biodegradable and will be diluted during use. No special procedures for controlling environmental exposure are recommended

9. PHYSICAL AND CHEMICAL PROPERTIES

9.1 Information on basic physical and chemical properties

a)	Appearance form:	Granular solid, powder, or concentrated solution; white to light tan
b)	Odor	No specific odor
c)	Odor threshold	No odor threshold identified
d)	pH	pH 5-8, depending on the formulation
e)	Melting point/freezing point	No data available
f)	Initial boiling point and boiling range	No data available
g)	Flash point	No data available
h)	Evaporation rate	No data available
i)	Flammability (solid, gas)	No data available
j)	Upper/lower flammability or explosive limits	No data available
k)	Vapor pressure	No data available
l)	Vapor density	No data available
m)	Relative density	No data available
n)	Water solubility	>10 g/L
o)	Partition coefficient: n-octanol/water	No data available
p)	Auto-ignition temperature	No data available
q)	Decomposition temperature	No data available
r)	Viscosity	No data available
s)	Explosive properties	No data available
t)	Oxidizing properties	No data available

9.2 Other safety information
No additional information available

10. STABILITY AND REACTIVITY

10.1 Reactivity
No data available

10.2 Chemical stability
Stable under recommended storage conditions

10.3 Possibility of hazardous reactions
No data available

10.4 Conditions to avoid
No data available

10.5 Incompatible materials
No data available

10.6 Hazardous decomposition products
Other decomposition products - No data available. In the event of fire: See Section 5

11. TOXICOLOGICAL INFORMATION

11.1 Information on toxicological effects

Acute toxicity
No data available

Inhalation
No data available

Dermal
No data available

Skin corrosion/irritation
No data available

Serious eye damage/eye irritation
No data available

Respiratory or skin sensitization
No data available; estimated to have low allergenicity potential

Germ cell mutagenicity
No data available

Carcinogenicity

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as a probable, possible or confirmed human carcinogen by IARC

ACGIH: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH

NTP: No component of this product present at levels greater than or equal to 0.1% is identified as a known or anticipated carcinogen by NTP

OSHA: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by OSHA

Reproductive toxicity
No data available

Specific target organ toxicity - single exposure
No data available

Specific target organ toxicity - repeated exposure
No data available

Aspiration hazard
No data available

Additional Information
RTECS: Not available. Product is not a hazardous substance or mixture

12. ECOLOGICAL INFORMATION

12.1 Toxicity
No data available

12.2 Persistence and degradability
Active ingredients are destroyed by heat, acid, and by digestive and microbial enzymatic activity

12.3 Bioaccumulative potential
None anticipated

12.4 Mobility in soil
No data available; product is water-soluble and biodegradable

12.5 Results of PBT and vPvB assessment
PBT/vPvB assessment not available as chemical safety assessment not required/not conducted

12.6 Other adverse effects
No data available

13. DISPOSAL CONSIDERATIONS

13.1 Waste treatment methods

Product
Offer surplus and non-recyclable solutions to a licensed disposal company

Contaminated packaging
Dispose of as unused product

14. TRANSPORT INFORMATION

DOT (US)
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

SARA 302 Components
SARA 302: No chemicals in this material are subject to the reporting requirements of SARA Title III, Section 302

SARA 313 Components
SARA 313: This material does not contain any chemical components with known CAS numbers that exceed the threshold (De Minimis) reporting levels established by SARA Title III, Section 313

SARA 311/312 Hazards
No SARA Hazards

Massachusetts Right to Know Components
No components are subject to the Massachusetts Right to Know Act

Pennsylvania Right to Know Components
Salmocins (*Salmonella*-specific antibacterial proteins from plants). See Section 1.1

New Jersey Right to Know Components
Salmocins (*Salmonella*-specific antibacterial proteins from plants). See Section 1.1

California Prop. 65 Components
This product does not contain any chemicals known to State of California to cause cancer, birth defects, or any other reproductive harm

16. OTHER INFORMATION

HMIS Rating

Health hazard:	0
Chronic Health Hazard:	0
Flammability:	0
Physical Hazard:	0

NFPA Rating

Health hazard:	0
Fire Hazard:	0
Reactivity Hazard:	0

Additional information

The information contained in this Safety Data Sheet is believed to be correct as of the time of its release. It should be used as a guide for safe handling, storage, preparation, and disposal of the product. Assessment of product safety under conditions of normal use is based on information available at the time. Because information in some categories is lacking, this SDS is not all-inclusive and is subject to periodic updates. Nomad Bioscience GmbH and its Affiliates shall not be held liable for any damage resulting from handling, use, disposal or from contact with the above product.

APPENDIX B. SALMOCIN Manufacturing Process Summary

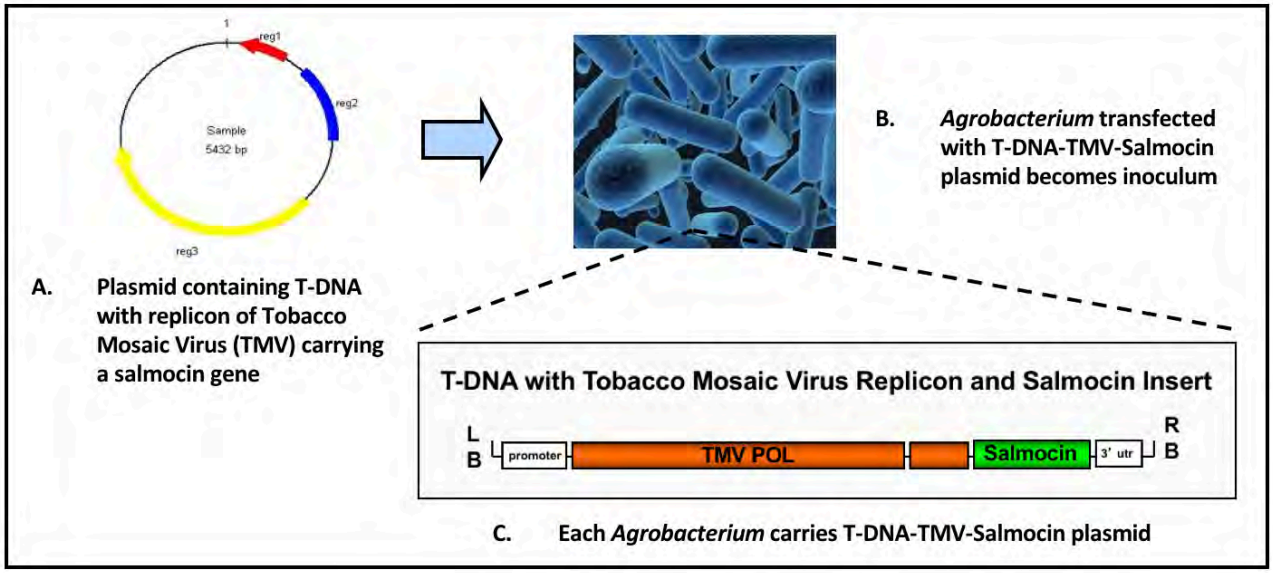
B.1 Introduction and Rationale

Notifier manufactures salmocin proteins recombinantly using a plant-based process very similar to the process described in GRN 775 and Schneider et al. (2018), modified for the current proteins. This approach minimizes concerns over toxicity of salmocins to the producing host and offers a more scalable and cost-effective manufacturing option relative to fermentation. In Notifier's process, leaf tissue of *Nicotiana benthamiana* can be induced to express salmocins by transient expression of a plant viral vector, such as tobacco mosaic virus (TMV) or potato virus X (PVX), containing the gene for the antimicrobial protein. The vector can be introduced into the host plant by vacuum-assisted infiltration, or by spraying the leaves with the vector admixed with a surfactant. The components of the expression system and host plants are prepared independently and subsequently combined. Alternatively, salmocins can be produced in the same host plant carrying transgenically the salmocin gene and an ethanol-inducible promoter, with induction by dilute ethanol. After induction with either method, salmocin protein is allowed to accumulate in leaf tissues for several days. Plants are subsequently harvested and the protein is extracted and concentrated from the plant material. Each salmocin is manufactured independently to meet its own active ingredient specification. Notifier's SALMOCIN product may be formulated to contain a single salmocin protein or blended as a mixture of two or more salmocins that act synergistically to control targeted pathogens.

B.2 Organism Used and Gene Expression Cassette

In the agroinduction method, the production organism *Agrobacterium tumefaciens* harboring a binary plasmid vector containing a TMV or PVX replicon with inserted salmocin gene is depicted in Figure B-1 (Source: Nomad Bioscience GmbH; TMV vector shown). Vectors are constructed by conventional molecular biology methods and maintained as Master and Working Plasmid Banks in *E. coli* (Figure B-1-**A**). The T-DNA vector encoding the TMV-salmocin replicon is introduced into *A. tumefaciens* to prepare the inoculum (Figure B-1-**B**). Each bacterium in the inoculum contains the T-DNA-TMV-salmocin plasmid (Figure B-1-**C**).

Figure B-1. Schematic of vector for salmocin expression in plants



SALMOCIN contains no live biological materials that were introduced in the upstream steps of the process (e.g. when using *Agrobacterium* and viral replicons). The process is generic in that it is applicable to the expression and isolation of a wide range of salmocins and other antimicrobials.

B.3 Procedure

A flow diagram summarizing the key steps in producing salmocin proteins is shown in Figure B-2. Summary descriptions of key process steps follow; step numbers correspond to the steps indicated in Figure B-2. The induction of gene expression can be accomplished by one of two alternative methods (described below), which share common downstream purification unit operations.

Step 1a. Inoculum production for *Agrobacterium* induction method

Proprietary industrial strains of *Agrobacterium tumefaciens* harboring binary plasmid vectors each containing a TMV replicon with an inserted gene for each salmocin are grown in defined medium under aseptic conditions following strict quality SOPs; this bacterial suspension constitutes the inoculum. Notifier's *Agrobacterium* strain is grown in medium containing de-mineralized water, yeast extract, peptones, minerals, kanamycin and rifampicin. The removal of residual antibiotics and fermentation chemicals is achieved by high dilution of the bacterial suspension before inoculation of plants and the ultra- and dia-filtration procedures during plant biomass extraction and processing. All raw materials and processing aids are food grade. A multi-vial Master Vector Bank of the vector is prepared and stored at -80°C, from which aliquots are removed as Working Vector Banks of the inoculum for each manufacturing batch.

Each Working Bank of *Agrobacterium* is handled in a way to reduce the risk of contamination by foreign microorganisms. This includes use of sterile materials for bacterial cultivation, quality control checks to ensure axenic culture, and confirmation of strain identity before plant inoculation. Samples not meeting criteria are rejected and disposed, and new aliquots are drawn from the Master Bank. If a problem is identified at the Master Bank level, a new Master Bank is generated and subjected to quality control procedures before further use.

Step 1b. Ethanol induction of transgenic plants

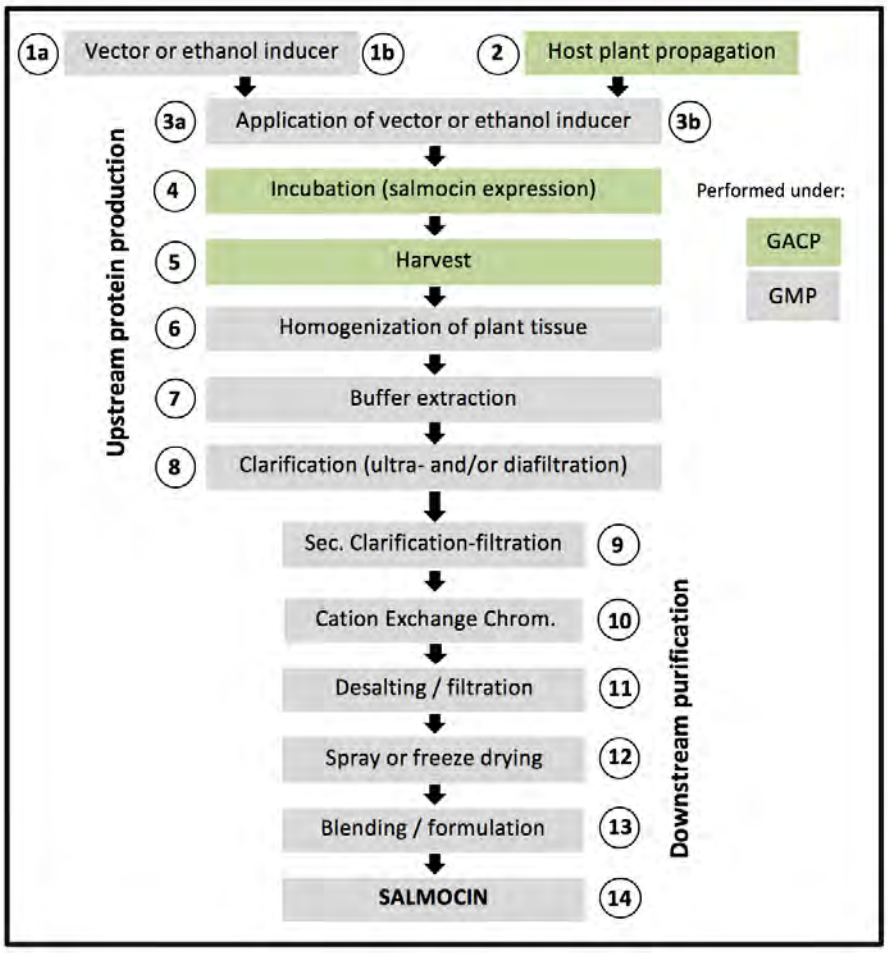
In this variation of the method, transgenic plants carrying an ethanol-inducible promoter are used. The procedure was developed by Notifier and described by Werner (2011). The process is based on inducible release of viral RNA replicons from stably integrated DNA pro-replicons. A simple treatment with dilute ethanol releases the replicon leading to RNA amplification and high-level production of the desired salmocin protein.

Step 2. Host plant propagation and preparation

For agroinduction, normal seeds of *Nicotiana benthamiana* are produced internally from stock plants. For ethanol induction, transgenic seeds of these host plants developed by Notifier are used, which contain the gene insert for the desired salmocin driven by an ethanol-inducible promoter.

With either method of induction, plants are propagated in trays using a food-crop compatible soil based substrate, fertilizer and water. For seeding, plant propagation, target expression and plant harvest, the principles of Good Agriculture and Collection Practices (GACP) are applied. All used materials underlie a quality management system ensuring a predefined quality.

Figure B-2. Summarized process diagram for SALMOCIN production in plants



Step 3a. Inoculation of host plants with agrobacterial vector

The *A. tumefaciens* inoculum carrying the selected salmocin replicon is applied to greenhouse-grown and quality tested host plants through the stomata (pores) in the leaves. The plant hence takes the place of a conventional "fermenter" in the production of the product. The *Agrobacterium* inoculum and the host plants are cultured under predefined and controlled conditions. At a specified time after seeding (e.g. 4-6 weeks), the plants are treated with a defined concentration of *Agrobacterium* in dilution buffer.

Inoculation of plants is accomplished by either vacuum-mediated infiltration after immersing the plant leaves in a suspension of the inoculum, or via a procedure wherein the inoculum is sprayed onto plant leaves mixed with a surfactant (Gleba 2014; Tusé 2014). Via either method, the agrobacteria are efficiently internalized into the plant and gain systemic distribution.

The agrobacteria infect the plant cells and insert the T-DNA plasmid into the nucleus, which initiates synthesis of salmocin-encoding RNA transcripts. Amplification of the transcript and translation of the salmocin RNA message into salmocin protein occurs in the cytoplasm of each plant cell. Neither the vector nor salmocin genes are integrated into seed or passed on to subsequent generations (i.e. no stable integration); thus, the expression of proteins via viral vectors is transient and the host plant is not genetically modified (not GMO).

Step 3b. Ethanol induction

In this variation of the method, a simple treatment of the transgenic plants carrying the salmocin gene with dilute ethanol (2.5% v/v) releases the replicon leading to RNA amplification and high-level salmocin expression. To achieve tight control of replicon activation and spread in the non-induced state, the viral vector is deconstructed, and its two components, the replicon and the cell-to-cell movement protein, have each been placed separately under the control of an inducible promoter (Werner 2011). Throughout the induction period, salmocin protein accumulates in the tissues of the host plant. The inducer (ethyl alcohol) is evaporated, metabolized and diluted during plant growth and is not found in the final product.

Step 4. Incubation

After agro-inoculation or ethanol induction, the plants are incubated for 5-10 days under controlled temperature, humidity, and light condition to allow for accumulation of the desired protein. During this incubation period, there is rapid systemic replication of the vector and expression and accumulation of the induced product.

Step 5. Harvest

Plants producing salmocin protein are harvested typically 4-9 days post inoculation/induction; the time-to-harvest can vary among expressed proteins. Samples of plant biomass are taken for analyses of salmocin protein content, general health and other process QC procedures prior to large-scale extraction. Plants in trays are transported to the cutting operation. The plants' aerial biomass (i.e. leaves and part of the stems) are mechanically cut and harvested into bins, which are transported to the extraction room.

Step 6. Homogenization of plant tissue

Cut plant biomass is disintegrated by homogenization in a grinder; the course plant material and fibers are removed, and the protein-containing soluble stream is further purified through a series of pH-assisted precipitations and filtration steps.

Step 7. Buffer extraction

The homogenate generated in Step 6 is extracted with specially formulated buffers to help precipitate major host cell proteins, resulting in a partially purified stream enriched for the salmocin protein.

Step 8. Primary clarification

Precipitated proteins and other impurities are removed by centrifugation and/or filtration.

Step 9. Secondary clarification

After clarification in Step 8, the process stream is pH-adjusted and can be further clarified. Additional impurities are removed by ultrafiltration and diafiltration; typically, impurities that are less than 5-10 kDa in mass are eliminated at this step.

Step 10. Chromatographic purification

The downstream processing (DSP) procedure employed when manufacturing salmocins (or other proteins) using *Nicotiana benthamiana* as the host plant includes chromatographic purification of the product-enriched stream from Step 9. For salmocins, cation-exchange chromatography (CIEX) is used. This step is

followed by desalting, buffer adjustment or additional filtration depending on the protein (**Step 11**). These extra purification steps remove additional residual host-cell proteins and plant metabolites such as polyphenols and host alkaloids, resulting in a clarified, enriched product with fewer impurities. The chromatographic step can be eliminated when manufacturing salmocins or other proteins using food species hosts such as spinach, leafy beet or lettuce, as those species have no pyridine alkaloids and their host proteins can be consumed in unlimited quantities.

Steps 12. Drying

After the precursor solution from Step 11 is stabilized and standardized by the addition of water, food-compatible pH regulators and sodium chloride, as needed, the solution is filter-sterilized and filled as a bulk liquid concentrate, or spray or freeze dried to produce a dry, off-white to light tan powdered product.

Step 13. Formulation, fill and finish

Salmocin mixes are blended into a final bulk product and packaged. Prior to release, the bulk products are tested to ensure compliance with the respective final product specification for SALMOCIN.

Step 14. SALMOCIN

The final, released salmocin mix from Step 13 constitutes the **SALMOCIN antimicrobial product**.

In-Process controls and quality assurance

In-process controls help manage the quality of process intermediates and final products throughout the manufacturing process. Materials not meeting pre-determined specifications are rejected. Product release is done after each batch passes stringent identity and potency tests. A Quality Management system ensures conformance with industry standards and federal and local regulatory guidelines.

B.4 Specification

The target Specification of the SALMOCIN product produced by this process is shown in Table B-1 (identical to Table 2-2).

Table B-1. Target Specification for SALMOCIN (mixed salmocin) Product

SALMOCIN Antimicrobial Dry Powder Formulation			
Parameter	**Method**	**Specification limit**	**Results of analyses[1]**
Appearance	Visual	Powder, white to beige	Conforms
Specific Activity (Potency; salmocin protein basis)[2]	Viability inhibition; *S. enterica* ssp. *enterica* serovar Enteritidis reference strain ATCC® 13076™	Minimum, based on least potent salmocin in blend: $\geq 1 \times 10^6$ AU/g protein	Average potency from salmocin blend: $3.52 \pm 4.14 \times 10^9$ AU/g protein
pH of a 1% solution in water	Potentiometric	6.0-7.5	Average 7.0 ± 0.5
Nicotine (per total salmocin blend)[3]	HPLC/MS	$\leq$ 0.4 μg/mg	Average 0.24 ± 09 μg/mg
Anabasine (per total salmocin blend)[3]	HPLC/MS	$\leq$ 0.1 μg/mg	Average 0.056 ± 0.05 μg/mg
Bioburden	USP32<61>	$\leq$ 10 CFU/25 g sample	0 (absent)
Agrobacterium (CFU/10 g sample)	Selective plate based assay	0 (absent)	0 (absent)
Undesirable microorganisms: *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp.* or coagulase-positive *Staphylococcus spp.*, per 25 g final product	USP32<1111>	0 (absent)	0 (absent)
Stability of dry concentrate product (0-10°C storage)[4]	Specific activity at T_n vs. T_0; plate-based assay	> 6 months	> 6.25 months (average) at time of GRN submission

[1]Results of analyses for a dry SALMOCIN (mixed-salmocin) product are based on average results obtained from analyses of individual salmocin proteins blended at a ratio (dwb) of 1x each SalE1b and SalE7.

[2]Measured potency values for Specification are based on potency averages $\pm$ s.d. of multiple non-consecutive developmental batches of salmocins SalE1b (4 batches) and SalE7 (5 batches).

[3]Alkaloid impurity values are averages derived from a minimum of 3 non-consecutive developmental batches of protein.

[4]Stability results are interim; all salmocins are in a continuing stability program. Stability is calculated from potency vs. time of storage for dry salmocins and salmocin solutions under different storage conditions and are expressed as averages from a minimum of 3 non-consecutive developmental batches.

B.5 Manufacturing Facilities

Notifier can manufacture SALMOCIN at various locations in Europe and the United States. For commercial manufacture, semi-automated plant cultivation, inoculation, incubation and harvesting systems can be applied. Depending on the scale needed, Notifier can manufacture at its own facilities or use a contract manufacturing organization to produce and formulate salmocin proteins meeting Notifier's specification. Features of an existing US facility's upstream and downstream processing capabilities include:

Upstream

- 80,000 sq ft of controlled growth space with 672 tables holding 30,240 plant trays in 3 levels. Each tray holds 104 plants
- Controlled conditions for the growth and harvest of transfected plants
- An automated plant movement system allowing movement, irrigation, lighting and environmental control (temperature and humidity) of trays for plant growth

Downstream

- 32,000 sq ft manufacturing area
- Linear scalability: 1 metric ton (mt)/shift pilot – 68 mt/shift commercial
- 75 L of Green Juice (post-grind/pre-clarification extract) per minute
- Continuous processing prior to UF
- 35,000 L of tank storage capacity
- Heating, cooling of in-process material
- Manufacturing clean rooms with controlled environments
- Computer-controlled processing and data collection
- Clarification options (UF/DF/Microfiltration/Nanofiltration/Reverse Osmosis)

Regardless of manufacturing venue, all substances, materials and reagents used in manufacturing SALMOCIN by Notifier's process conform to food grade or higher standards. All processing equipment is high-grade stainless-steel meeting food-industry criteria. All cleaning and sterilization procedures are validated with FDA guidelines for food-grade materials.

B.6 Waste Handling and Disposal

Waste streams containing plant-derived residuals are treated per local regulations and discarded.

No by-products or residuals of the process are used in food or feed products, supplements, additives or treatment aids.

APPENDIX C. Methodology

The methods employed to assess the properties and characteristics of plant-made salmocin proteins that are candidate components of the SALMOCIN product are presented herein. The methods used were described in detail in Schneider (2018) and are summarized here for convenience.

C.1 Bacterial Strains and Growth Conditions

Salmonella enterica ssp. *enterica* strains listed in Table 2-3 were cultivated at 37°C in LB medium (lysogeny broth). *Agrobacterium tumefaciens* ICF320 cells were cultivated at 28°C in LBS medium (modified LB medium containing 1% soya peptone (Duchefa)).

C.2 Expression Vector Construction

All methods used to construct and apply TMV- and PVX-based magnICON® vectors and TMV-based vectors for EtOH-inducible expression were described in Schneider (2018). A list of *Salmonella* bacteriocins (salmocins) used in these studies is shown in Table C-1, including cytotoxic mode of action, protein molecular weight (MW) and accession numbers.

Table C-1. Salmonella-specific bacteriocins (salmocins) used in these studies

No.	Salmocin	Activity	MW (Da)	GenBank Accession No.
1	SalE2	DNase	61960	KTM78572.1
2	SalE3	RNase	61710	GAS18013.1
3	SalE7	DNase	62260	KSU39545.1
4	SalE1a	Pore-forming	52812	OIN35410.1
5	SalE1b	Pore-forming	57584	OIN32443.1

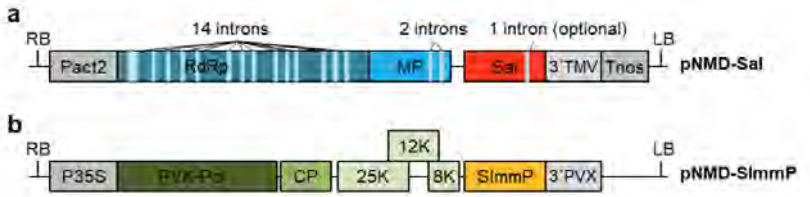
Coding sequences for the salmocins shown in Table C-1 and salmocin immunity proteins (if applicable) were codon-optimized for *Nicotiana benthamiana*, synthesized by Thermo Fisher Scientific and were cloned into the BsaI sites of the respective destination vectors.

A schematic representation of T-DNA regions of plasmid constructs used for transient *Agrobacterium*-mediated expression of salmocins is shown in Figure C-1. Panel (**a**) shows a salmocin-expressing TMV-based vector capable of cell-to-cell movement (pNMD-Sal).

Panel (**b**) PVX-based vector capable of systemic movement for the expression of salmocin immunity proteins (pNMD-SImmP). LB and RB, binary left and right borders, respectively; Pact2, *Arabidopsis thaliana* actin 2 promoter; Tnos, nos terminator; RdRp, RNA-dependent RNA polymerase of TVCV (Turnip Vein-Clearing Virus); MP, movement protein; 3´TMV, 3´untranslated region of TMV; P35S, CaMV 35S promoter; PVX-Pol, PVX RdRp; CP, PVX coat protein; 25K, 12K, 8K, PVX triple gene block; 3´PVX, 3´CP coding sequence and 3´untranslated region of PVX.

Panel (**c**) is a list of plasmid constructs used in this study. Position of intron in salmocin refers to amino acid codon in which the intron (from *Ricinus communis* cat 1 gene for catalase CAT1 (GenBank #D21161.1, base pairs 679-867)) was inserted and position of encoded amino acid in the sequence.

Figure C-1. Schematic representation of T-DNA regions of salmocin expression vectors



c

No.	Salmocin	Construct		Position of intron in salmocin
		Name	Internal name	
1	SalE2	pNMD-SalE2	pNMD28161	V510
2	SalE3	pNMD-SalE3	pNMD28151	G554
3	SalE7	pNMD-SalE7	pNMD28172	V532
4	SalE1a	pNMD-SalE1a	pNMD28191	-
5	SalE1b	pNMD-SalE1b	pNMD28204	-

No.	Immunity protein	Construct	
		Name	Internal name
1	SlmmE2	pNMD-SlmmE2	pNMD28222
2	SlmmE7	pNMD-SlmmE7	pNMD28232

C.3 Plant Material and Inoculation

C.3.1 Plant material for *Agrobacterium*-mediated transient gene expression

Non-recombinant *Nicotiana benthamiana* and food species such as *Spinacia oleracea* cv. Frühes Riesenblatt (spinach) plants were grown in the greenhouse (day and night temperatures of 19–23 °C and 17–20 °C, respectively, with 12 h light and 35–70% humidity). Six-week-old plants were used for inoculations. Plant biomass of each species for transfection were cultivated as described in Schulz (2015).

C.3.2 Stable plant transformation for ethanol-induced transgene expression

Nicotiana benthamiana was transformed by *Agrobacterium*-mediated leaf disk transformation using vectors for EtOH-inducible transgene expression. Transformation procedure and induction of detached leaves of T0 generation transgenic plants for salmocin expression were performed as described in Schulz (2015).

C.3.3 Inoculation and induction

Inoculation with *Agrobacterium*. Briefly, saturated *Agrobacterium* overnight cultures were adjusted to OD600=1.3 (~1.2 × 10^9 CFU/mL) with *Agrobacterium* inoculation solution (10 mM MES, pH 5.5, 10 mM MgSO4) and diluted with same solution 1:100 for inoculation using a needleless syringe. For agrospray inoculation, 1:100 diluted *Agrobacterium* cultures were supplemented with 0.1% (vol/vol) of the surfactant Sil-wet L-77 (Kurt Obermeier), and inoculation was carried out using a hand sprayer (Carl Roth).

Induction with ethanol. Detached leaves of T0 generation transgenic *N. benthamiana* plants (primary transformants; construction described in Schulz (2015)) were dipped into 4% (vol/vol) EtOH and incubated in 12 × 12-cm Petri dishes with the abaxial leaf surface on one layer of Whatman filter paper moisturized with 5 mL 4% (vol/vol) EtOH and one layer of glass fiber mesh and the petiole wrapped with tissue paper moisturized with 5 mL 4% (vol/vol) EtOH for 1 d with fluorescent light at 22 °C. Leaves were transferred to new Petri dishes composed as before but moisturized with 15 mL water and further incubated for 3 d with fluorescent light at 22 °C. Plant material was harvested 4 d post induction.

C.4 Protein Analysis

For small-scale analytical studies, plant leaf material was ground in liquid nitrogen and protein extracts were either prepared with 5 vol. 2· Laemmli buffer (crude extracts) or different buffers, e.g. 50 mM HEPES pH 7.0, 10 mM K acetate, 5 mM Mg acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 300 mM NaCl (total soluble protein (TSP) extracts). The protein concentration of TSP extracts was determined by Bradford or BCA assay using Bio-Rad Protein Assay (Bio-Rad Laboratories) or Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and BSA (Sigma-Aldrich) as a standard.

For analysis by SDS-PAGE and Coomassie-staining using PageBlue Protein Staining Solution (Thermo Fisher Scientific), protein extracts were denatured at 95°C for 5 min before loading. Estimation of recombinant salmocins as % of TSP was done by comparison of TSP extracts to known amounts of BSA standard (Sigma-Aldrich) on Coomassie-stained SDS-PAA gels. Additional details are presented in Schneider (2018).

C.5 Salmocin Expression in Non-Transgenic and Transgenic Plants

Spinacea oleracea (spinach) plants as a representative food species host, and *Nicotiana benthamiana* plants as a representative non-food species host, were used to assess expression of each salmocin, as detailed in Schneider (2018). Figure C-2 summarizes expression of salmocins by various gene expression methods in different host plants.

Figure C-2. Salmocins expression in spinach and *N. benthamiana* by various gene expression methods

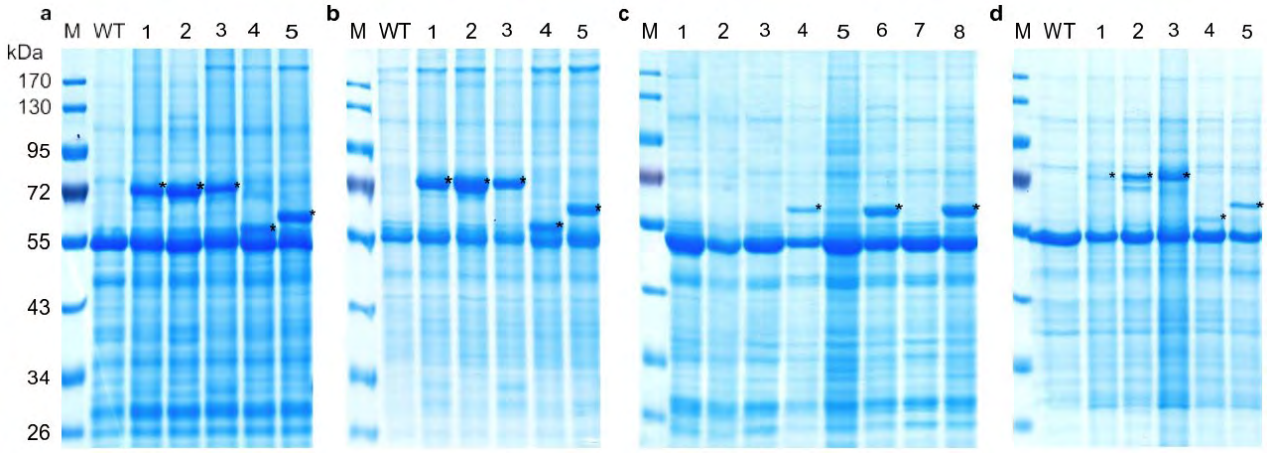


Figure C-2 Expression of salmocins in plants. Small-scale transient expression in *N. benthamiana* upon vacuum infiltration (**a**) or syringe infiltration (**b**) with agrobacteria carrying TMV or TMV and PVX vectors. Coomassie-stained SDS protein gels loaded with samples (**a,b**) corresponding to 3 mg FW plant material, (**a**) crude extracts prepared with 2 × Laemmli buffer or (**b**) TSP extracts prepared with 50 mM HEPES pH 7.0, 10 mM K acetate, 5 mM Mg acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween 20®, 300 mM NaCl. (**c**) Inducible expression of salmocin SalE1b in stable transgenic *Nicotiana benthamiana* plants. Loading with crude extracts corresponds to 3 mg FW extracted with 2 × Laemmli buffer from (lanes 1, 3, 5, 7) non-induced plant material or (lanes 2, 4, 6, 8) plant material 4 dp induction with ethanol. (Lanes 1, 2) *N. benthamiana* WT plant, (lanes 3, 4), (lanes 5, 6), (lanes 7, 8) different transgenic plant candidates for single copy T-DNA insertion of T0 generation (#4, 12, 37 for SalE1b). (**d**) Transient expression in *Spinacia oleracea* cv. Frühes Riesenblatt upon syringe infiltration with agrobacteria carrying TMV or TMV and PVX vectors. Loading of TSP extracts corresponds to 3 mg FW plant material extracted with 5 vol. 150 mM NaCl. Plant material was harvested (a) 5 dpi (days post infiltration) for SalE1b, 6 dpi for SalE3, SalE7 and SalE1a or 7 dpi for SalE2 or (b) 4 dpi for SalE1b, 5 dpi for SalE3, SalE7 and SalE1a and 6 dpi for SalE2 or (d) 8 dpi for SalE2, SalE3, SalE7, SalE1a and SalE1b. (a,b,d) Analyzed extracts were prepared from plant material expressing SalE2 (lane 1), SalE3 (lane 2), SalE7 (lane 3), SalE1a (lane 4) and SalE1b (lane 5) or from (WT) non-transfected leaf tissue. SalE2 and SalE7 were co-expressed with their respective immunity proteins. Asterisks mark recombinant proteins.

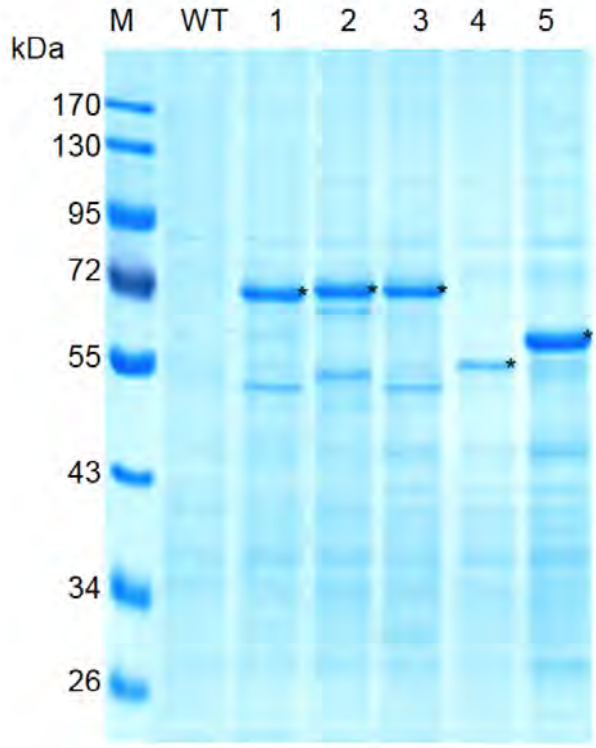
C.6 Purification of Salmocins

Purification of salmocins from plant host biomass was detailed in Schneider (2018). Briefly, for small-scale analytical studies, plant TSP extracts were prepared by supplementation of leaf material ground in liquid nitrogen with 5 vol. pre-chilled extraction buffers as 20 mM citric acid pH 4, 20 mM NaH_2PO_4, 30 mM NaCl, 0.05% Tween-80 for SalE1a and SalE7, or 20 mM citric acid pH 5.5, 20 mM NaH_2PO_4, 30 mM NaCl, 0.05% Tween-80 for SalE1b. Homogenates were incubated for 15 min on ice. Extracts were clarified by centrifugation for 15 min at 3515 × g and filtration using Miracloth. For SalE7 and SalE1a, clarified extracts were supplemented with 10 mg/ml diatomaceous earth. All extracts were incubated for 30 min at room temperature with constant agitation and were clarified again by centrifugation for 15 min at 3515 × g and filtered through filter discs of 8–12 µm pore size before loading for column-purification by cation exchange chromatography (CIEX) using SP-Sepharose FF for SalE1a and SalE1b and by CIEX using CM-Sepharose for SalE7.

SalE1a was step-eluted with 20 mM citric acid pH 4, 20 mM Na_2HPO_4, 1 M NaCl, 0.05% Tween-80 upon column wash with extraction and 45% elution buffer. Buffer exchange of SalE1a eluate was performed using U-tube concentrators (Sartorius) at molecular cut off of 10 kDa with 20 mM Na_2HPO_4, 10 mM citric acid, 50 mM NaCl, pH 6. SalE1b was step-eluted with 10 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl upon column-wash with extraction buffer and 8% elution buffer. SalE7 was step-eluted using 50 mM Na_2HPO_4, 10 mM citric acid, 50 mM NaCl upon column-wash with extraction buffer and 50% elution buffer. Eluted fractions of SalE1b and SalE7 or of SalE1a upon buffer exchange were snap-frozen in liquid nitrogen and freeze-dried.

Figure C-3 shows results of soluble selective extraction of salmocins from *N. benthamiana* plant biomass.

Figure C-3. Extraction of salmocins from *N. benthamiana* biomass



Soluble selective extraction of salmocins from *N. benthamiana* plant material upon transient expression. Leaf material was vacuum-infiltrated with agrobacteria carrying TMV or TMV and PVX vectors. Coomassie-stained SDS protein gel loaded with TSP extracts corresponding to 3 mg fresh weight plant material, prepared with 20 mM citric acid pH 4.0, 20 mM NaH2PO4, 30 mM NaCl. Plant material was harvested 5 dpi (days post infiltration) for SalE1b and 6 dpi for SalE3, SalE7 and SalE1a. Analyzed extracts were prepared from plant material expressing **SalE2 (lane 1), SalE3 (lane 2), SalE7 (lane 3), SalE1a (lane 4) and SalE1b (lane 5)** or from (WT) non-transfected leaf tissue. SalE2 and SalE7 were co-expressed with their respective immunity proteins. Asterisks mark recombinant proteins.

C.7 Methods to Assess Bactericidal Activity of Salmocins

Semi-quantitative determination of salmocin antimicrobial activity was done by spot-on-lawn/radial diffusion assay on serial dilutions of plant TSP extracts containing salmocins as employed in Schulz (2015). Salmocin-specific antimicrobial activity was calculated in arbitrary Activity Units (AU) per µg recombinant protein using the reciprocal of the highest dilution showing visible growth reduction of bacterial cells and the recombinant protein content of the solutions analyzed.

Salmocin antibacterial activity was determined *in vitro* as well as on various food matrices including mammalian, avian and marine meat samples and raw whole egg product to assess suitability, as further described in APPENDIX D. Representative results of the antibacterial activity of various salmocins against *S. enterica* pathovars are shown and discussed in Section 2.4.

C.8 Methods to Determine Salmocin Protein Mass and Amino Acid Sequences

Notifier commissioned the Fraunhofer Institute for Cell Therapy and Immunology (IZI; Halle, Germany) to independently analyze the amino acid sequences of its plant-made salmocin proteins. For all proteins, the molecular mass was determined, and the termini analyzed. Analytical methods and results are detailed in Schneider (2018) and are summarized here for convenience.

For proteolytic digestion, plant-derived TSP extracts in 20 mM Na citrate, 20 mM NaH_2PO_4, 30 mM NaCl, pH 5.5 were subjected to SDS-PAGE and Coomassie-stained SDS gel bands containing 5 µg of protein were excised and destained by consecutive washing with 100 mM NH_4HCO_3 and 100 mM NH_4HCO_3 in acetonitrile (ACN)/H_2O (50; 50, v/v). Disulfide bonds were reduced with 10 mM DTT for 45 min at 50 °C followed by alkylation with 10 mg/ml of iodoacetamide for 60 min.

Destained and alkylated gel bands were then subjected to proteolytic digestion with different sequencing grade endoproteinases (Promega, Madison, USA). Protease:protein ratio in the digestion solutions was adjusted to 1:20 (w/w) and digestions were carried out for 12 h at 25 °C (chymotrypsin) or 37 °C (Asp-N, Glu-C, Lys-C, trypsin). Proteolytic peptides were extracted by consecutive washing with H_2O, ACN/H_2O/trifluoroacetic acid (50; 45; 5, v/v/v) and ACN, respectively. Extraction solutions were combined, concentrated and resolubilized in H_2O/acetic acid (90:10, v/v). These proteolytic salmocin peptides or purified intact plant-produced salmocin SalE1a, SalE1b and SalE7 proteins were purified for mass spectrometry by solid-phase extraction using C4 or C18 bonded silica material (ZipTip, Millipore, Darmstadt, Germany) and elution solutions were co-crystallized on a MALDI ground steel target with 2,5-dihydroxyacetophenone as well as 2,5-dihydroxybenzoic acid matrix (Bruker Daltonics, Bremen, Germany).

Mass spectra were acquired on a MALDI-TOF/TOF mass spectrometer (Autoflex SpeedTM, Bruker Daltonics, Bremen, Germany) with positive polarity in linear mode for molecular mass determination and in reflector mode for protein sequencing by **in-source decay** (**ISD**) analysis. The matrix crystals were irradiated with a Nd:YAG laser (Smart beam-IITM, Bruker Daltonics, Bremen, Germany) at an emission wavelength of 355 nm and set to a pulse rate of 1 kHz. MS and MS/MS spectra were recorded with FlexControl and spectra processing was carried out with FlexAnalysis (version 3.4, Bruker Daltonics, Bremen, Germany). Determination of the intact molecular mass was based on the mass-to-charge-ratios (*m/z*) of single and multiple charged molecular ions.

Sequencing of protein termini was carried out by ISD analysis. The annotation of ISD fragment spectra was carried using BioTools (version 3.2, Bruker Daltonics, Bremen, Germany) by *in silico* generation of *m/z* values for fragment ions and their comparison with the *m/z* values of the fragment signals observed within

the acquired ISD spectra. This approach enabled the identification of the terminal amino acid sequences as well as of any modifications that were present.

For protein sequencing analysis, only fragment (MS/MS) spectra were used for the identification of proteolytic peptides. Identification of proteins and verification of their amino acid sequences was performed by searching the MS/MS data against the NCBI non-redundant database and the UniProt/SwissProt database to which the sequences of the salmocins were appended, respectively. The maximum number for both missed cleavages as well as post-translational modifications for one proteolytic fragment was set to 3. Non-specific cleavage was allowed for both protein termini.

C.9 Molecular Analysis of Salmocin Proteins

Results of protein analyses of plant-produced salmocins by the methods described above and in Schneider (2018) are summarized in Table C-2.

Table C-2. Identity and integrity of salmocins as determined by mass spectrometry

salmocin-containing TSP extracts of *N. benthamiana*						
No.	salmocin	peptide mass fingerprinting				
		Peptides annotated	Amino acid coverage (trypsin, Asp-N, chymotrypsin, Glu-C, Lys-C, combined)	Proteoform	N-terminus (aa and PTM)	C-terminus (aa and PTM)
1	SalE2	44	56,3%	1 2	H_2N-SGGDGIGHNS[…] (M cleaved, no PTM) Acetyl-SGGDGIGHNS[…] (M cleaved, acetylation)	[…]KLHIDIHRGK-OH (intact, no PTM)
2	SalE7	31	33,3%	-	ND	[…]KRHIDIHRGQ-OH (intact, no PTM)
3	SalE3	18	31,5%	-	ND	ND
4	SalE1a	14	34,2%	1	Acetyl-ADNTIAYYED[…] (M cleaved, acetylation)	ND
5	SalE1b	25	47,1%	-	ND	ND

salmocins purified from *N. benthamiana*							
No.	salmocin	Batch	Theoretical mass of intact protein (Da)	Molecular mass		in-source decay	
				Average mass (Da) (PTM)	Proteoform	N-terminus (aa and PTM)	C-terminus (aa and PTM)
2	SalE7	1 2 3	62259.4	62111.0 62126.9 62137.8 (N-terminus: M cleaved)	1 1 1	H_2N-SGGDGG[…] (M cleaved, no PTM) H_2N-SGGDGG[…] (M cleaved, no PTM) ND	[…]KRHIDIHRGQ-OH (intact, no PTM) […]KRHIDIHRGQ-OH (intact, no PTM) ND
4	SalE1a	1 2 3	52811.3	ND ND 52722.1 (N-terminus: M cleaved, acetylation)	1 1 1	ND ND ND	ND ND ND
5	SalE1b	1 2 3	57583.1	57486.3 57470.0 57480.7 (N-terminus: M cleaved)	1 1 1	ND ND ND	ND ND ND

Table C-2. Identity and integrity studies on plant-produced salmocins. MALDI-TOF/TOF mass spectrometry analysis of salmocin-containing TSP extracts of *N. benthamiana* or purified salmocins by peptide mass fingerprinting or by sequencing of protein termini by in-source decay and molecular mass determination, respectively. Proteases used for generation of peptide fragments are indicated. The identity of salmocins was confirmed by searching MS/MS datasets obtained against NCBI non-redundant database. Obtained molecular masses indicate that the proteins were intact. ND, not detected; PTM post-translational modification; aa, amino acid sequence.

C.10 Determination of Salmocins' Digestibility

The instability of salmocins in simulated gastric and duodenal environments was determined to confirm protein degradability upon ingestion. As we reported in Schneider (2018), gastric (phase I) and duodenal (phase II) digestion *in vitro* was performed with plant-produced lyophilized purified SalE7, SalE1a or SalE1b dissolved in Millipore water by the method described in Schulz (2015).

Briefly, individual salmocins were incubated in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) containing pepsin or trypsin and chymotrypsin in physiological concentrations, respectively. Incubation with pepsin in SGF for up to 60 min was followed by incubation with trypsin and chymotrypsin in SIF for up to 3 h.

Aliquots of the reactions were evaluated for antimicrobial activity and protein degradation pattern by SDS-PAGE and Coomassie-staining upon different intervals of incubation. For SDS-PAGE analysis, pre-cast 4-20% Mini-PROTEAN TGX gels (Bio-Rad Laboratories) with loading corresponding to 1.5 µg SalE1a and SalE7 or 1 µg SalE1b proteins per lane were used.

Results are shown in Table C-3. It is clear from the results of digestion experiments in simulated gastrointestinal environment that pore-forming salmocins such as SalE1a and SalE1b and a DNase-active salmocin such as SalE7 would be degraded in the stomach or upper intestinal tract upon ingestion, and that their activity is almost totally lost after 1 – 3 h of incubation in simulated digestive passage (residual bactericidal activity of 0%-001% of non-digested native proteins.

Because of their similarities to colicins, we expect that other salmocins not tested in this series or candidates for future inclusion in the SALMOCIN product would also be susceptible to digestive action. Additional data from SDS-PAGE analysis are shown in Figure 2-15.

Table C-3. Digestibility of salmocins in simulated gastroduodenal environment

A	**Mean percent residual antimicrobial activity**						
		Phase I – gastric digestion			**Phase II – duodenal digestion**		
Salmocin	Not digested	0 min -/- SGF	60 min -/- SGF	60 min P/- SGF	0 min P/- SIF	180 min P/- SIF	180 min P/T, C SIF
SalE1a	100%	60%	60%	0.26%	0.06%	0.06%	0.001%
SalE1b	100%	100%	100%	75%	5%	5%	0%
SalE7	100%	55%	55%	0.1%	6%	6%	0%

C.11 Batch-to-Batch Salmocin Manufacturing Consistency

For the proteins defined above, multiple batches were produced and the results compared, using a minimum of three (3) independent developmental runs in each. Table C-4 shows results of batch-to-batch **protein yield consistency** obtained. The plant-based process for salmocin manufacturing yields consistent results from batch to batch.

Table C-4. Batch-to-batch yield comparability of plant-made salmocins

Salmocin	Yield of purified protein (mg/kg FW plant material)		Number of non-consecutive batches analyzed	Purity of salmocin protein (% of total protein)		Number of non-consecutive batches analyzed
	Average	STDEV		Average	STDEV	
SalE1a	50	30	3	Not analyzed		
SalE1b	460	390	3	55.63	9.46	3
SalE7	490	140	3	56.6	25.47	3

Yield of recovered protein at the indicated plant harvest times (days post inoculation; dpi). Protein yields for each salmocin for N=3 developmental batches are expressed as averages ± standard deviation as mg protein/kg fresh weight (FW) plant biomass and as salmocin protein as % of total protein. Purity of purified salmocins was determined by capillary gel electrophoresis (CGE) method using Agilent 2100 Bioanalyzer (Agilent Technologies).

Similarly, the **potency consistency** of representative salmocins from three (3) independent manufacturing runs can be determined to assess manufacturing process control and control of product quality. Table C-5 shows average potency values ± SD for each of 3 sets of salmocins tested. Because the salmocins may be blended into a final SALMOCIN product, the average of means was used in setting the activity (Potency) minimum in the Specification (Table B-1).

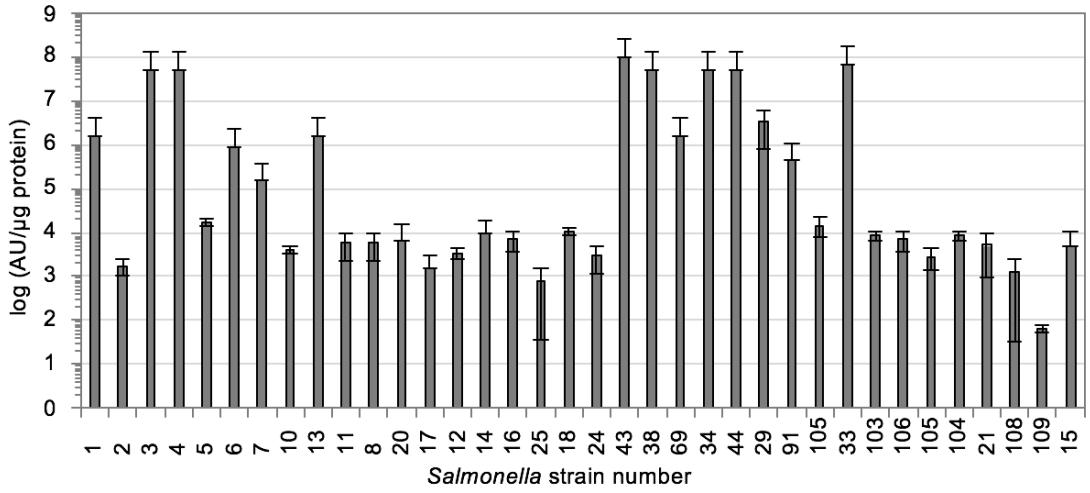
Table C-5. Batch-to-batch consistency of potency of purified salmocins

Salmocin	**Specific antimicrobial activity** (AU/mg purified protein) Assayed on *S. enterica* ssp. *enterica* serovar Enteritidis strain ATCC® 13076™*		Number of non-consecutive batches analyzed
	Average	Std Dev	
SalE1a	6.29×10^7	3.57×10^7	3
SalE1b	6.45×10^6	4.24×10^6	4
SalE7	5.98×10^5	3.77×10^5	5

As seen in the results of multiple potency studies against a reference target strain of *S. enterica*, SalE1a and SalE1b are considerably more potent than SalE7. For practical reasons, the potency averages are themselves averaged to define a minimum potency for the Specification in a blended salmocin product. Hence, the minimum potency value is set at 1×10^8 AU/g protein for product functionality, whereas the average measured potency from the data in Table C-5 exceeds the minimum value at 23.3×10^9 AU/g protein (Table B-1).

An additional method to show consistency of potency on a broader spectrum is to list the activity of each salmocin in a screen against several *S. enterica* pathovars. This data set appears in Section 2.4.1. As an example, Figure C-4 shows consistency of antibacterial activity of salmocin SalE1b across major *Salmonella enterica* ssp *enterica* pathovars. Results of N=3 studies ± SD are plotted.

Figure C-4. Batch-to-batch consistency of potency of SalE1b on *S. enterica* ssp *enterica* pathovars



Antibacterial potency shown as average log AU/µg protein ± SD for salmocin SalE1b from 3 manufacturing batches.

The results of studies on the consistency of antibacterial activity for all salmocins are shown in Section 2.4.1, Figure 2-2 (**SalE2**), Figure 2-3 (**SalE3**), Figure 2-4 (**SalE7**), Figure 2-5 (**SalE1a**) and Figure 2-6 (**SalE1b**).

All salmocins produced by the plant-based process described show a high degree of consistency with respect to bactericidal activity against respective pathovars per unit weight of protein (i.e. potency).

C.12 Analysis of Heavy Metal Content

Elemental analyses were conducted to estimate the residual heavy metal content of developmental batches of the most potent and broad-spectrum salmocins with complementary activity, SalE1b and SalE7.

In these studies, approximately 6-week-old *N. benthamiana* host plant biomass from 3 independant and non-consecutive salmocin developmental batches were analyzed after infiltration with *Agrobacterium* ICF320 vector carrying either SalE1b or SalE7 genetic constructs. Sections of leaves from each plant were harvested and incubated at 37°C for 24-48 h until dry. The dried biomass was blended to a fine powder (RMBL, Rotor Lips AG) at maximum velocity for 2 x 15 seconds. The powders containing salmocins were stored at RT until metal determination.

Analyses for elemental impurities were conducted on behalf of Notifier by Wolfener Analytik GmbH (Bitterfeld-Wolfen, Germany; Prüfbericht Nr. 05878/18, 14 September 2018). Developmental batches were analyzed for the most toxic heavy metals; additional elemental analyses are in progress.

The metals Cd, Pb and As were determined according to DIN 38406-29 (Determination of Selected Elements by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)), which is similar to USP38<2332, "Elemental Contaminants in Dietary Supplements." The metal Hg was determined according to Wolfener Analytik GmbH in-house SOP also using ICP-MS, as above.

Results of these analyses are summarized in Table C-6. The individual levels from 3-batch analyses are shown for each salmocin, as well as the average values for a SalE1b + SalE7 blended preparation. Also shown is the projected per capita daily exposure from consumption of 0.8 mg residual SALMOCIN in food.

Table C-6. Calculated residual heavy metal content in salmocins SalE1b and SalE7

Salmocin (Mode of Action)	Elemental Impurity	Elemental Impurity (ng) per mg of SALMOCIN protein [1]	Estimated Per Capita Exposure (ng/day) from 0.8 mg Residual SALMOCIN in Treated Food [2]
		Average (N=3)	Average (N=3)
SalE1b (Pore former)	Pb	49.59	39.7
	Cd	86.17	68.9
	Hg	9.83	7.90
	As	54.42	43.5
SalE7 (DNase)	Pb	41.93	33.5
	Cd	85.36	68.3
	Hg	13.32	10.6
	As	60.94	48.7
Average in 1:1 blend of the 2 salmocins in SALMOCIN product	Pb	45.76 ± 3.83	36.6 ± 3.10
	Cd	85.76 ± 0.31	68.6 ± 0.47
	Hg	11.6 ± 1.75	9.28 ± 1.35
	As	57.68 ± 3.62	46.1 ± 2.6

Table C-6 residual heavy metal impurities. Legend:

[1]Measured levels of each metal species based on salmocin content, expressed as nanogram (ng) metal per mg of salmocin protein powder (ng/mg). Values are averages derived from 3 non-consecutive developmental batches (N=3).

[2]Calculated per capita daily heavy metal intake from SALMOCIN-treated food products based on daily ingestion of 0.8 mg salmocin/person-day (see Section 3.2), expressed as nanogram element/person-day (ng/person-day).

The conclusion that can be drawn from these analyses is that ingestion of the projected maximum amount of 0.8 mg salmocins/person-day from SALMOCIN-treated foods, based on measured levels of heavy metals, would expose consumers to low amounts of heavy metals relative to reported ambient and food-borne levels of exposure (see Section 6.3 for risk assessment).

C.13 Stability of Salmocins

To assess the stability of salmocins upon storage, samples of purified salmocins SalE1b and SalE7, selected for their complementary bactericidal activities and for being the most likely candidates for formulation in a SALMOCIN blend, were analyzed under different storage conditions for various durations of storage. The samples were stored as (a) dry (lyophilized) powders or as (b) 0.01% (w/v) solutions in water, both held at either 4°C or at room temperature (RT).

Potency stability was determined by quantifying specific activity by soft-agar overlay assay using *Salmonella enterica* ssp. *enterica* serovar Enteritidis strain ATCC® 13076™* as the tester strain. Protein integrity was analyzed by SDS-PAGE analysis of the proteins to detect potential degradation under each storage condition. Specifically, samples analyzed for antimicrobial activity were mixed 1:1 with 2x Laemmli buffer and 7.5 µl of each sample were analyzed by 12% SDS-PAGE and Coomassie-staining.

Figure C-5 shows summary results of stability analyses of selected salmocins **SalE1b** (Panel **A**) and **SalE7** (Panel **B**). At the time of this submission all salmocins were undergoing a stability program and the results obtained are interim. Results are provided for 3 non-consecutive batches each of SalE1b and SalE7. Both of these salmocins were stable for a minimum of 234 days when stored as dry powders. The final Specification is for a targeted minimum stability of **6 months** for all dry salmocin powders comprising the commercial SALMOCIN product.

Figure C-5. Stability of dry powders and solutions of salmocins SalE1b and SalE7

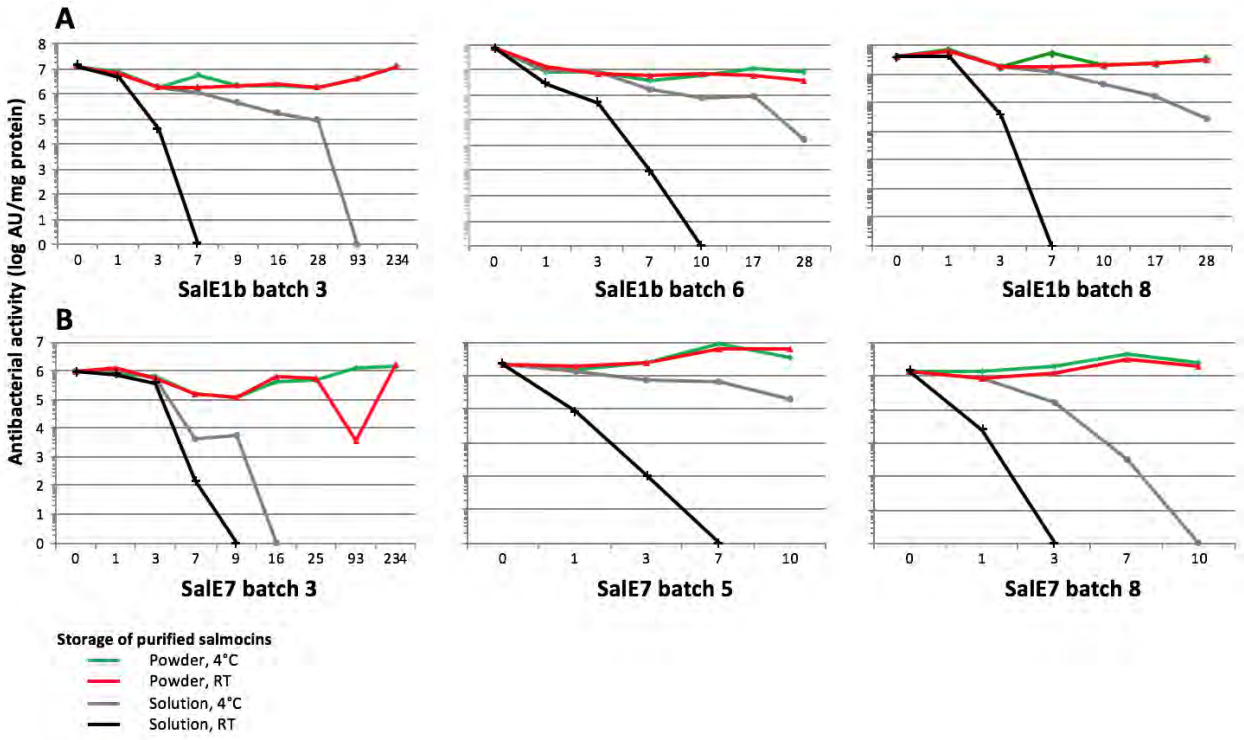


Figure C-5 Interim stability of purified salmocins SalE1b and SalE7. Potency was analyzed semiquantitatively by radial diffusion assay via spot-on-lawn-method analyzing serial dilutions of salmocin solutions for antimicrobial activity (log AU/mg purified protein) on *S. enterica* ssp. *enterica* serovar Enteritidis strain ATCC® 13076™* Protein integrity was evaluated by detecting breakdown product in 12% SDS-PAGE followed by Coomassie-staining.

In Figure C-5, the line graphs show potency (antibacterial activity/mg salmocin protein) versus storage time and formulation (dry vs. liquid) and storage temperature (RT vs. 4°C). These interim results show that dry salmocin powders stored at 4°C (——) have the longest shelf life, followed by salmocins powders stored at RT (——), which have nearly equivalent stability.

Although salmocin solutions are stable for 7 days or more after preparation if stored at 4°C (——), the integrity of the salmocin proteins degrades substantially after a few days in most cases, especially in solutions stored at room temperature (——).

Hence, dry storage of the bulk product followed by application within a few days of preparation (i.e. preparation of SALMOCIN solution from the dry bulk powder) should enable practical storage, handling and application of the antimicrobial in various food treatment environments.

The fact that salmocin protein solutions degrade over several days introduces an even greater measure of environmental safety into the product profile and minimizes the impact of product or waste solution disposal.

C.14 Statistical Analyses

Standard statistical treatment of data generated in the studies described in this GRN were applied to determine significance. Typically, the measured parameters of salmocin proteins were compared against negative controls consisting of equivalent vehicle or carrier solutions containing no salmocin proteins.

In some experiments the parameters were compared between and/or among results obtained for individual salmocins or for mixtures of salmocins. Results within data sets were typically evaluated by one-way ANOVA (Tukey´s multiple comparisons test) and unpaired parametric t-test using GraphPad Prism v. 6.01. Criterion for null hypothesis rejection = $p \leq 0.05$.

Results among data sets were averaged to find the mean of populations and standard deviation (s.d.) among sets. The uncorrected sample standard deviation method was applied, for example, when comparing mean values of potency, protein yield, impurity levels, etc., for different salmocins obtained from multiple non-sequential manufacturing batches. In such analyses, the following equation was used to determine the s.d. among averaged sets (population), which is defined as the square root of the sample variance of a set of N values (http://mathworld.wolfram.com/StandardDeviation.html):

$$s_N = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \overline{x})^2}$$

In the equation, "N" is the number of a sample set, “i" is the starting number, “x_I” is the individual variance from the mean, “$\overline{x}$” is the mean, and "S_N" is the standard deviation of the population.

This method was particularly helpful in reporting measured values in different analyses entered in the target Specification and helped define workable release criteria for the SALMOCIN product.

APPENDIX D. On-Matrix Determination of Antibacterial Activity

	Standard Operating Procedure	
	Determination of Efficacy and Duration of Bactericidal Effect of SALMOCIN on Pathogenic Strains of *Salmonella enterica* ssp. *enterica* Applied to Food Matrices	**NMD 1101-01**

D.1 Purpose of Standard Operating Procedure

This protocol (Standard Operating Procedure) describes the methods for evaluating the efficacy and suitability of SALMOCIN in reducing contamination by pathogenic *S. enterica* ssp. *enterica* strains on various food matrices (e.g. skinless and skin-on chicken breast, beef, tuna and whole egg) and for evaluating residual technical effect (duration of activity) on these foods.

Chicken meat matrices are used to illustrate procedures. However, this SOP is applicable for determining the bactericidal effects of SALMOCIN on other meats such as turkey, or other foods susceptible to contamination by Salmonellae. In this SOP, the term salmocins applies to individual salmocin proteins, whereas SALMOCIN refers to a formulated product containing a specified mix of salmocins.

D.2 Scope

Assays for SALMOCIN's efficacy and continued technical effect

Evaluation of efficacy encompasses the analysis of pathogenic *S. enterica* ssp. *enterica* populations on contaminated food samples subsequently treated with mixtures of various plant-made recombinant salmocins (plant extracts containing salmocins or purified salmocin proteins), or a control carrier solution consisting of plant extract from the same production host or buffer in case of purified salmocins but without salmocins, and stored for various time periods.

Evaluation of continued technical effect encompasses the analysis of time-dependent re-growth of pathogenic *S. enterica* ssp. *enterica* strains on contaminated food matrices after SALMOCIN or carrier control application during prolonged storage of food matrices.

One level of on-matrix (food) microbial contamination is analyzed for efficacy determination: A level close to realistic or typical contamination level of 3 log CFU/g meat to substantiate the reduction of bacterial load with timed sampling and evaluation of re-growth.

The effect of SALMOCIN on the bacterial populations upon storage periods of 1-48 or 1-72 h are analyzed at a temperature of 10 °C which is suboptimal for but allowing bacterial growth.

Each treatment is performed in three replicates; each replicate is defined as an independent experiment starting with a new pathogen culture.

In each replicate experiment, each sample is analyzed in 4 replicates.

D.3 Definitions

LB Luria Bertani medium
OD_{600} Optical density of bacterial solution at 600 nm
RT Room temperature
TSP Total soluble protein
CFU Colony forming unit
XLD Xylose Lysine Desoxycholate Agar

D.4 Consumables

Disposable plastic cuvettes for spectrophotometric measurement of OD_{600}
Sterile Petri dish, ~94x16 mm
Sterile 5 mL disposable plastic syringes with Luer lock connector
Sterile disposable 50 mL centrifuge (Falcon) tubes
Sterile disposable 15 mL centrifuge (Falcon) tubes
Sterile 1.5 mL disposable reaction tubes
Sterile disposable forceps 25 cm (RMP-med Steffen Roßberg, cat.# 720183)
Sterile disposable plastic spatulas
Sterile disposable 25 mL, 10 mL serological pipettes
Sterile lateral filter bags BagFilter®400 P (Interscience, cat.# 111 425)
Atomizer flasks (Carl Roth GmbH & Co. KG, cat.#N145.1)

D.5 Equipment

Sterile 100 mL wide neck Erlenmeyer flasks for cultivation of *S. enterica* ssp. *enterica* in liquid culture
Incubator shaker (150 rpm, 37°C) for cultivation of *S. enterica* ssp. *enterica* strains
Spectrophotometer for measurement of OD_{600} of bacterial culture
Pipetting aid for serological pipettes
250 mL sterile measuring cylinder
500 mL sterile measuring cylinder
5 L sterile beaker
3 L sterile beaker
2 L sterile beaker
Laminar flow cabinet
Microwave oven
Closing clip BagClip®400 (Interscience, cat.# 231 040)
Lab blender BagMixer® 400 CC® (Interscience, cat.# 024 230)
Incubator (37°C)
Autoclave
Refrigerator (10 °C)
Freezer -80 °C
Freezer -20 °C
Personal protective equipment

D.6 Chemicals/Media/Solutions

LB medium (sterile, liquid): For cultivation of *S. enterica* ssp. *enterica* strains

1% (w/v) Bacto-tryptone (pancreatic digest of casein; Duchefa T1332)
0.5% (w/v) Yeast extract (Duchefa Biochemie, cat. #Y1333)
1% (w/v) NaCl (AppliChem GmbH, cat. #A4661)
pH 7.5, Autoclaved

LB medium (sterile, solid): For cultivation of *S. enterica* ssp. *enterica* strains

1% (w/v) Bacto-tryptone (pancreatic digest of casein; Duchefa T1332)
0.5% (w/v) Yeast extract (Duchefa Biochemie, cat. #Y1333)
1% (w/v) NaCl (AppliChem GmbH, cat. #A4661)
pH 7.5
1.5% (w/v) Agar, bacteriology grade (AppliChem, cat. #0949)
Autoclaved

Xylose Lysine Desoxycholate Agar (XLD Agar, sterile solid): Selective medium for recovery of *Salmonella* from food samples (Sifin GmbH, cat. #TN1170)

55.2 g/L, not autoclaved, melted in microwave oven

Buffered peptone water (liquid, sterile): Isotonic diluent for examination of foodstuff, homogenization of food samples for sample preparation prior to microbiological analysis (Carl Roth GmbH, cat.#X917.1)

20 g/L, Autoclaved

Nalidixic acid (sterile): For selection of resistant *S. enterica* ssp. *enterica* mutant strains

25 mg/mL Stock solution

Nalidixic acid sodium salt (Sigma, cat. #N4382-1G) is dissolved in deionized water to 25 mg/mL, sterile filtered, aliquoted and stored at -20°C

D.7 Biologicals

Bacterial tester strains used in efficacy and technical effect experiments

The *S. enterica* ssp. *enterica* strains used in the experiments conducted within this SOP are shown in Table D-1. These targets represent various serovars of *Salmonella* responsible for food-borne outbreaks.

For better differentiation between pathogenic vs naturally resident non-pathogenic strains, nalidixic acid resistant derivatives of pathogenic strains are used. Nalidixic acid resistant spontaneous mutants are selected by cultivation of original isolates on LB agar plates supplemented with nalidixic acid concentrations of 25 µg/mL.

Comparable susceptibility of original strains and nalidixic acid resistant mutants thereof towards salmocins are confirmed. These strains are stored in liquid nitrogen in LB broth supplemented with 15% (v/v) glycerol and 25 µg/mL nalidixic acid. When using nalidixic acid resistant derivatives of *Salmonella* strains, all media employed for bacterial growth are supplemented with 25 µg/mL nalidixic acid.

Table D-1. *Salmonella enterica* ssp. *enterica* strains used in these studies

Original strain: Culture Collection Reference #	source	Nalidixic acid resistant derivative generated for and used in these studies	Serovar	CFU Appearance on XLD agar
ATCC®13076™*	Microbiologics Inc., St. Cloud, MN, USA	ATCC®13076™*nalR#6	Enteritidis	White with black center
ATCC®14028™*		ATCC®14028™*nalR#3	Typhimurium	
ATCC®6962™*		ATCC®6962™*nalR#4	Newport	
ATCC®10721™*	LGC Standards, Teddington, UK	ATCC®10721™*nalR#4	Javiana	
ATCC®8326™*	Microbiologics Inc., St. Cloud, MN, USA	ATCC®8326™*nalR#5	Heidelberg	
ATCC®BAA-1675™*	LGC Standards, Teddington, UK	ATCC®BAA-1675™*nalR#6	Infantis	
ATCC®8388™*		ATCC®8388™*nalR#1	Muenchen	

D.8 Precautions

All work with pathogenic *S. enterica* ssp. *enterica* is done under sterile conditions and in biocontainment laboratories that are compliant with their respective national and regional biosafety requirements.

D.9 Procedure for Determining Efficacy and Duration of Technical Effect

D.9.1 Salmocins

Salmocin proteins are produced in plants as described for colicins in GRN 593. The intended product contains individual plant-made salmocins or a blend of several salmocin proteins selected from the list that includes salmocins SalE7 (GenBank KSU39545.1), SalE1a (GenBank OIN35410.1), SalE1b (GenBank OIN32443.1), SalE2 (GenBank KTM78572.1) and SalE3 (GenBank GAS18013.1).

Plant-made salmocins blended into the SALMOCIN product can be supplied in various forms, including: 1) salmocin-containing plant total soluble protein (TSP) extracts; 2) dry (e.g. lyophilized) salmocin-containing plant TSP; or 3) dry (e.g. lyophilized) purified salmocin proteins. SALMOCIN formulations may be delivered to the customer as dry powder, ready-to-use solution, or concentrated liquid with defined concentrations of salmocins. Before use, the supplied salmocin formulations should be diluted/dissolved in the appropriate volume of deionized water and stored at low temperature (4°C).

Salmocin-containing TSP extracts or TSP carrier control extracts not containing salmocins of production host were prepared with 5 vol. of 50 mM HEPES pH7.0, 10 mM K acetate, 5 mM Mg acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 300 mM NaCl. The same buffer was also used for dilutions of TSP extracts. Salmocin-containing solutions prepared with lyophilized purified salmocins were prepared and diluted with 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl. The same buffer was used as carrier control in that case.

D.9.2 Preparation of devices for spray application of salmocin blend or carrier solution

Atomizer flasks are sterilized by rinsing and spraying with 70% (v/v) EtOH and dried under a laminar flow cabinet. SALMOCIN-containing or carrier solutions are filled into flasks and stored at 4 °C until use.

D.9.3 Preparation of food test matrices

Purchase of meat and eggs in local retail outlets

No special sourcing of meat samples is used to ensure that SALMOCIN activity is evaluated in representative consumer products. Raw skinless and skin-on chicken breast fillet, raw fresh or frozen tuna fillet, raw untrimmed beef round roast or raw eggs are purchased at retail outlets (for these studies, ALDI, Kaufland or EDEKA supermarkets, respectively, Halle, Germany), one day before the experiment. The fresh meat, tuna or eggs are stored at 4 °C, the frozen tuna is thawed at 4°C for overnight. The packaging is disinfected with 70% ethanol before opening, and the chicken, beef or tuna meat is not washed or pre-treated before experimental exposures. For preparation of whole egg from fresh raw eggs, eggs are washed with 70% ethanol before opening of the egg shell. A matrix summarizing how food samples are prepared for these experiments is shown in Table D-2.

Table D-2. Matrix for preparation of chicken breast and tuna cuts or whole egg

Test matrix	**Whole cuts**				**Whole egg**
	Raw skinless chicken breast	Raw skin-on chicken breast	Raw beef	Raw tuna	Raw whole egg
Initial meat block	Chicken breast fillet of ~200-300 g	Chicken breast fillet of ~200-300 g	Untrimmed round roast	Tuna fillet steak	Raw eggs
Preparation	Fillets are trimmed using a knife and cutting board disinfected with Bacillol® AF (Bode Chemie) to obtain cuts of ~20 g weight	Fillets are trimmed using a knife and cutting board disinfected with Bacillol® AF (Bode Chemie) to obtain cuts of ~20 g weight	The meat block is trimmed using a knife and cutting board cleaned with Bacillol® AF (Bode Chemie) to obtain cuts of ~20 g weight	steaks are trimmed using a knife and cutting board disinfected with Bacillol® AF (Bode Chemie) to obtain cuts of ~20 g weight	Eggshells are opened and eggwhite and eggyolk is collected and homogenized using a lab blender for 2 min at 1000 rpm

D.9.4 Preparation of bacterial cultures used to experimentally contaminate food matrices

The food test matrices are experimentally contaminated with a 1:1:1:1:1:1:1 or a 1:1 mixture of 7 (ATCC®13076™*nalR#6, Enteritidis; ATCC®14028™*nalR#3, Typhimurium; ATCC®6962™*nalR#4, Newport; ATCC®10721™*nalR#4, Javiana; ATCC®8326™*nalR#5, Heidelberg; ATCC®BAA-1675™*nalR#6, Infantis and ATCC®8388™*nalR#1, Muenchen) or 2 (ATCC®13076™*nalR#6, Enteritidis and ATCC®14028™*nalR#3, Typhimurium) nalidixic acid resistant derivatives of *S. enterica* ssp. *enterica* strains representing 7 or 2 different serotypes, as shown in Table in 7-1. *Salmonella* strains were chosen with respect to their serotype being most commonly reported for laboratory-confirmed human *Salmonella* infections reported to CDC in 2003-2012 (CDC, June 2016; https://www.cdc.gov/nationalsurveillance/pdfs/salmonella-annualreport-2013-508c.pdf).

Before inoculation the strains are thawed from liquid nitrogen permanent culture, individually grown on LB agar medium supplemented with 25 µg/mL nalidixic acid for overnight at 37°C and stored at 4°C. One day before contamination of food matrices, strains are individually inoculated to LB broth supplemented with 25 µg/mL nalidixic acid. Individual saturated LB broth overnight cultures (37 °C, 150 rpm) are diluted to OD_{600}=0.05 with fresh LB broth supplemented with 25 µg/mL nalidixic acid and grown to OD_{600}=~0.3 which corresponds to ~$6x10^7$ CFU/mL (~7.75 log CFU/mL).

Individual cultures are diluted with LB broth to OD_{600}=0.3 and mixed 1:1:1:1:1:1:1 or 1:1. The strain mix is further diluted to the desired cell number (see Table 9-2) with LB broth for use as meat contamination suspension. Subsequently, 100 µl aliquots of serial dilutions of the bacterial suspensions are plated on XLD supplemented with 25 µg/mL nalidixic acid in order to determine the cell density.

D.9.5 Contamination of meat

Chicken breast, beef and tuna cuts are supplemented with 10 mL/kg bacterial contamination suspension while being tumbled and hand kneaded to ensure uniform exposure. Whole egg is also supplemented with 10 mL/kg bacterial contamination suspension, uniform exposure is ensured by intermixing bacterial solution using a stirrer at 620 rpm for 1 min. Contaminated meat and bacteria are allowed to dry and colonize matrix samples, respectively, for 30 min at RT, during which time cuts are tumbled and whole egg is stirred for 1 min. at 750 rpm every 15 min. A summary matrix of the process is shown in Table D-3. For each food matrix, non-treated samples for detection of potential "background microbes" that could disturb enumeration of *Salmonella* were prepared and incubated as the other treated samples, but were neither contaminated with *Salmonella* nor treated with salmocins.

Table D-3. Matrix for experimental contamination of foods with mixed pathogen suspension

Test matrix – general	**Whole cuts**				**Whole egg**
	Raw skinless chicken breast	Raw skin-on chicken breast	Raw beef	Raw tuna	Raw whole egg
Test matrix – specific	Pool of meat cuts with each cut of ~20 g weight				Pool of whole egg homogenate
Containment	5 L Beaker, sterile				3 L Beaker, sterile
Density of *Salmonella* suspension	~$2x10^5$ CFU/mL (OD_{600}= 0.001; 5.3 logs/mL)		~$2x10^5$ or ~$5x10^6$ CFU/mL (OD_{600}= 0.001 or 0.005; 5.3 or 6.7 logs/mL)		
Composition of pathogenic *Salmonella* suspension	1:1:1:1:1:1:1 mixture of serotypes Enteritidis, Typhimurium, Javiana Heidelberg, Infantis, Muenchen		1:1 mixture of serotypes Enteritidis, Typhimurium		
Application and **dose** of *Salmonella* suspension	10 mL/kg; Equal distribution, tumbling, hand mixing				10 mL/kg; Equal distribution using a stirrer at 620 rpm for 1 min
Bacterial load of contaminated food matrix for salmocin efficacy tests	1-3x10^3 CFU/g (3-3.5 logs/g)	0.8-1.5x10^3 CFU/g (2.9-3.2 logs/g)	1-3x10^3 CFU/g (3-3.5 logs/g) or ~$2x10^4$ CFU/g (~4.3 logs/g)	1-2x10^3 CFU/g (3-3.3 logs/g) or ~$2x10^4$ CFU/g (~4.3 logs/g)	3-4x10^3 CFU/g (3.5-3.6 logs/g) or ~$2x10^4$ CFU/g (~4.3 logs/g)

D.9.6 Application of salmocin (blend) solution

The contaminated food samples are divided into different groups for different treatments by aliquoting meat cuts or whole egg into new sterile beakers. Contaminated food is either treated with carrier or salmocin blend solution by low-pressure spraying (2-4 bar) using atomizer flasks or by application without spraying, as shown in Table D-4.

Reasonably even coverage of the entire surface is ensured. The food is further incubated for 30 min at RT while meat cubes are tumbled and hand-massaged and whole egg is stirred every 15 min.

Table D-4. Matrix for application of SALMOCIN to food samples

Test matrix – general	**Whole cuts**				**Whole egg**
	Raw skinless chicken breast	Raw skin-on chicken breast	Raw beef	Raw tuna	Raw whole egg
Test matrix – specific	Pool of meat cuts with each cut of ~20 g weight				Pool of whole egg homogenate
containment	3 L Beaker, sterile				2 L Beaker, sterile
Application of SALMOCIN/carrier solution	Spraying, equal distribution by tumbling, hand mixing	Addition, equal distribution by tumbling, hand mixing			Addition, equal distribution using a stirrer at 750 rpm for 1 min
Type of SALMOCIN/carrier solution	TSP extract of production host	Purified protein			
Application rate	28.71 mL/kg 3 mg/kg SalE1b 3+1+1+1 mg/kg SalE1b+SalE1a+SalE2+SalE7 0.3+0.1+0.1+0.1 mg/kg SalE1b+SalE1a+SalE2+SalE7	20 mL/kg 5 mg/kg SalE1b 1 mg/kg SalE1b 0.5 mg/kg SalE1b 0.1 mg/kg SalE1b	20 mL/kg 5 or 0.5 mg/kg SalE1b		

D.9.7 Aliquoting and packaging of food samples

Thirty (30) min after SALMOCIN application, 2 meat cuts ~20 g each are combined for one sample. Replicate meat samples are placed into sterile sample bags (BagFilter®400 P), the exact weight of each sample is recorded, and sample bags are closed using a closing clip (BagClip®400).

For whole egg, 40 mL of whole egg homogenate are placed into sterile sample bags (BagFilter®400 P), the samples are further processed as described for meat samples. A summary matrix of these steps in the process is shown in Table D-5.

D.9.8 Storage of food samples

The sealed food samples from Step 9.6 are then stored at 10 °C and sampled for counts of *Salmonella* contamination at different timepoints of storage as indicated in Table D-5. Images of various foods

contaminated with *Salmonella*, treated with salmocins, and incubated are shown in Figure D-1 prior to and post homogenization in BagFilter®400 P.

D.9.9 Analysis of viable populations of pathogenic *Salmonella* on meat samples

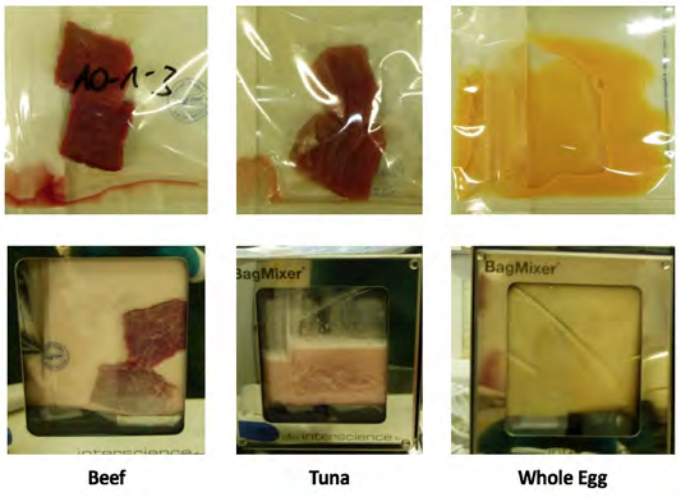
Preparation of sample homogenates

For recovery of pathogenic *Salmonella* from food samples, to each ~40 g aliquot of meat or whole egg samples ~160 mL buffered peptone water is added using a sterile 250 mL measuring cylinder, respectively. The volume of medium used for each sample is recorded. The samples are homogenized in a laboratory blender (BagMixer® 400 CC®; settings: gap 0, time 30 s, speed 4).

Table D-5. Matrix for aliquoting and packaging of SALMOCIN-treated food samples

Test matrix – general	**Whole cuts**				**Whole egg**
	Raw skinless chicken breast	Raw skin-on chicken breast	Raw beef	Raw tuna	Raw whole egg
Test matrix – specific	Pool of meat cuts with each cut of ~20 g weight				Pool of whole egg homogenate
Containment	3 L Beaker, sterile				2 L Beaker, sterile
Aliquoting and **packaging**	Two meat cuts of each ~20 g placed into a sterile bag BagFilter®400 P using sterile forceps; for each treatment group, 4 replicates are prepared				40 mL whole egg homogenate placed into a sterile bag BagFilter®400 P using a sterile serological pipette; for each treatment group, 4 replicates are prepared
Incubation at RT upon SALMOCIN application	1 h (including time for aliquoting and packaging)		2 h (including time for aliquoting and packaging)		
Sampling times for microbial counts upon storage at 10°C	1 h 24 h 48 h 72 h	1 h 24 h 72 h	1 h 48 h		

Figure D-1. Homogenization of foods contaminated with *Salmonella* and salmocin treatment



Quantification of pathogenic *Salmonella* population density on food samples by dilution plating and CFU enumeration

In SALMOCIN efficacy tests with all tested food matrices, 10 mL microbial suspension from the filtered part of the storage bag resulting from sample homogenization is transferred into a 15 mL Falcon tube using a serological pipet. A 1:10 dilution series of microbial suspension (100 µl microbial suspension + 900 µl peptone water) is prepared.

Subsequently, 100 µl aliquots of up to 1 mL undiluted or diluted microbial suspensions are plated on XLD agar supplemented with 25 µg/mL nalidixic acid. The plates are incubated for 16-20 h at 37 °C and the CFU are enumerated.

The CFU per g food sample is calculated as follows:

$$\frac{\text{Total CFU}}{\text{g Food}} = \frac{\text{Actual CFU x Dilution Factor}}{\text{0.1 mL Plating Volume}} \times \frac{\text{Actual mL Peptone Water}}{\text{Actual g Sample}}$$

For plated aliquots of the same sample, the average number of CFU/g food is calculated.

D.9.10 Statistical analysis

The efficacy of the SALMOCIN treatment in reducing the number of viable pathogenic *Salmonella* in the experimentally contaminated food samples, and the duration of (residual) technical effect of SALMOCIN treatment, are evaluated by comparing the data obtained with the carrier-treated control samples and SALMOCIN-treated samples by one-way ANOVA (Tukey´s multiple comparisons test) and unpaired parametric t-test using GraphPad Prism v. 6.01. Criterion for null hypothesis rejection = $p \leq 0.05$.

END OF NOTICE.

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Appendix 1 to Notifier's Responses to FSIS Questions

GRN 824 SALMOCIN

Bacteriocin Preparations Specific to *Salmonella*

Statistical Analyses – On-Matrix Studies

24 April 2019

FOR CFSAN/FSIS INTERNAL DISCUSSION ONLY

Efficacy – skinless poultry matrix (chicken breast fillet)

Meat matrix

→raw chicken skinless chicken breast fillets trimmed to ~20 g pieces

Introduction of *Salmonella* contamination

→*S. enterica* strains used were nalidixic acid resistant mutants

→grown in LB medium supplemented with 25 µg/ml nalidixic acid to OD_{600}~0.3

S. enterica ssp. *enterica*, serotype Enteritidis (ATCC®13076™*nalR#6)
S. enterica ssp. *enterica*, serotype Typhimurium (ATCC®14028™*nalR#3)
S. enterica ssp. *enterica*, serotype Newport (ATCC®6962™*nalR#4)
S. enterica ssp. *enterica*, serotype Javiana (ATCC®10721™*nalR#4)
S. enterica ssp. *enterica*, serotype Heidelberg (ATCC®8326™*nalR#5)
S. enterica ssp. *enterica*, serotype Infantis (ATCC®BAA-1675™*nalR#6)
S. enterica ssp. *enterica*, serotype Muenchen (ATCC®8388™*nalR#1)

→Contamination level: ~$1x10^3$-$1x10^4$ cfu/g food (intended)

→food matrix contaminated with 1:1:1:1:1:1:1 mixture of 7 *Salmonella* strains representing 7 different serotypes seryotypes (OD_{600}=0.001 (~$1.5x10^5$ cfu/ml) in LB, 10 ml/kg application rate of bacterial solution)

→bacterial solution was added to meat cubes and equally distributed on meat pieces by hand-massage

→contaminated meat was incubated for 30 min at RT for attachment of bacteria and hand-massaged upon 15 and 30 min. of incubation

Salmocin treatment

→salmocin solutions used were TSP extracts of *N. benthamiana* plant material expressing salmocins prepared using 5 vol. of 50 mM HEPES pH7.0, 10 mM K acetate, 5 mM Mg acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 300 mM NaCl

→carrier solution used was TSP extract of *N. benthamiana* WT plant material prepared using 5 vol. of 50 mM HEPES pH7.0, 10 mM K acetate, 5 mM Mg acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 300 mM NaCl

→treatments: Carrier
3 mg/kg SalE1a
3+1+1+1 mg/kg SalE1a+SalE1b+SalE2+SalE7
0.3+0.1+0.1+0.1 mg/kg SalE1a+SalE1b+SalE2+SalE7

→ Salmocin or carrier solution were applied with a rate of 28.71 ml/kg by spraying in puffs of each 100 µl on contaminated meat cubes using atomizer flasks and equally distributed by hand-massage in between spray application

→treated meat was incubated for 30 min at RT and hand-massaged upon 15 and 30 min. of incubation

Sample preparation and analysis for bacterial counts

→40 g aliquots of meat pieces were packed into bags and stored at 10°C

→total incubation time of meat at RT upon salmocin treatment: 1.5 h

→meat samples were supplemented with 4 vol. peptone water and homogenized in Bag Mixer

→serial dilutions of recovered bacterial solution were plated on XLD Agar supplemented with 25 µg/ml nalidixic acid

→plates were incubated at 37°C for 16-20h and cfu were counted

Efficacy – skinless poultry matrix (chicken breast fillet)

Fig. 2-8

medium contamination level
intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

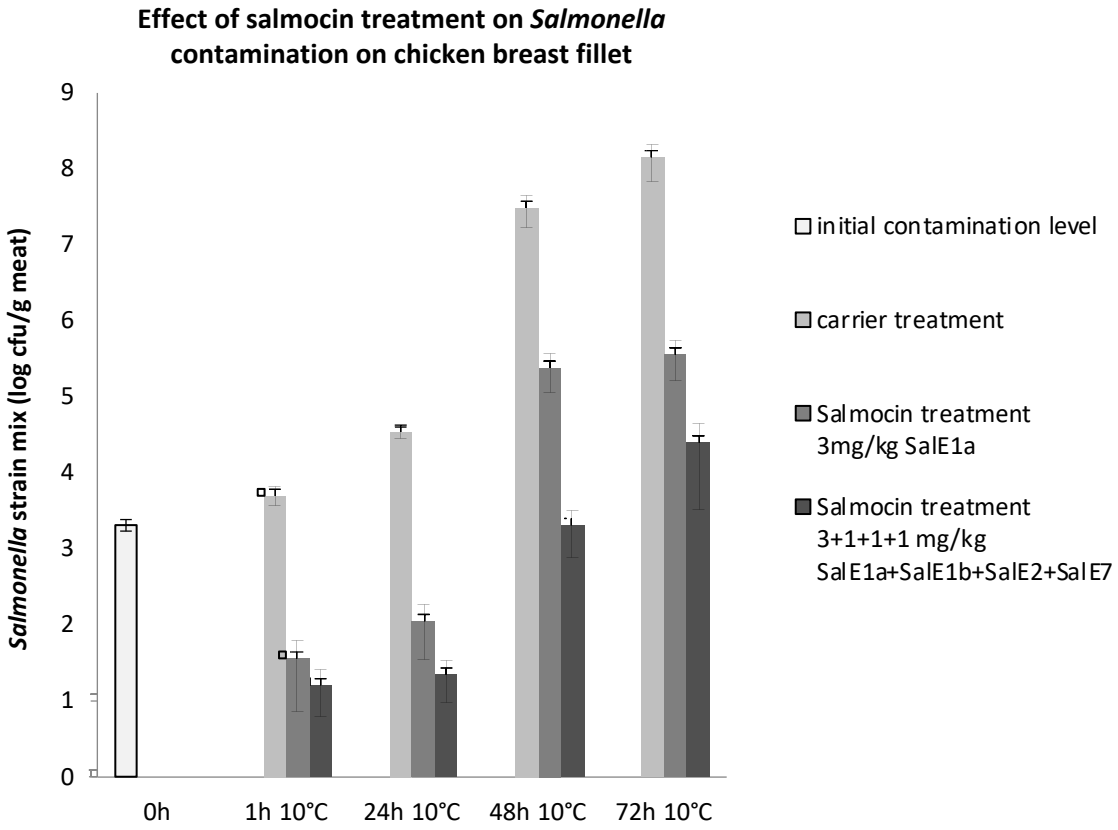
25-04-2017

Inoculum:
S. enterica ssp. *enterica*, 7-strain mix, nalidixic acid resistant mutants of serotypes:

Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
Newport (ATCC®6962™*nalR#4)
Javiana (ATCC®10721™*nalR#4)
Heidelberg (ATCC®8326™*nalR#5)
Infantis (ATCC®BAA-1675™*nalR#6)
Muenchen (ATCC®8388™*nalR#1)
(1.7x10^5 cfu/ml, 10 ml/kg)

Replicates:
N=4

Initial contamination:
2.1x10^3



Efficacy – skinless poultry matrix (chicken breast fillet)

Fig. 2-8

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

25-04-2017

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)
1 h	yes	7160 4120 4960 4200	3.85 3.61 3.70 3.62	Carrier	N=4	5.11x10^3	5.08x10^3	2.16	99.32	Yes (0.2065)	Yes (<0.0001)	Yes (0.1387)
1 h	yes	16 28 76 20	1.20 1.45 1.88 1.30	Salmocin 3 mg/kg	N=4	3.50x10^1				Yes (0.4003)		
1 h	yes	7160 4120 4960 4200	3.85 3.61 3.70 3.62	Carrier	N=4	5.11x10^3	5.09x10^3	2.50	99.69	Yes (0.2065)	Yes (<0.0001)	Yes (0.0849)
1 h	yes	4 16 28 16	0.60 1.20 1.45 1.20	Salmocin 3+1+1+1 mg/kg	N=4	1.60x10^1				Yes (0.2841)		
24 h	yes	40533 28667 29067 38000	4.61 4.46 4.46 4.58	Carrier	N=4	3.40x10^4	3.40x10^4	2.49	99.68	Yes (0.1612)	Yes (<0.0001)	Yes (0.0667)
24 h	yes	108 216 64 52	2.03 2.33 1.81 1.72	Salmocin 3 mg/kg	N=4	1.10x10^2				Yes (0.6472)		
24 h	yes	40533 28667 29067 38000	4.61 4.46 4.46 4.58	Carrier	N=4	3.40x10^4	3.40x10^4	3.19	99.94	Yes (0.1612)	Yes (<0.0001)	Yes (0.1178)
24 h	yes	40 20 16 12	1.60 1.30 1.20 1.08	Salmocin 3+1+1+1 mg/kg	N=4	2.20x10^1				Yes (0.6865)		
48 h	yes	17000000 32460000 44560000 29640000	7.23 7.51 7.65 7.47	Carrier	N=4	3.04x10^7	3.02x10^7	2.10	99.20	Yes (0.7197)	Yes (<0.0001)	Yes (0.3664)
48 h	yes	224000 380000 292000 74460	5.35 5.58 5.47 4.87	Salmocin 3 mg/kg	N=4	2.43x10^5				Yes (0.3421)		
48 h	yes	17000000 32460000 44560000 29640000	7.23 7.51 7.65 7.47	Carrier	N=4	3.04x10^7	3.02x10^7	2.10	99.20	Yes (0.7197)	Yes (<0.0001)	Yes (0.6341)
48 h	yes	1708 3772 1064 1424	3.23 3.58 3.03 3.15	Salmocin 3+1+1+1 mg/kg	N=4	2.43x10^5				Yes (0.5394)		

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

Yes= Normal probability distribution

Yes= Statistically significantly different

Yes= Equal variances

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy – skinless poultry matrix (chicken breast fillet)

Fig. 2-8

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

25-04-2017

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)
72 h	yes	120000000 190000000 44800000 190000000	8.08 8.28 7.65 8.28	Carrier	N=4	1.36x10^8	1.36x10^8	2.58	99.74	Yes (0.1468)	Yes (<0.0001)	Yes (0.7811)
72 h	yes	194000 464000 570000 192000	5.29 5.67 5.76 5.28	Salmocin 3 mg/kg	N=4	3.55x10^5				Yes (0.1256)		
72 h	yes	120000000 190000000 44800000 190000000	8.08 8.28 7.65 8.28	Carrier	N=4	1.36x10^8	1.36x10^8	3.75	99.98	Yes (0.1468)	Yes (<0.0001)	Yes (0.3672)
72 h	yes	50000 3120 30200 12600	4.70 3.49 4.48 4.10	Salmocin 3+1+1+1 mg/kg	N=4	2.40x10^4				Yes (0.7207)		
										Yes= Normal probability distribution	Yes= Statistically significantly different	Yes= Equal variances

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy (re-growth) – skinless poultry matrix (chicken breast fillet)

Fig. 2-8

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

25-04-2017

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	replicates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	Analysis of statistical significance log_{10} values: F-test for equality of variances (p-value)
1 h	7160 4120 4960 4200	3.85 3.61 3.70 3.62	Carrier	N=4	5.11x10^3	2.90x10^4	0.82	567	Yes (<0.0001)	Yes (0.5735)
24 h	40533 28667 29067 38000	4.61 4.46 4.46 4.58	Carrier	N=4	3.41x10^4					
1 h	7160 4120 4960 4200	3.85 3.61 3.70 3.62	Carrier	N=4	5.11x10^3	3.04x10^7	3.77	159230594811	Yes (<0.0001)	Yes (0.4805)
48 h	17000000 32460000 44560000 29640000	7.23 7.51 7.65 7.47	Carrier	N=4	3.04x10^7					
1 h	7160 4120 4960 4200	3.85 3.61 3.70 3.62	Carrier	N=4	5.11x10^3	1.36x10^8	4.43	2665262	Yes (<0.0001)	Yes (0.1424)
72 h	120000000 190000000 44800000 190000000	8.08 8.28 7.65 8.28	Carrier	N=4	1.36x10^8					
1 h	16 28 76 20	1.20 1.45 1.88 1.30	Salmocin 3 mg/kg	N=4	3.50x10^1	7.50x10^1	0.50	214	Yes (0.0447)	Yes (0.8980)
24 h	108 216 64 52	2.03 2.33 1.81 1.72	Salmocin 3 mg/kg	N=4	1.10x10^2					
1 h	16 28 76 20	1.20 1.45 1.88 1.30	Salmocin 3 mg/kg	N=4	3.50x10^1	2.43x10^5	3.84	693086	Yes (<0.0001)	Yes (0.9492)
48 h	224000 380000 292000 74460	5.35 5.58 5.47 4.87	Salmocin 3 mg/kg	N=4	2.43x10^5					
1 h	16 28 76 20	1.20 1.45 1.88 1.30	Salmocin 3 mg/kg	N=4	3.50x10^1	3.55x10^5	4.01	1014186	Yes (<0.0001)	Yes (0.7686)
72 h	194000 464000 570000 192000	5.29 5.67 5.76 5.28	Salmocin 3 mg/kg	N=4	3.55x10^5					

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

→statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 72 h, different kinetics of regrowth when compared to carrier treatment due to different contamination levels created upon treatment

Efficacy (re-growth) – skinless poultry matrix (chicken breast fillet)

Fig. 2-8

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

25-04-2017

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	replicates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	Analysis of statistical significance log_{10} values: F-test for equality of variances (p-value)
1 h	4 16 28 16	0.60 1.20 1.45 1.20	Salmocin 3+1+1+1 mg/kg	N=4	1.60x10^1	6.00x10^0	0.14	38	No (0.4227)	Yes (0.4515)
24 h	40 20 16 12	1.60 1.30 1.20 1.08	Salmocin 3+1+1+1 mg/kg	N=4	2.20x10^1					
1 h	4 16 28 16	0.60 1.20 1.45 1.20	Salmocin 3+1+1+1 mg/kg	N=4	1.60x10^1	1.98x10^3	2.10	12350	Yes (<0.0001)	Yes (0.5021)
48 h	1708 3772 1064 1424	3.23 3.58 3.03 3.15	Salmocin 3+1+1+1 mg/kg	N=4	1.99x10^3					
1 h	4 16 28 16	0.60 1.20 1.45 1.20	Salmocin 3+1+1+1 mg/kg	N=4	1.60x10^1	2.40x10^4	3.18	149775	Yes (<0.0001)	Yes (0.5468)
72 h	50000 3120 30200 12600	4.70 3.49 4.48 4.10	Salmocin 3+1+1+1 mg/kg	N=4	2.4x10^4					

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

→statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 72 h, different kinetics of regrowth when compared to carrier treatment due to different contamination levels created upon treatment

Efficacy – skinless poultry matrix (chicken breast fillet)

Fig. 2-9

medium contamination level
intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

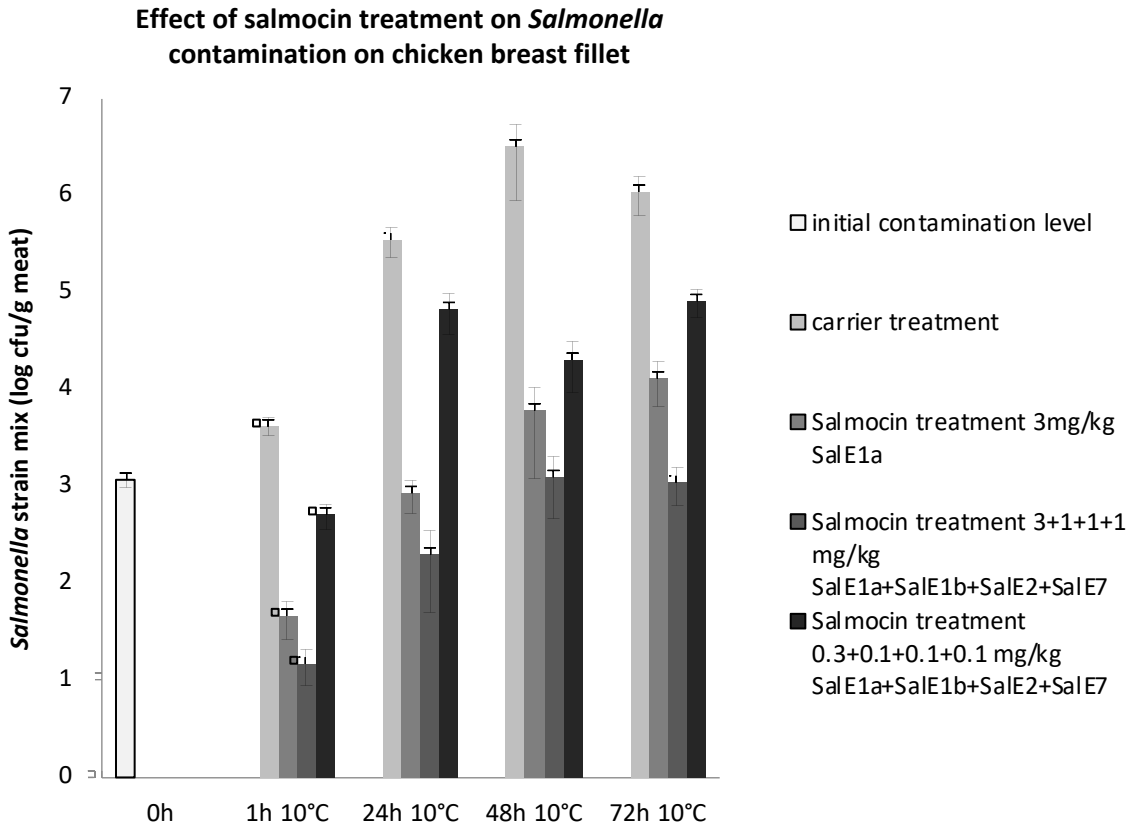
08-08-2017

Inoculum:
S. enterica ssp. *enterica*, 7-strain mix, nalidixic acid resistant mutants of serotypes:

Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
Newport (ATCC®6962™*nalR#4)
Javiana (ATCC®10721™*nalR#4)
Heidelberg (ATCC®8326™*nalR#5)
Infantis (ATCC®BAA-1675™*nalR#6)
Muenchen (ATCC®8388™*nalR#1)
(2.43x10^5 cfu/ml, 10 ml/kg)

Replicates:
N=4

Initial contamination:
1.18x10^3



Efficacy – skinless poultry matrix (chicken breast fillet)

Fig. 2-9

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

08-08-2017

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)
1 h	yes	3160 4440 4200 5360	3.50 3.65 3.62 3.73	Carrier	N=4	4.29x10^3	4.24x10^3	1.97	99.93	Yes (0.8207)	Yes (<0.0001)	Yes (0.2981)
1 h	yes	28 32 56 68	1.45 1.51 1.75 1.83	Salmocin 3 mg/kg	N=4	4.60x10^1				Yes (0.4135)		
1 h	yes	3160 4440 4200 5360	3.50 3.65 3.62 3.73	Carrier	N=4	4.29x10^3	4.28x10^3	2.46	99.65	Yes (0.8207)	Yes (<0.0001)	Yes (0.2751)
1 h	yes	20 12 8 20	1.30 1.08 0.90 1.30	Salmocin 3+1+1+1 mg/kg	N=4	1.50x10^1				Yes (0.2739)		
1 h	yes	3160 4440 4200 5360	3.50 3.65 3.62 3.73	Carrier	N=4	4.29x10^4	3.78x10^3	0.93	89.09	Yes (0.8207)	Yes (<0.0001)	Yes (0.6238)
1 h	yes	344 692 448 552	2.54 2.84 2.65 2.74	Salmocin 0.3+0.1+01+0.1 mg/kg	N=4	5.09x10^2				Yes (0.9852)		
24 h	yes	228000 302000 516000 362000	5.38 5.48 5.71 5.56	Carrier	N=4	3.52x10^5	3.51x10^5	2.62	99.76	Yes (0.9849)	Yes (<0.0001)	Yes (0.8534)
24 h	yes	804 489 1250 829	2.91 2.69 3.10 2.92	Salmocin 3 mg/kg	N=4	8.43x10^2				Yes (0.7417)		
24 h	yes	228000 302000 516000 362000	5.38 5.48 5.71 5.56	Carrier	N=4	3.52x10^5	3.52x10^5	3.24	99.94	Yes (0.9849)	Yes (<0.0001)	Yes (0.2806)
24 h	yes	420 140 164 80	2.62 2.15 2.21 1.90	Salmocin 3+1+1+1 mg/kg	N=4	2.01x10^2				Yes (0.7470)		
24 h	yes	228000 302000 516000 362000	5.38 5.48 5.71 5.56	Carrier	N=4	3.52x10^5	2.85x10^5	0.72	81.10	Yes (0.9849)	Yes (0.0009)	Yes (0.6859)
24 h	yes	106000 74000 50000 38800	5.03 4.87 4.70 4.59	Salmocin 0.3+0.1+01+0.1 mg/kg	N=4	6.72x10^4				Yes (0.8862)		
										Yes= Normal probability distribution	Yes= Statistically significantly different	Yes= Equal variances

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy – skinless poultry matrix (chicken breast fillet)

Fig. 2-9

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

08-08-2017

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)
48 h	yes	1580000 3240000 2100000 5680000	6.20 6.51 6.32 6.75	Carrier	N=4	3.12x10^6	3.11x10^6	2.72	98.81	Yes (0.8516)	Yes (<0.0001)	Yes (0.3051)
48 h	yes	2667 10320 9600 1093	3.43 4.01 3.98 3.04	Salmocin 3 mg/kg	N=4	5.92x10^3				Yes (0.3340)		
48 h	yes	1580000 3240000 2100000 5680000	6.20 6.51 6.32 6.75	Carrier	N=4	3.12x10^6	3.12x10^6	3.39	99.96	Yes (0.8516)	Yes (<0.0001)	Yes (0.7175)
48 h	yes	543 1896 617 1980	2.73 3.28 2.79 3.30	Salmocin 3+1+1+1 mg/kg	N=4	1.26x10^3				Yes (0.0827)		
48 h	yes	1580000 3240000 2100000 5680000	6.20 6.51 6.32 6.75	Carrier	N=4	3.12x10^6	3.10x10^6	2.19	99.54	Yes (0.8516)	Yes (<0.0001)	Yes (0.9560)
48 h	yes	34800 22000 10000 14400	4.54 4.34 4.00 4.16	Salmocin 0.3+0.1+01+0.1 mg/kg	N=4	2.03x10^4				Yes (0.9543)		
72 h	yes	800000 840000 960000 1800000	5.90 5.92 5.98 6.26	Carrier	N=4	1.10x10^6	1.09x10^6	1.92	98.81	Yes (0.0958)	Yes (<0.0001)	Yes (0.7586)
72 h	yes	13400 22000 8800 8200	4.13 4.34 3.94 3.91	Salmocin 3 mg/kg	N=4	1.31x10^4				Yes (0.4414)		
72 h	yes	800000 840000 960000 1800000	5.90 5.92 5.98 6.26	Carrier	N=4	1.10x10^6	1.10x10^6	3.00	99.90	Yes (0.0958)	Yes (<0.0001)	Yes (0.8520)
72 h	yes	1120 920 640 1760	3.05 2.96 2.81 3.25	Salmocin 3+1+1+1 mg/kg	N=4	1.11x10^3				Yes (0.9780)		
72 h	yes	800000 840000 960000 1800000	5.90 5.92 5.98 6.26	Carrier	N=4	1.10x10^6	1.02x10^6	1.13	93.70	Yes (0.0958)	Yes (<0.0001)	Yes (0.9097)
72 h	yes	51200 104000 67000 104000	4.71 5.02 4.83 5.02	Salmocin 0.3+0.1+01+0.1 mg/kg	N=4	8.16x10^4				Yes (0.2521)		
										Yes= Normal probability distribution	Yes= Statistically significantly different	Yes= Equal variances

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy (re-growth) – skinless poultry matrix (chicken breast fillet)

Fig. 2-9

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

08-08-2017

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	replicates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	Analysis of statistical significance log_{10} values: F-test for equality of variances (p-value)
1 h	3160 4440 4200 5360	3.50 3.65 3.62 3.73	Carrier	N=4	4.29x10^3	3.48x10^5	1.91	8105	Yes (<0.0001)	Yes (0.4813)
24 h	228000 302000 516000 362000	5.38 5.48 5.71 5.56	Carrier	N=4	3.52x10^5					
1 h	3160 4440 4200 5360	3.50 3.65 3.62 3.73	Carrier	N=4	4.29x10^3	3.12x10^6	2.86	72627	Yes (<0.0001)	Yes (0.1587)
48 h	1580000 3240000 2100000 5680000	6.20 6.51 6.32 6.75	Carrier	N=4	3.12x10^6					
1 h	3160 4440 4200 5360	3.50 3.65 3.62 3.73	Carrier	N=4	4.29x10^3	1.10x10^6	2.41	25541	Yes (<0.0001)	Yes (0.3994)
72 h	800000 840000 960000 1800000	5.90 5.92 5.98 6.26	Carrier	N=4	1.10x10^6					
1 h	28 32 56 68	1.45 1.51 1.75 1.83	Salmocin 3 mg/kg	N=4	4.60x10^1	7.97x10^2	1.26	1733	Yes (<0.0001)	Yes (0.8617)
24 h	804 489 1250 829	2.91 2.69 3.10 2.92	Salmocin 3 mg/kg	N=4	8.43x10^2					
1 h	28 32 56 68	1.45 1.51 1.75 1.83	Salmocin 3 mg/kg	N=4	4.60x10^1	5.87x10^3	2.11	12770	Yes (0.0002)	Yes (0.1628)
48 h	2667 10320 9600 1093	3.43 4.01 3.98 3.04	Salmocin 3 mg/kg	N=4	5.92x10^3					
1 h	28 32 56 68	1.45 1.51 1.75 1.83	Salmocin 3 mg/kg	N=4	4.60x10^1	1.31x10^4	2.45	28378	Yes (<0.0001)	Yes (0.9246)
72 h	13400 22000 8800 8200	4.13 4.34 3.94 3.91	Salmocin 3 mg/kg	N=4	1.31x10^4					

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

→statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 72 h, different kinetics of regrowth when compared to carrier treatment due to different contamination levels created upon treatment

Efficacy (re-growth) – skinless poultry matrix (chicken breast fillet)

Fig. 2-9

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

08-08-2017

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	replicates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	Analysis of statistical significance log_{10} values: F-test for equality of variances (p-value)
1 h	20 12 8 20	1.30 1.08 0.90 1.30	Salmocin 3+1+1+1 mg/kg	N=4	1.50x10^1	8.86x10^2	1.13	1240	Yes (0.0009)	Yes (0.4895)
24 h	420 140 164 80	2.62 2.15 2.21 1.90	Salmocin 3+1+1+1 mg/kg	N=4	2.01x10^2					
1 h	20 12 8 20	1.30 1.08 0.90 1.30	Salmocin 3+1+1+1 mg/kg	N=4	1.50x10^1	1.24x10^3	1.92	8293	Yes (<0.0001)	Yes (0.4746)
48 h	543 1896 617 1980	2.73 3.28 2.79 3.30	Salmocin 3+1+1+1 mg/kg	N=4	1.26x10^3					
1 h	20 12 8 20	1.30 1.08 0.90 1.30	Salmocin 3+1+1+1 mg/kg	N=4	1.50x10^1	1.10x10^3	1.87	7300	Yes (<0.0001)	Yes (0.9339)
72 h	1120 920 640 1760	3.05 2.96 2.81 3.25	Salmocin 3+1+1+1 mg/kg	N=4	1.11x10^3					
1 h	344 692 448 552	2.54 2.84 2.65 2.74	Salmocin 0.3+0.1+01+0.1 mg/kg	N=4	5.09x10^2	6.67x10^4	2.12	13102	Yes (<0.0001)	Yes (0.5352)
24 h	106000 74000 50000 38800	5.03 4.87 4.70 4.59	Salmocin 0.3+0.1+01+0.1 mg/kg	N=4	6.72x10^4					
1 h	344 692 448 552	2.54 2.84 2.65 2.74	Salmocin 0.3+0.1+01+0.1 mg/kg	N=4	5.09x10^2	1.98x10^4	1.60	3888	Yes (<0.0001)	Yes (0.3572)
48 h	34800 22000 10000 14400	4.54 4.34 4.00 4.16	Salmocin 0.3+0.1+01+0.1 mg/kg	N=4	2.03x10^4					
1 h	344 692 448 552	2.54 2.84 2.65 2.74	Salmocin 0.3+0.1+01+0.1 mg/kg	N=4	5.09x10^2	8.10x10^4	2.20	15922	Yes (<0.0001)	Yes (0.8006)
72 h	51200 104000 67000 104000	4.71 5.02 4.83 5.02	Salmocin 0.3+0.1+01+0.1 mg/kg	N=4	8.16x10^4					

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

→statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 72 h, different kinetics of regrowth when compared to carrier treatment due to different contamination levels created upon treatment

Efficacy – skin-on poultry matrix (chicken breast fillet)

Meat matrix

→raw chicken skin-on chicken breast fillets trimmed to ~20 g pieces

Introduction of *Salmonella* contamination

→*S. enterica* strains used were nalidixic acid resistant mutants

→grown in LB medium supplemented with 25 µg/ml nalidixic acid to OD_{600}~0.3

S. enterica ssp. *enterica*, serotype Enteritidis (ATCC®13076™*nalR#6)
S. enterica ssp. *enterica*, serotype Typhimurium (ATCC®14028™*nalR#3)
S. enterica ssp. *enterica*, serotype Newport (ATCC®6962™*nalR#4)
S. enterica ssp. *enterica*, serotype Javiana (ATCC®10721™*nalR#4)
S. enterica ssp. *enterica*, serotype Heidelberg (ATCC®8326™*nalR#5)
S. enterica ssp. *enterica*, serotype Infantis (ATCC®BAA-1675™*nalR#6)
S. enterica ssp. *enterica*, serotype Muenchen (ATCC®8388™*nalR#1)

→Contamination level: ~$1x10^3$-$1x10^4$ cfu/g food (intended)

→food matrix contaminated with 1:1:1:1:1:1:1 mixture of 7 *Salmonella* strains representing 7 different serotypes seryotypes (OD_{600}=0.001 (~$1.5x10^5$ cfu/ml) in LB, 10 ml/kg application rate of bacterial solution)

→bacterial solution was added to meat cubes and equally distributed on meat pieces by hand-massage

→contaminated meat was incubated for 30 min at RT for attachment of bacteria and hand-massaged upon 15 and 30 min. of incubation

→note: skin is partially separated from meat during mixing process

Salmocin treatment

→salmocin solutions used were purified protein powders in 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl

→carrier solution was 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl

→treatments: Carrier

5 mg/kg SalE1b, 1 mg/kg SalE1b, 0.5 mg/kg SalE1b, 0.1 mg/kg SalE1b

→ Salmocin or carrier solution were added with a rate of 20 ml/kg on contaminated meat cubes and equally distributed by hand-massage

→treated meat was incubated for 30 min at RT and hand-massaged upon 15 and 30 min. of incubation

Sample preparation and analysis for bacterial counts

→40 g aliquots of meat pieces were packed into bags and stored at 10°C

→total incubation time of meat at RT upon salmocin treatment: 1.5 h

→meat samples were supplemented with 4 vol. peptone water and homogenized in Bag Mixer

→serial dilutions of recovered bacterial solution were plated on XLD Agar supplemented with 25 µg/ml nalidixic acid

→plates were incubated at 37°C for 16-20h and cfu were counted

Efficacy – skin-on poultry matrix (chicken breast fillet)

Fig. 2-10

medium contamination level
intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

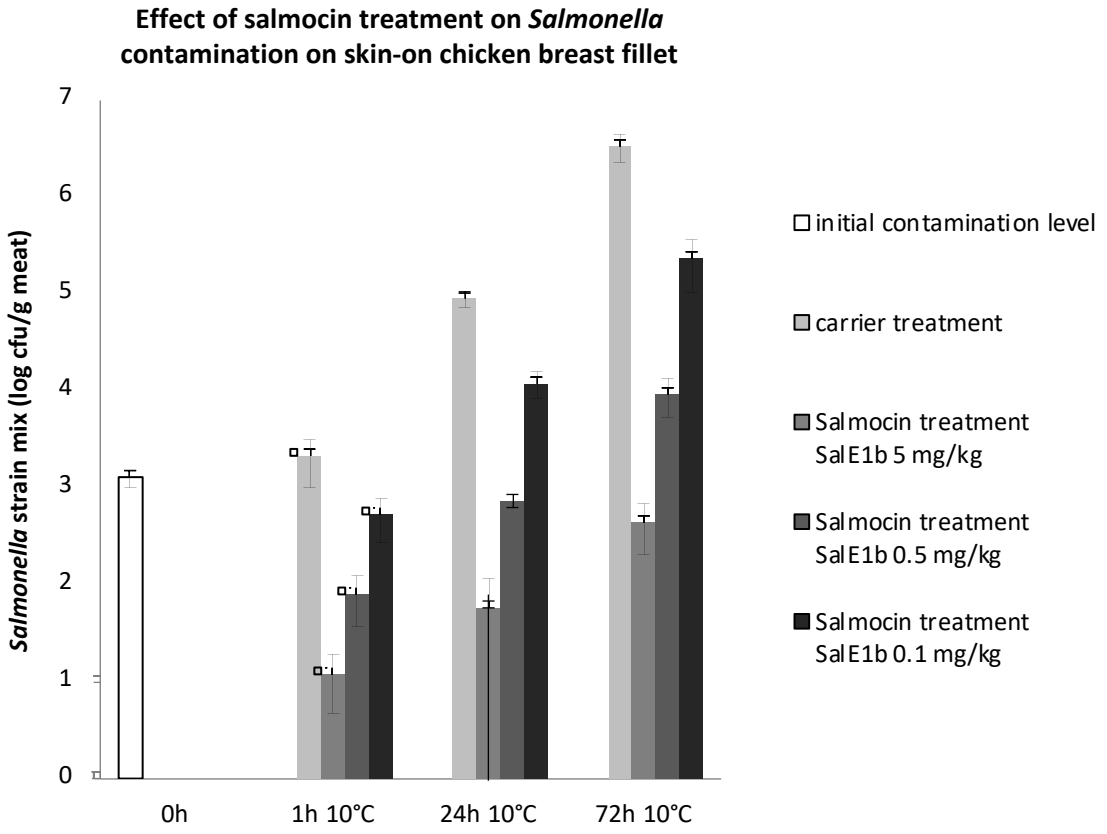
21-08-2018

Inoculum:
S. enterica ssp. *enterica*, 7-strain mix, nalidixic acid resistant mutants of serotypes:

Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
Newport (ATCC®6962™*nalR#4)
Javiana (ATCC®10721™*nalR#4)
Heidelberg (ATCC®8326™*nalR#5)
Infantis (ATCC®BAA-1675™*nalR#6)
Muenchen (ATCC®8388™*nalR#1)
(3.40x10^5 cfu/ml, 10 ml/kg)

Replicates:
N=4

Initial contamination:
1.26x10^3 cfu/g



Efficacy – skin-on poultry matrix (chicken breast fillet)

Fig. 2-10

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 1.26x10^3 cfu/g 21-08-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)	Non-parametric Mann-Whitney test (p-value)
1 h	yes	1053 1253 3093 2960	3.023 3.098 3.490 3.471	Carrier	N=4	2.09x10^3	2.08x10^3	2.24	99.43	Yes (0.1388) Yes (0.6416)	Yes (<0.0001)	Yes (0.6847)	Yes (<0.0001)	Yes (0.0286)
1 h	yes	4 16 20 8	0.602 1.204 1.301 0.903	Salmocin SalE1b 5 mg/kg	N=4	1.20x10^1								
1 h	yes	1053 1253 3093 2960	3.023 3.098 3.490 3.471	Carrier	N=4	2.09x10^3	2.01x10^3	1.41	96.12	Yes (0.1388) No (0.0485)	Yes (0.0005)	Yes (0.5532)	Yes (0.0009)	Yes (0.0286)
1 h	yes	112 80 20 112	2.049 1.903 1.301 2.049	Salmocin SalE1b 0.5 mg/kg	N=4	8.10x10^1								
1 h	yes	1053 1253 3093 2960	3.023 3.098 3.490 3.471	Carrier	N=4	2.09x10^3	1.57x10^3	0.6	74.98	Yes (0.1388) Yes (0.4393)	Yes (0.01550)	Yes (0.9257)	Yes (0.0151)	Yes (0.0286)
1 h	yes	500 804 200 588	2.699 2.905 2.301 2.769	Salmocin SalE1b 0.1 mg/kg	N=4	5.23x10^2								
24 h	yes	86800 69200 87200 105400	4.939 4.840 4.941 5.023	Carrier	N=4	8.72x10^4	8.71x10^4	3.18	99.93	Yes (0.6905) Yes (0.9796)	Yes (<0.0001)	No (0.0146)	Yes (0.0050)	Yes (0.0286)
24 h	yes	144 12 28 48	2.158 1.079 1.447 1.681	Salmocin SalE1b 5 mg/kg	N=4	5.80x10^1								
24 h	yes	86800 69200 87200 105400	4.939 4.840 4.941 5.023	Carrier	N=4	8.72x10^4	8.64x10^4	2.07	99.14	Yes (0.6905) Yes (0.5190)	Yes (<0.0001)	Yes (0.8646)	Yes (<0.0001)	Yes (0.0286)
24 h	yes	916 756 648 676	2.962 2.879 2.812 2.830	Salmocin SalE1b 0.5 mg/kg	N=4	7.49x10^2								
24 h	yes	86800 69200 87200 105400	4.939 4.840 4.941 5.023	Carrier	N=4	8.72x10^4	7.50x10^4	0.86	86.06	Yes (0.6905) Yes (0.8272)	Yes (<0.0001)	Yes (0.3677)	Yes (0.0001)	Yes (0.0286)
24 h	yes	17400 8600 12600 10000	4.241 3.934 4.100 4.000	Salmocin SalE1b 0.1 mg/kg	N=4	1.22x10^4								

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy – skin-on poultry matrix (chicken breast fillet)

Fig. 2-10

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 1.26x10^3 cfu/g 21-08-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)	Non-parametric Mann-Whitney test (p-value)
72 h	yes	2760000 4480000 3880000 2200000	6.441 6.651 6.589 6.342	Carrier	N=4	3.33x10^6	3.33x10^6	3.87	99.99	Yes (0.7261)	Yes (<0.0001)	Yes (0.4670)	Yes (<0.0001)	Yes (0.0286)
72 h	yes	420 350 790 230	2.623 2.544 2.898 2.362	Salmocin SalE1b 5 mg/kg	N=4	4.48x10^2				Yes (0.8882)				
72 h	yes	2760000 4480000 3880000 2200000	6.441 6.651 6.589 6.342	Carrier	N=4	3.33x10^6	3.32x10^6	2.55	99.72	Yes (0.7261)	Yes (<0.0001)	Yes (0.6294)	Yes (<0.0001)	Yes (0.0286)
72 h	yes	11600 13800 5800 6200	4.064 4.140 3.763 3.792	Salmocin SalE1b 0.5 mg/kg	N=4	9.35x10^3				Yes (0.2339)				
72 h	yes	2760000 4480000 3880000 2200000	6.441 6.651 6.589 6.342	Carrier	N=4	3.33x10^6	3.10x10^6	1.14	92.99	Yes (0.7261)	Yes (0.0001)	Yes (0.4248)	Yes (0.0004)	Yes (0.0286)
72 h	yes	122000 248000 410000 154000	5.086 5.394 5.613 5.188	Salmocin SalE1b 0.1 mg/kg	N=4	2.34x10^5				Yes (0.7834)				

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy (re-growth) – skin-on poultry matrix (chicken breast fillet)

Fig. 2-10

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 1.26x10^3 cfu/g 21-08-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	replicates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)	Non-parametric Mann-Whitney test (p-value)
1 h	3160	3.50	Carrier	N=4	2.09x10^3	8.51x10^4	1.62	4069.86	Yes (<0.0001)	Yes (0.0823)	Yes (0.0004)	Yes (0.0286)
	4440	3.65										
	4200	3.62										
	5360	3.73										
24 h	228000	5.38	Carrier	N=4	8.72x10^4							
	302000	5.48										
	516000	5.71										
	362000	5.56										
1 h	3160	3.50	Carrier	N=4	2.09x10^3	3.33x10^6	3.20	159230	Yes (<0.0001)	Yes (0.3841)	Yes (<0.0001)	Yes (0.0286)
	4440	3.65										
	4200	3.62										
	5360	3.73										
72 h	800000	5.90	Carrier	N=4	3.33x10^6							
	840000	5.92										
	960000	5.98										
	1800000	6.26										
1 h	28	1.45	Salmocin 5 mg/kg	N=4	1.20x10^1	4.60x10^1	0.68	383.33	No (0.0766)	Yes (0.5725)	No (0.0820)	No (0.1143)
	32	1.51										
	56	1.75										
	68	1.83										
24 h	804	2.91	Salmocin 5 mg/kg	N=4	5.80x10^1							
	489	2.69										
	1250	3.10										
	829	2.92										
1 h	28	1.45	Salmocin 5 mg/kg	N=4	1.20x10^1	4.32x10^2	1.57	3629.17	Yes (0.0002)	Yes (0.5795)	Yes (0.0072)	Yes (0.0286)
	32	1.51										
	56	1.75										
	68	1.83										
72 h	13400	4.13	Salmocin 5 mg/kg	N=4	4.48x10^2							
	22000	4.34										
	8800	3.94										
	8200	3.91										

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

→statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 72 h, different kinetics of regrowth when compared to carrier treatment due to different contamination levels created upon treatment

Efficacy (re-growth) – skin-on poultry matrix (chicken breast fillet)

Fig. 2-10

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 1.26x10^3 cfu/g 21-08-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	replicates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)	Non-parametric Mann-Whitney test (p-value)
1 h	112	2.049	Salmocin SalE1b 0.5 mg/kg	N=4	8.10x10^1	6.68x10^2	0.97	177.52	Yes (0.0012)	No (0.0213)	Yes (0.0086)	Yes (0.0286)
	80	1.903										
	20	1.301										
	112	2.049										
24 h	916	2.962	Salmocin SalE1b 0.5 mg/kg	N=4	7.49x10^2							
	756	2.879										
	648	2.812										
	676	2.830										
1 h	112	2.049	Salmocin SalE1b 0.5 mg/kg	N=4	8.10x10^1	9.27x10^3	2.06	9063.26	Yes (<0.0001)	Yes (0.3292)	Yes (0.0002)	No (0.0286)
	80	1.903										
	20	1.301										
	112	2.049										
72 h	11600	4.064	Salmocin SalE1b 0.5 mg/kg	N=4	9.35x10^3							
	13800	4.140										
	5800	3.763										
	6200	3.792										
1 h	500	2.699	Salmocin SalE1b 0.1 mg/kg	N=4	5.23x10^2	1.16x10^4	1.37	1446.78	Yes (<0.0001)	Yes (0.3022)	Yes (0.0004)	Yes (0.0286)
	804	2.905										
	200	2.301										
	588	2.769										
24 h	17400	4.241	Salmocin SalE1b 0.1 mg/kg	N=4	1.22x10^4							
	8600	3.934										
	12600	4.100										
	10000	4.000										
1 h	500	2.699	Salmocin SalE1b 0.1 mg/kg	N=4	5.23x10^2	2.33x10^5	2.95	51524.29	Yes (<0.0001)	Yes (0.8652)	Yes (<0.0001)	Yes (0.0286)
	804	2.905										
	200	2.301										
	588	2.769										
72 h	122000	5.086	Salmocin SalE1b 0.1 mg/kg	N=4	2.34x10^5							
	248000	5.394										
	410000	5.613										
	154000	5.188										

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

→statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 72 h, different kinetics of regrowth when compared to carrier treatment due to different contamination levels created upon treatment

Efficacy – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, left

medium contamination level
intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

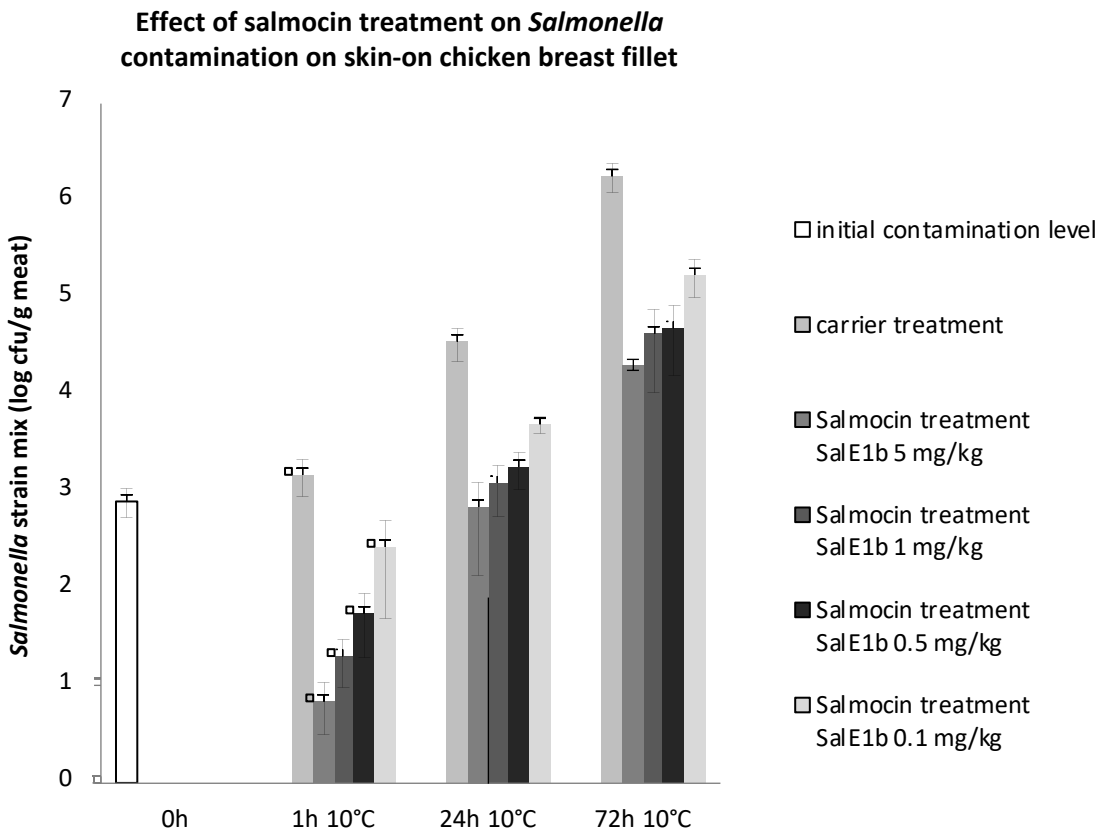
28-08-2018

Inoculum:
S. enterica ssp. *enterica*, 7-strain mix, nalidixic acid resistant mutants of serotypes:

Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
Newport (ATCC®6962™*nalR#4)
Javiana (ATCC®10721™*nalR#4)
Heidelberg (ATCC®8326™*nalR#5)
Infantis (ATCC®BAA-1675™*nalR#6)
Muenchen (ATCC®8388™*nalR#1)
(2.15x10^5 cfu/ml, 10 ml/kg)

Replicates:
N=4

Initial contamination:
0.81x10^3



Efficacy – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, left

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 0.81x10^3 cfu/g 28-08-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)
1 h	yes	1640 1213 907 2373	3.22 3.08 2.96 3.38	Carrier	N=4	1.53x10^3	1.53x10^3	2.34	99.54	Yes (0.9707)	Yes (<0.0001)	Yes (0.6619)
1 h	yes	4 12 8 4	0.60 1.08 0.90 0.62	Salmocin SalE1b 5 mg/kg	N=4	7.00x10^0				Yes (0.2465)		
1 h	yes	1640 1213 907 2373	3.22 3.08 2.96 3.38	Carrier	N=4	1.53x10^3	1.51x10^3	1.88	98.70	Yes (0.9707)	Yes (<0.0001)	Yes (0.5514)
1 h	yes	16 8 24 32	1.20 0.90 1.38 1.51	Salmocin SalE1b 1 mg/kg	N=4	2.00x10^1				Yes (0.7787)		
1 h	yes	1640 1213 907 2373	3.22 3.08 2.96 3.38	Carrier	N=4	1.53x10^3	1.48x10^3	1.44	96.41	Yes (0.9707)	Yes (0.0001)	Yes (0.4368)
1 h	yes	52 104 44 20	1.72 2.02 1.64 1.30	Salmocin SalE1b 0.5 mg/kg	N=4	5.50x10^1				Yes (0.9012)		
1 h	yes	1640 1213 907 2373	3.22 3.08 2.96 3.38	Carrier	N=4	1.53x10^3	1.26x10^3	0.74	81.87	Yes (0.9707)	Yes (0.0049)	Yes (0.3432)
1 h	yes	256 132 608 166	2.41 2.12 2.78 2.06	Salmocin SalE1b 0.1 mg/kg	N=4	2.78x10^2				Yes (0.4457)		

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, left

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 0.81x10^3 cfu/g 28-08-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)
24 h	yes	35400 19600 33400 50800	4.55 4.29 4.52 4.71	Carrier	N=4	3.84x10^4	3.41x10^4	1.70	98.02	Yes (0.7394)	Yes (<0.0001)	Yes (0.3415)
24 h	yes	1504 264 480 512	3.18 2.42 2.68 2.71	Salmocin SalE1b 5 mg/kg	N=4	6.90x10^2				Yes (0.5668)		
24 h	yes	35400 19600 33400 50800	4.55 4.29 4.52 4.71	Carrier	N=4	3.84x10^4	3.36x10^4	1.46	96.53	Yes (0.7394)	Yes (<0.0001)	Yes (0.6110)
24 h	yes	2096 1156 560 1016	3.32 3.06 2.75 3.01	Salmocin SalE1b 1 mg/kg	N=4	1.21x10^3				Yes (0.9052)		
24 h	yes	35400 19600 33400 50800	4.55 4.29 4.52 4.71	Carrier	N=4	3.84x10^4	3.30x10^4	1.29	94.83	Yes (0.7394)	Yes (<0.0001)	Yes (0.7446)
24 h	yes	853 2560 2147 1636	2.93 3.41 3.33 3.21	Salmocin SalE1b 0.5 mg/kg	N=4	1.80x10^3				Yes (0.5324)		
24 h	yes	35400 19600 33400 50800	4.55 4.29 4.52 4.71	Carrier	N=4	3.84x10^4	2.99x10^4	0.85	86.02	Yes (0.7394)	Yes (0.0001)	Yes (0.1942)
24 h	yes	4800 6000 4667 4000	3.68 3.78 3.67 3.60	Salmocin SalE1b 0.1 mg/kg	N=4	4.87x10^3				Yes (0.7676)		

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, left

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 0.81x10^3 cfu/g 28-08-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)
72 h	yes	1080000 2160000 1540000 2440000	6.03 6.33 6.19 6.39	Carrier	N=4	1.81x10^6	1.78x10^6	1.95	98.87	Yes (0.6728)	Yes (<0.0001)	Yes (0.1425)
72 h	yes	16600 21000 21600 22600	4.22 4.32 4.33 4.35	Salmocin SalE1b 5 mg/kg	N=4	2.05x10^4				Yes (0.1527)		
72 h	yes	1080000 2160000 1540000 2440000	6.03 6.33 6.19 6.39	Carrier	N=4	1.81x10^6	1.76x10^6	1.62	97.63	Yes (0.6728)	Yes (0.0001)	Yes (0.2178)
72 h	yes	12000 31200 88000 40200	4.08 4.49 4.94 4.60	Salmocin SalE1b 1 mg/kg	N=4	4.29x10^4				Yes (0.9258)		
72 h	yes	1080000 2160000 1540000 2440000	6.03 6.33 6.19 6.39	Carrier	N=4	1.81x10^6	1.76x10^6	1.56	97.27	Yes (0.6728)	Yes (0.0001)	Yes (0.2281)
72 h	yes	72000 25200 16000 84000	4.86 4.40 4.20 4.92	Salmocin SalE1b 0.5 mg/kg	N=4	4.93x10^4				Yes (0.3671)		
72 h	yes	1080000 2160000 1540000 2440000	6.03 6.33 6.19 6.39	Carrier	N=4	1.81x10^6	1.63x10^6	1.02	90.50	Yes (0.6728)	Yes (0.0002)	Yes (0.7285)
72 h	yes	126000 226000 240000 94000	5.10 5.35 5.38 4.97	Salmocin SalE1b 0.1 mg/kg	N=4	1.72x10^5				Yes (0.3451)		

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy (re-growth) – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, left

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 0.81x10^3 cfu/g 28-08-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	replicates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	1640	3.22	Carrier	N=4	1.53x10^3	3.33x10^4	1.36	2170	Yes (<0.0001)	Yes (0.9380)	Yes (<0.0001)
	1213	3.08									
	907	2.96									
	2373	3.38									
24 h	35400	4.55	Carrier	N=4	3.48x10^4						
	19600	4.29									
	33400	4.52									
	50800	4.71									
1 h	1640	3.22	Carrier	N=4	1.53x10^3	1.80x10^6	3.07	117617	Yes (<0.0001)	Yes (0.8511)	Yes (<0.0001)
	1213	3.08									
	907	2.96									
	2373	3.38									
72 h	1080000	6.03	Carrier	N=4	1.81x10^6						
	2160000	6.33									
	1540000	6.19									
	2440000	6.39									
1 h	4	0.60	Salmocin SalE1b 5 mg/kg	N=4	7.00x10^0	6.83x10^2	1.99	9757	Yes (<0.0001)	Yes (0.6481)	Yes (<0.0001)
	12	1.08									
	8	0.90									
	4	0.62									
24 h	1504	3.18	Salmocin SalE1b 5 mg/kg	N=4	6.9x10^2						
	264	2.42									
	480	2.68									
	512	2.71									
1 h	4	0.60	Salmocin SalE1b 5 mg/kg	N=4	7.00x10^0	2.04x10^4	3.47	292043	Yes (<0.0001)	No (0.0496)	Yes (<0.0001)
	12	1.08									
	8	0.90									
	4	0.62									
72 h	16600	4.22	Salmocin SalE1b 5 mg/kg	N=4	2.05x10^4						
	21000	4.32									
	21600	4.33									
	22600	4.35									
1 h	16	1.20	Salmocin SalE1b 1 mg/kg	N=4	2.00x10^1	1.19x10^3	1.78	6145	Yes (<0.0001)	Yes (0.8676)	Yes (<0.0001)
	8	0.90									
	24	1.38									
	32	1.51									
24 h	2096	3.32	Salmocin SalE1b 1 mg/kg	N=4	1.21x10^3						
	1156	3.06									
	560	2.75									
	1016	3.01									
1 h	16	1.20	Salmocin SalE1b 1 mg/kg	N=4	2.00x10^1	4.28x10^4	3.33	312799	Yes (<0.0001)	Yes (0.6209)	Yes (<0.0001)
	8	0.90									
	24	1.38									
	32	1.51									
72 h	12000	4.08	Salmocin SalE1b 1 mg/kg	N=4	4.29x10^4						
	31200	4.49									
	88000	4.94									
	40200	4.60									

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

→statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 72 h, different kinetics of regrowth when compared to carrier treatment due to different contamination levels created upon treatment

Efficacy (re-growth) – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, left

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 0.81x10^3 cfu/g 28-08-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	replicates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	52 104 44 20	1.72 2.02 1.64 1.30	Salmocin SalE1b 0.5 mg/kg	N=4	5.50x10^1	1.74x10^3	1.51	1977	Yes (0.0001)	Yes (0.5906)	Yes (0.0002)
24 h	853 2560 2147 1636	2.93 3.41 3.33 3.21	Salmocin SalE1b 0.5 mg/kg	N=4	1.80x10^3						
1 h	52 104 44 20	1.72 2.02 1.64 1.30	Salmocin SalE1b 0.5 mg/kg	N=4	5.50x10^1	4.92x10^4	2.95	95170	Yes (0.0001)	Yes (0.7815)	Yes (<0.0001)
72 h	72000 25200 16000 84000	4.86 4.40 4.20 4.92	Salmocin SalE1b 0.5 mg/kg	N=4	4.93x10^4						
1 h	256 132 608 166	2.41 2.12 2.78 2.06	Salmocin SalE1b 0.1 mg/kg	N=4	2.78x10^2	4.59x10^3	1.24	1651	Yes (0.0002)	No (0.0333)	Yes (0.0030)
24 h	4800 6000 4667 4000	3.68 3.78 3.67 3.60	Salmocin SalE1b 0.1 mg/kg	N=4	4.87x10^3						
1 h	256 132 608 166	2.41 2.12 2.78 2.06	Salmocin SalE1b 0.1 mg/kg	N=4	2.78x10^2	1.71x10^5	2.79	61591	Yes (<0.0001)	Yes (0.4251)	Yes (<0.0001)
72 h	126000 226000 240000 94000	5.10 5.35 5.38 4.97	Salmocin SalE1b 0.1 mg/kg	N=4	1.72x10^5						

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

→statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 72 h, different kinetics of regrowth when compared to carrier treatment due to different contamination levels created upon treatment

Efficacy – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, right

medium contamination level
intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

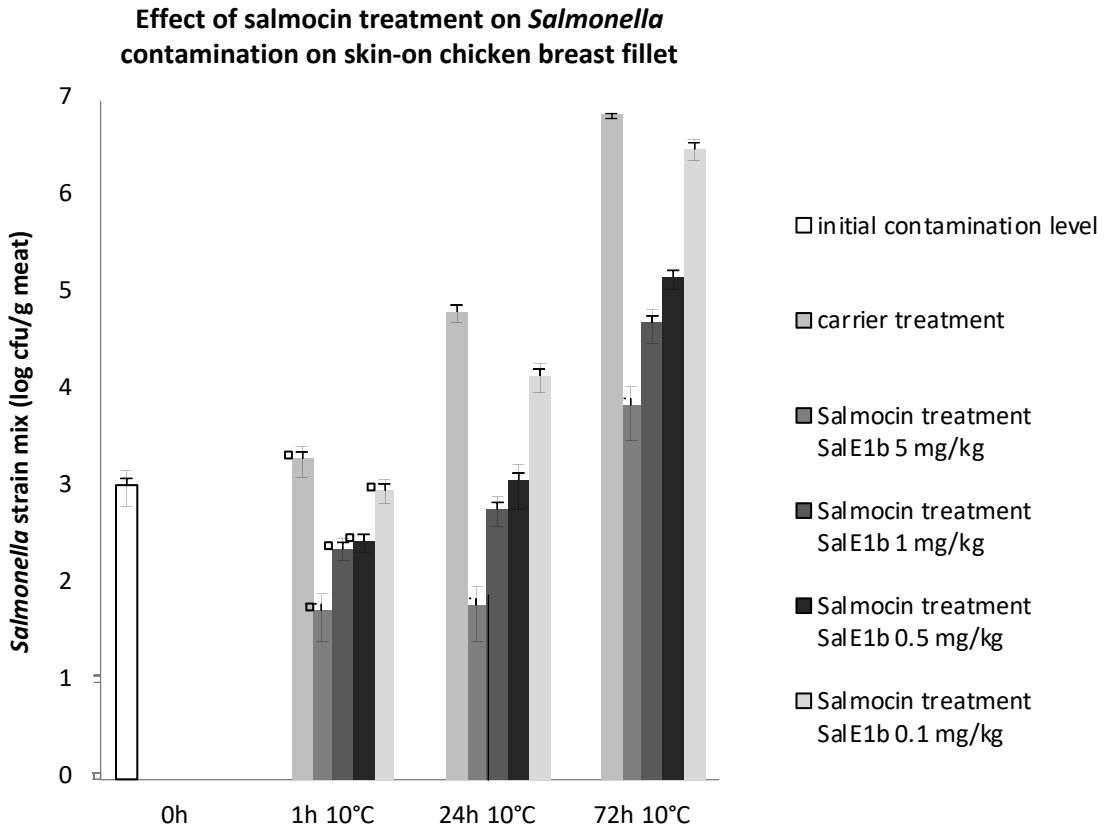
04-09-2018

Inoculum:
S. enterica ssp. *enterica*, 7-strain mix, nalidixic acid resistant mutants of serotypes:

Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
Newport (ATCC®6962™*nalR#4)
Javiana (ATCC®10721™*nalR#4)
Heidelberg (ATCC®8326™*nalR#5)
Infantis (ATCC®BAA-1675™*nalR#6)
Muenchen (ATCC®8388™*nalR#1)
(1.75x10^5 cfu/ml, 10 ml/kg)

Replicates:
N=4

Initial contamination:
1.1x10^3



Efficacy – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, right

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 1.1x10^3 cfu/g 04-09-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	yes	1667 1533 1773 3107	3.22 3.19 3.25 3.49	Carrier	N=4	2.02x10^3	1.97x10^3	1.56	97.28	Yes (0.0970)	Yes (<0.0001)	Yes (0.6847)	Yes (0.0286)	Yes (0.0001)
1 h	yes	24 92 56 48	1.38 1.96 1.75 1.68	Salmocin SalE1b 5 mg/kg	N=4	5.50x10^1				Yes (0.8696)				
1 h	yes	1667 1533 1773 3107	3.22 3.19 3.25 3.49	Carrier	N=4	2.02x10^3	1.78x10^3	0.92	87.92	Yes (0.0970)	Yes (0.0005)	Yes (0.5532)	Yes (0.0286)	Yes (<0.0001)
1 h	yes	268 148 280 280	2.43 2.17 2.45 2.45	Salmocin SalE1b 1 mg/kg	N=4	2.44x10^2				No (0.0064)				
1 h	yes	1667 1533 1773 3107	3.22 3.19 3.25 3.49	Carrier	N=4	2.02x10^3	1.74x10^3	0.85	85.89	Yes (0.0970)	Yes (0.01550)	Yes (0.9257)	Yes (0.0286)	Yes (0.0001)
1 h	yes	280 372 268 220	2.45 2.57 2.43 2.34	Salmocin SalE1b 0.5 mg/kg	N=4	2.85x10^2				Yes (0.7999)				
1 h	yes	1667 1533 1773 3107	3.22 3.19 3.25 3.49	Carrier	N=4	2.02x10^3	1.04x10^3	0.32	52.63	Yes (0.0970)	Yes (<0.0001)	No (0.0146)	Yes (0.0286)	Yes (0.0166)
1 h	yes	1252 660 1144 852	3.10 2.82 3.06 2.93	Salmocin SalE1b 0.1 mg/kg	N=4	9.77x10^2				Yes (0.6370)				

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, right

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 1.1x10^3 cfu/g 04-09-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)
24 h	yes	75600 76600 58800 49400	4.88 4.88 4.77 4.69	Carrier	N=4	6.51x10^4	6.50x10^4	3.02	99.90	Yes (0.3285)	Yes (<0.0001)	Yes (0.1147)	Yes (0.0286)	Yes (<0.0001)
24 h	yes	32 100 84 32	1.51 2.00 1.92 1.51	Salmocin SalE1b 5 mg/kg	N=4	6.20x10^1				Yes (0.0907)				
24 h	yes	75600 76600 58800 49400	4.88 4.88 4.77 4.69	Carrier	N=4	6.51x10^4	6.45x10^4	2.03	99.05	Yes (0.3285)	Yes (<0.0001)	Yes (0.5422)	Yes (0.0286)	Yes (<0.0001)
24 h	yes	920 600 480 471	2.96 2.78 2.68 2.67	Salmocin SalE1b 1 mg/kg	N=4	6.18x10^2				Yes (0.2268)				
24 h	yes	75600 76600 58800 49400	4.88 4.88 4.77 4.69	Carrier	N=4	6.51x10^4	6.39x10^4	1.73	98.16	Yes (0.3285)	Yes (<0.0001)	Yes (0.1749)	Yes (0.0286)	Yes (0.0001)
24 h	yes	949 577 1977 1291	2.98 2.76 3.30 3.11	Salmocin SalE1b 0.5 mg/kg	N=4	1.20x10^3				Yes (0.9950)				
24 h	yes	75600 76600 58800 49400	4.88 4.88 4.77 4.69	Carrier	N=4	6.51x10^4	5.04x10^4	0.65	77.46	Yes (0.3285)	Yes (0.0002)	Yes (0.5306)	Yes (0.0286)	Yes (0.0004)
24 h	yes	14300 21700 10400 12300	4.16 4.34 4.02 4.09	Salmocin SalE1b 0.1 mg/kg	N=4	1.47x10^4				Yes (0.6948)				

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, right

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 1.1x10^3 cfu/g 04-09-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)
72 h	yes	6760000 6880000 6620000 7540000	6.83 6.84 6.82 6.88	Carrier	N=4	6.95x10^6	6.94x10^6	2.99	99.90	Yes (0.2757)	Yes (<0.0001)	No (0.0030)	Yes (0.0286)	Yes (0.0001)
72 h	yes	6900 12700 3000 6100	3.84 4.10 3.48 3.79	Salmocin SalE1b 5 mg/kg	N=4	7.18x10^3				Yes (0.8703)				
72 h	yes	6760000 6880000 6620000 7540000	6.83 6.84 6.82 6.88	Carrier	N=4	6.95x10^6	6.90x10^6	2.13	99.26	Yes (0.2757)	Yes (<0.0001)	No (0.0056)	Yes (0.0286)	Yes (0.0002)
72 h	yes	52400 60533 23467 68267	4.72 4.78 4.37 4.83	Salmocin SalE1b 1 mg/kg	N=4	5.12x10^4				Yes (0.1523)				
72 h	yes	6760000 6880000 6620000 7540000	6.83 6.84 6.82 6.88	Carrier	N=4	6.95x10^6	6.80x10^6	1.67	97.87	Yes (0.2757)	Yes (<0.0001)	No (0.0339)	Yes (0.0286)	Yes (<0.0001)
72 h	yes	170000 146000 100000 176000	5.23 5.16 5.00 5.25	Salmocin SalE1b 0.5 mg/kg	N=4	1.48x10^5				Yes (0.2341)				
72 h	yes	6760000 6880000 6620000 7540000	6.83 6.84 6.82 6.88	Carrier	N=4	6.95x10^6	3.76x10^6	0.34	54.10	Yes (0.2757)	Yes (0.0009)	No (0.0350)	Yes (0.0286)	Yes (0.0066)
72 h	yes	2240000 2960000 3480000 4080000	6.35 6.47 6.54 6.61	Salmocin SalE1b 0.1 mg/kg	N=4	3.19x10^6				Yes (0.9085)				

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment) for all timepoints analyzed

Efficacy (re-growth) – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, right

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 1.1x10^3 cfu/g 04-09-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	replicates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)	Non-parametric Mann-Whitney test (p-value)
1 h	1667 1533 1773 3107	3.22 3.19 3.25 3.49	Carrier	N=4	2.02x10^3	6.31x10^4	1.51	3123	Yes (<0.0001)	Yes (0.5128)	Yes (<0.0001)	Yes (0.0286)
24 h	75600 76600 58800 49400	4.88 4.88 4.77 4.69	Carrier	N=4	6.51x10^4							
1 h	1667 1533 1773 3107	3.22 3.19 3.25 3.49	Carrier	N=4	2.02x10^3	6.95x10^6	3.54	343959	Yes (<0.0001)	No (0.0184)	Yes (<0.0001)	Yes (0.0286)
72 h	6760000 6880000 6620000 7540000	6.83 6.84 6.82 6.88	Carrier	N=4	6.95x10^6							
1 h	24 92 56 48	1.38 1.96 1.75 1.68	Salmocin SalE1b 5 mg/kg	N=4	5.50x10^1	7.00x10^0	0.05	13	No (0.8297)	Yes (0.8770)	No (0.8298)	Yes (0.8286)
24 h	32 100 84 32	1.51 2.00 1.92 1.51	Salmocin SalE1b 5 mg/kg	N=4	6.20x10^1							
1 h	24 92 56 48	1.38 1.96 1.75 1.68	Salmocin SalE1b 5 mg/kg	N=4	5.50x10^1	7.12x10^3	2.12	12945	Yes (<0.0001)	Yes (0.9183)	Yes (<0.0001)	Yes (0.0286)
72 h	6900 12700 3000 6100	3.84 4.10 3.48 3.79	Salmocin SalE1b 5 mg/kg	N=4	7.18x10^3							
1 h	268 148 280 280	2.43 2.17 2.45 2.45	Salmocin SalE1b 1 mg/kg	N=4	2.44x10^2	3.74x10^2	0.40	227	Yes (0.0058)	Yes (0.9962)	Yes (0.0058)	Yes (0.0286)
24 h	920 600 480 471	2.96 2.78 2.68 2.67	Salmocin SalE1b 1 mg/kg	N=4	6.18x10^2							
1 h	268 148 280 280	2.43 2.17 2.45 2.45	Salmocin SalE1b 1 mg/kg	N=4	2.44x10^2	5.09x10^4	2.32	30359	Yes (<0.0001)	Yes (0.4940)	Yes (<0.0001)	Yes (0.0286)
72 h	52400 60533 23467 68267	4.72 4.78 4.37 4.83	Salmocin SalE1b 1 mg/kg	N=4	5.12x10^4							

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

→statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 72 h, different kinetics of regrowth when compared to carrier treatment due to different contamination levels created upon treatment

Efficacy (re-growth) – skin-on poultry matrix (chicken breast fillet)

Fig. 2-11, right

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g); **Initial contamination:** 1.1x10^3 cfu/g 04-09-2018

Evaluation of data – contamination of chicken breast fillet meat with a 7-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	replicates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)	Non-parametric Mann-Whitney test (p-value)
1 h	280 372 268 220	2.45 2.57 2.43 2.34	Salmocin SalE1b 0.5 mg/kg	N=4	2.85x10^2	9.14x10^2	0.62	837	Yes (0.0029)	Yes (0.1855)	Yes (0.0084)	Yes (0.0286)
24 h	949 577 1977 1291	2.98 2.76 3.30 3.11	Salmocin SalE1b 0.5 mg/kg	N=4	1.20x10^3							
1 h	280 372 268 220	2.45 2.57 2.43 2.34	Salmocin SalE1b 0.5 mg/kg	N=4	2.85x10^2	1.48x10^5	2.72	54245	Yes (<0.0001)	Yes (0.7771)	Yes (<0.0001)	Yes (0.0286)
72 h	170000 146000 100000 176000	5.23 5.16 5.00 5.25	Salmocin SalE1b 0.5 mg/kg	N=4	1.48x10^5							
1 h	1252 660 1144 852	3.10 2.82 3.06 2.93	Salmocin SalE1b 0.1 mg/kg	N=4	9.77x10^2	1.37x10^4	1.18	1402	Yes (<0.0001)	Yes (0.9029)	Yes (<0.0001)	Yes (0.0286)
24 h	14300 21700 10400 12300	4.16 4.34 4.02 4.09	Salmocin SalE1b 0.1 mg/kg	N=4	1.47x10^4							
1 h	1252 660 1144 852	3.10 2.82 3.06 2.93	Salmocin SalE1b 0.1 mg/kg	N=4	9.77x10^2	3.19x10^6	3.51	326410	Yes (<0.0001)	Yes (0.8349)	Yes (<0.0001)	Yes (0.0286)
72 h	2240000 2960000 3480000 4080000	6.35 6.47 6.54 6.61	Salmocin SalE1b 0.1 mg/kg	N=4	3.19x10^6							

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

→statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 72 h, different kinetics of regrowth when compared to carrier treatment due to different contamination levels created upon treatment

Efficacy – raw beef

Meat matrix

→raw beef round roast trimmed to ~20 g pieces

Introduction of *Salmonella* contamination

→*S. enterica* strains used were nalidixic acid resistant mutants

→grown in LB medium supplemented with 25 µg/ml nalidixic acid to OD_{600}~0.3

S. enterica ssp. *enterica*, serotype Enteritidis (ATCC®13076™*nalR#6)
S. enterica ssp. *enterica*, serotype Typhimurium (ATCC®14028™*nalR#3)

→Contamination level: ~$1x10^3$-$1x10^4$ cfu/g food (intended)

→food matrix contaminated with 1:1 mixture of 2 *Salmonella* strains representing 2 different serotypes seryotypes (OD_{600}=0.001 (~$1.5x10^5$ cfu/ml) or OD_{600}=0.005 (~$7.5x10^5$ cfu/ml) in LB), 10 ml/kg application rate of bacterial solution)

→bacterial solution was added to meat cubes and equally distributed on meat pieces by hand-massage

→contaminated meat was incubated for 30 min at RT for attachment of bacteria and hand-massaged upon 15 and 30 min. of incubation

Salmocin treatment

→salmocin solutions used were purified protein powders in 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl

→carrier solution was 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl

→treatments: Carrier

5 mg/kg SalE1b or 0.5 mg/kg SalE1b

→ Salmocin or carrier solution were added with a rate of 20 ml/kg on contaminated meat cubes and equally distributed by hand-massage

→treated meat was incubated for 30 min at RT and hand-massaged upon 15 and 30 min. of incubation

Sample preparation and analysis for bacterial counts

→40 g aliquots of meat pieces were packed into bags and stored at 10°C

→total incubation time of meat at RT upon salmocin treatment: 2 h

→meat samples were supplemented with 4 vol. peptone water and homogenized in Bag Mixer

→serial dilutions of recovered bacterial solution were plated on XLD Agar supplemented with 25 µg/ml nalidixic acid

→plates were incubated at 37°C for 16-20h and cfu were counted

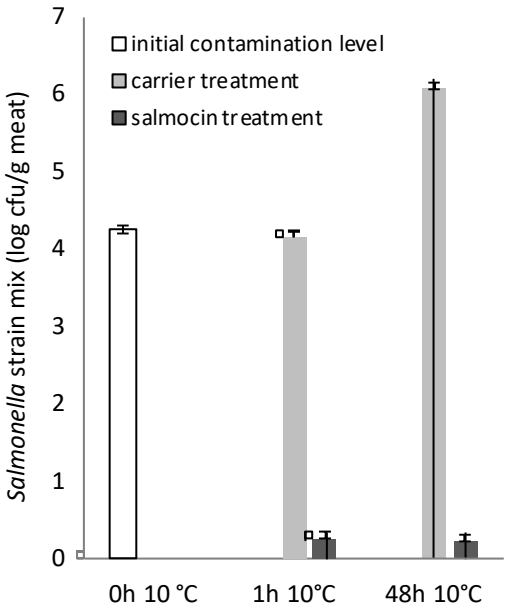
Efficacy – raw beef round roast

Fig. 2-12

medium contamination level
intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

A 29-05-2018

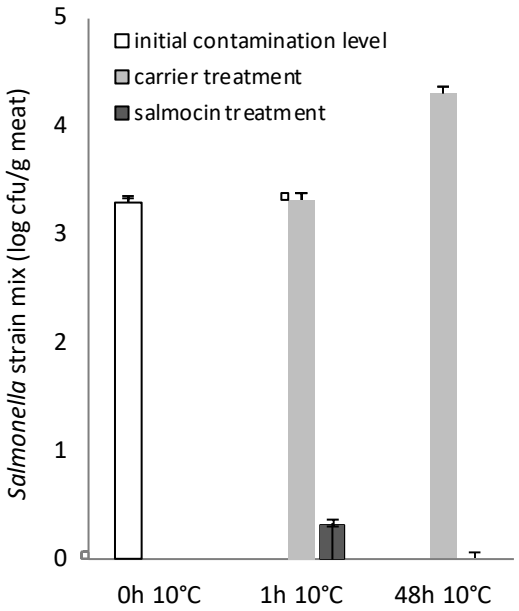
Effect of salmocin treatment on *Salmonella* contamination on raw beef



Inoculum:
S. enterica ssp. *enterica*, 2-strain mix, nalidixic acid resistant mutants of serotypes:
Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
(5.5x10^6 cfu/ml, 10 ml/kg)
Initial contamination: 1.81x10^4
Salmocin treatment: 5 mg/kg SalE1b (20 ml/kg)
Replicates: N=4

B 10-07-2018

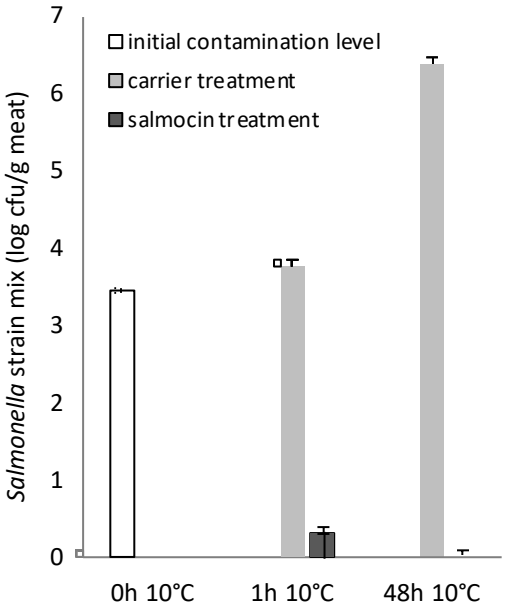
Effect of salmocin treatment on *Salmonella* contamination on raw beef



Inoculum:
S. enterica ssp. *enterica*, 2-strain mix, nalidixic acid resistant mutants of serotypes:
Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
(2.0x10^5 cfu/ml, 10 ml/kg)
Initial contamination: 1.93x10^3
Salmocin treatment: 0.5 mg/kg SalE1b (20 ml/kg)
Replicates: N=4

C 17-07-2018

Effect of salmocin treatment on *Salmonella* contamination on raw beef



Inoculum:
S. enterica ssp. *enterica*, 2-strain mix, nalidixic acid resistant mutants of serotypes:
Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
(4.7x10^5 cfu/ml, 10 ml/kg)
Initial contamination: 2.85x10^3
Salmocin treatment: 0.5 mg/kg SalE1b (20 ml/kg)
Replicates: N=4

Efficacy – raw beef round roast

Fig. 2-12

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

29-05-2018

Evaluation of data – contamination of beef round roast meat with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

A

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test
1 h	yes	15867 10400 16533 13067	4.20 4.02 4.22 4.12	Carrier	N=4	1.4x10^4	1.4x10^4	3.9	99.99	Yes (0.4915)	Yes (<0.0001)	Yes (0.3250)	Yes (0.0286)
1 h	yes	2 4 4 2	0.30 0.60 0.60 0.30	Salmocin SalE1b 5 mg/kg	N=4	1.8x10^0				No (0.0239)			
48 h	yes	912000 3280000 227333 246000	5.96 6.52 5.36 5.39	Carrier	N=4	1.2x10^6	1.2x10^6	5.8	99.99	Yes (0.3429)	Yes (<0.0001)	Yes (0.0911)	Yes (0.0286)
48 h	yes	2 4 4 2	0.30 0.60 0.60 0.30	Salmocin SalE1b 5 mg/kg	N=4	1.7x10^0				No (0.0239)			

Raw data = 0 were set to LOQ/2

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment)

Efficacy (re-growth) – raw beef round roast

Fig. 2-12

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

29-05-2018

Evaluation of data – contamination of beef round roast meat with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

A

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	15867 10400 16533 13067	4.20 4.02 4.22 4.12	Carrier	N=4	1.4x10^4	1.2x10^6	1.9	8250.84	Yes (0.0010)	No (0.0153)	Yes (0.0286)	Yes (0.0079)
48 h	912000 3280000 227333 246000	5.96 6.52 5.36 5.39	Carrier	N=4	1.2x10^6							
1 h	2 4 4 2	0.30 0.60 0.60 0.30	Salmocin SalE1b 5 mg/kg	N=4	1.8x10^0	1.5x10^{-1}	0.0	-8.33	No (>0.9999)	No (<0.05)	No (>0.9999)	No (>0.9999)
48 h	2 4 4 2	0.30 0.60 0.60 0.30	Salmocin SalE1b 5 mg/kg	N=4	1.7x10^0							

Raw data = 0 were set to LOQ/2

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

→statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 48 h

Efficacy – raw beef round roast

Fig. 2-12

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

10-07-2018

Evaluation of data – contamination of beef round roast meat with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

B

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test
1 h	yes	2547 2640 1947 1173	3.41 3.42 3.29 3.07	Carrier	N=4	2.1x10^3	2.1x10^3	3.0	99.90	Yes (0.2693) No (0.0239)	Yes (<0.0001)	Yes (0.9896)	Yes (0.0286)
1 h	yes	4 2 4 2	0.60 0.30 0.60 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	3.0x10^0							
48 h	yes	19600 30800 16800 15600	4.29 4.49 4.23 4.19	Carrier	N=4	2.1x10^4	2.1x10^4	4.3	99.99	Yes (0.3049) No (0.0012)	Yes (<0.0001)	Yes (0.8383)	Yes (0.0286)
48 h	yes	2 4 2 2	0.30 0.60 0.30 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	2.5x10^0							

Raw data = 0 were set to LOQ/2

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment)

Efficacy (re-growth) – raw beef round roast

Fig. 2-12

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

10-07-2018

Evaluation of data – contamination of beef round roast meat with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

B

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test
1 h	2547 2640 1947 1173	3.41 3.42 3.29 3.07	Carrier	N=4	2.1x10^3	1.9x10^4	1.0	897	Yes (<0.0001)	Yes (0.7445)	Yes (0.0286)
48 h	19600 30800 16800 15600	4.29 4.49 4.23 4.19	Carrier	N=4	2.1x10^4						
1 h	4 2 4 2	0.60 0.30 0.60 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	3.0x10^0	0.5x10^0	-0.3	-50	No (0.5370)	Yes (0.8187)	No (>0.9999)
48 h	2 4 2 2	0.30 0.60 0.30 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	2.5x10^0						

Raw data = 0 were set to LOQ/2

→no statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 48 h

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

Efficacy – raw beef round roast

Fig. 2-12

17-07-2018

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

Evaluation of data – contamination of beef round roast meat with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

C

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test
1 h	yes	5120 6973 6787 3600	3.71 3.84 3.83 3.56	Carrier	N=4	5.6x10^3	5.6x10^4	3.4	99.96	Yes (0.3498) No (0.0239)	Yes (<0.0001)	Yes (0.6778)	Yes (0.0286)
1 h	yes	4 4 2 2	0.60 0.60 0.30 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	3x10^0							
48 h	yes	3800000 2320000 2720000 840000	6.58 6.37 6.43 5.92	Carrier	N=4	2.4x10^6	2.4x10^6	6.4	99.99	Yes (0.3951) No (0.0012)	Yes (<0.0001)	Yes (0.3290)	Yes (0.0286)
48 h	yes	4 2 2 2	0.60 0.30 0.30 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	2.5x10^0							

Raw data = 0 were set to LOQ/2

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment)

Efficacy (re-growth) – raw beef round roast

Fig. 2-12

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

17-07-2018

Evaluation of data – contamination of beef round roast meat with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

C

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g $\log_{10}$ values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean $\log_{10}$ cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance $\log_{10}$ values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test
1 h	5120 6973 6787 3600	3.71 3.84 3.83 3.56	Carrier	N=4	5.6x10^3	2.4x10^6	2.6	42961	Yes (<0.0001)	Yes (0.2514)	Yes (0.0286)
48 h	3800000 2320000 2720000 840000	6.58 6.37 6.43 5.92	Carrier	N=4	2.4x10^6						
1 h	4 4 2 2	0.60 0.60 0.30 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	3x10^0	0.5x10^0	-0.3	-50	No (0.5370)	Yes (0.8187)	No (>0.9999)
48 h	4 2 2 2	0.60 0.30 0.30 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	2.5x10^0						

Raw data = 0 were set to LOQ/2

→no statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 48 h

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

Efficacy – raw tuna

Meat matrix

→raw frozen tuna fillets thawed for overnight at 4°C trimmed to ~20 g pieces

Introduction of *Salmonella* contamination

→*S. enterica* strains used were nalidixic acid resistant mutants

→grown in LB medium supplemented with 25 µg/ml nalidixic acid to OD_{600}~0.3

S. enterica ssp. *enterica*, serotype Enteritidis (ATCC®13076™*nalR#6)
S. enterica ssp. *enterica*, serotype Typhimurium (ATCC®14028™*nalR#3)

→Contamination level: ~$1x10^3$-$1x10^4$ cfu/g food (intended)

→food matrix contaminated with 1:1 mixture of 2 *Salmonella* strains representing 2 different serotypes seryotypes (OD_{600}=0.001 (~$1.5x10^5$ cfu/ml) or OD_{600}=0.005 (~$7.5x10^5$ cfu/ml) in LB), 10 ml/kg application rate of bacterial solution)

→bacterial solution was added to meat cubes and equally distributed on meat pieces by hand-massage

→contaminated meat was incubated for 30 min at RT for attachment of bacteria and hand-massaged upon 15 and 30 min. of incubation

Salmocin treatment

→salmocin solutions used were purified protein powders in 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl

→carrier solution was 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl

→treatments: Carrier

5 mg/kg SalE1b or 0.5 mg/kg SalE1b

→ Salmocin or carrier solution were added with a rate of 20 ml/kg on contaminated meat cubes and equally distributed by hand-massage

→treated meat was incubated for 30 min at RT and hand-massaged upon 15 and 30 min. of incubation

Sample preparation and analysis for bacterial counts

→40 g aliquots of meat pieces were packed into bags and stored at 10°C

→total incubation time of meat at RT upon salmocin treatment: 2 h

→meat samples were supplemented with 4 vol. peptone water and homogenized in Bag Mixer

→serial dilutions of recovered bacterial solution were plated on XLD Agar supplemented with 25 µg/ml nalidixic acid

→plates were incubated at 37°C for 16-20h and cfu were counted

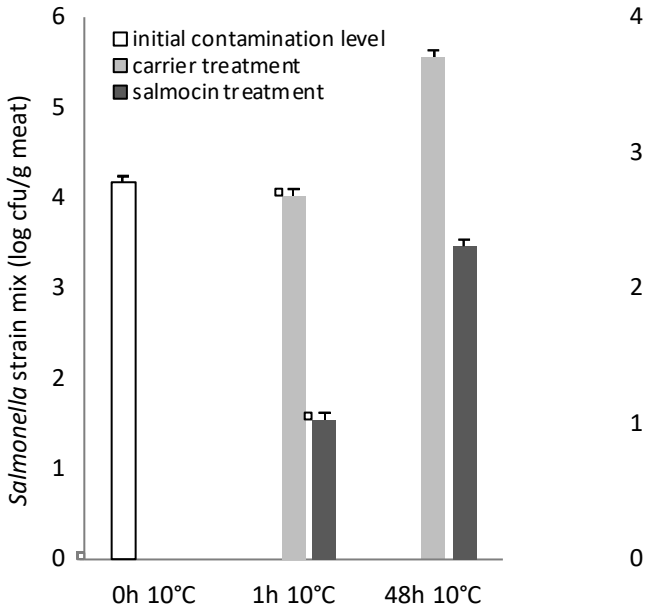
Efficacy – raw tuna fillet

Fig. 2-13

medium contamination level
intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

A

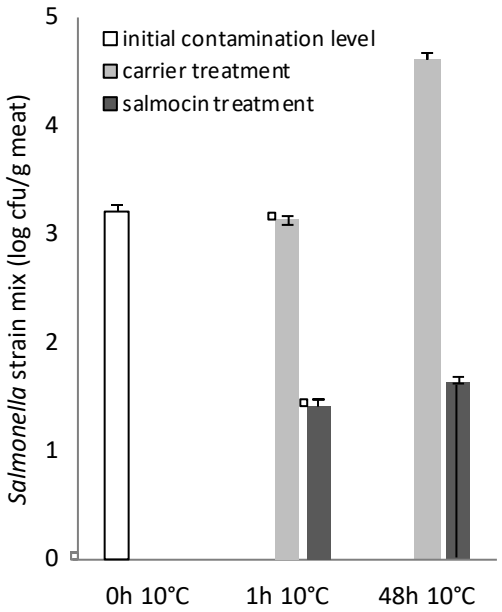
29-05-2018



Inoculum:
S. enterica ssp. *enterica*, 2-strain mix, nalidixic acid resistant mutants of serotypes:
Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
(5.5x10^6 cfu/ml, 10 ml/kg)
Initial contamination: 1.44x10^4
Salmocin treatment: 5 mg/kg SalE1b (20 ml/kg)
Replicates: N=4

B

17-07-2018



Inoculum:
S. enterica ssp. *enterica*, 2-strain mix, nalidixic acid resistant mutants of serotypes:
Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
(4.7x10^5 cfu/ml, 10 ml/kg)
Initial contamination: 1.6x10^3
Salmocin treatment: 0.5 mg/kg SalE1b (20 ml/kg)
Replicates: N=4

Efficacy – raw tuna fillet

Fig. 2-13 A

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

29-05-2018

Evaluation of data – contamination of tuna with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	yes	10267 12400 7867 11333	4.01 4.09 3.90 4.05	Carrier	N=4	1.1x10^4	1.0x10^4	2.5	99.66	Yes (0.6097)	Yes (<0.0001)	No (0.0292)	Yes (0.0007)
1 h	yes	65 33 7 36	1.82 1.51 0.86 1.56	Salmocin SalE1b 5 mg/kg	N=4	3.5x10^1				Yes (0.3717)			
48 h	yes	140000 148000 446000 700000	5.15 5.17 5.65 5.85	Carrier	N=4	3.6x10^5	3.6x10^5	2.1	99.21	Yes (0.2465)	Yes (0.0069)	Yes (0.0693)	Yes (0.0206)
48 h	yes	3987 13 1173 6173	3.60 1.12 3.07 3.79	Salmocin SalE1b 5 mg/kg	N=4	2.8x10^3				Yes (0.1581)			

Raw data = 0 were set to LOQ/2

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment)

Efficacy (re-growth) – raw tuna fillet

Fig. 2-13 A

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

29-05-2018

Evaluation of data – contamination of tuna with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	10267 12400 7867 11333	4.01 4.09 3.90 4.05	Carrier	N=4	1.1x10^4	3.5x10^5	1.5	3325	Yes (0.0002)	No (0.0447)	Yes (0.0027)
48 h	140000 148000 446000 700000	5.15 5.17 5.65 5.85	Carrier	N=4	3.6x10^5						
1 h	65 33 7 36	1.82 1.51 0.86 1.56	Salmocin SalE1b 5 mg/kg	N=4	3.5x10^1	2.8x10^3	1.9	7901	No (0.0638)	Yes (0.1041)	No (0.0921)
48 h	3987 13 1173 6173	3.60 1.12 3.07 3.79	Salmocin SalE1b 5 mg/kg	N=4	2.8x10^3						

Raw data = 0 were set to LOQ/2

→no statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 48 h

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

Efficacy – raw tuna fillet

medium contamination level
intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

Fig. 2-13 B

Evaluation of data – contamination of tuna with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars
Numerical representation of efficacy results

17-07-2018

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	yes	1267 1227 1507 1373	3.10 3.09 3.18 3.14	Carrier	N=4	1.3x10^3	1.3x10^3	1.7	98.06	Yes (0.6896)	Yes (<0.0001)	No (0.0232)	Yes (0.0003)
1 h	yes	16 32 16 40	1.20 1.51 1.20 1.60	Salmocin SalE1b 0.5 mg/kg	N=4	2.6x10^1				Yes (0.1562)			
48 h	yes	27600 14000 52000 72800	4.44 4.15 4.72 4.86	Carrier	N=4	4.2x10^4	4.2x10^4	3.0	99.90	Yes (0.8118)	Yes (0.0002)	Yes (0.1459)	Yes (0.0016)
48 h	yes	156 4 8 2	2.19 0.60 0.90 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	4.3x10^1				Yes (0.3253)			

Raw data = 0 were set to LOQ/2

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment)

Efficacy (re-growth) – raw tuna fillet

Fig. 2-13

17-07-2018

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

Evaluation of data – contamination of tuna with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	1267 1227 1507 1373	3.10 3.09 3.18 3.14	Carrier	N=4	1.3x10^3	4.0x10^4	1.5	2997	Yes (0.0001)	No (0.0066)	Yes (0.0027)
48 h	27600 14000 52000 72800	4.44 4.15 4.72 4.86	Carrier	N=4	4.2x10^4						
1 h	16 32 16 40	1.20 1.51 1.20 1.60	Salmocin SalE1b 0.5 mg/kg	N=4	2.6x10^1	1.6x10^1	0.2	62	No (0.4108)	No (0.0460)	No (0.4354)
48 h	156 4 8 2	2.19 0.60 0.90 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	4.3x10^1						

Raw data = 0 were set to LOQ/2

→no statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 48 h

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

Efficacy – raw whole egg

food matrix

→raw egg contents (eggwhite and eggyolk) are homogenized using a lab blender for 2 min. at 1000 rpm

Introduction of *Salmonella* contamination

→*S. enterica* strains used were nalidixic acid resistant mutants

→grown in LB medium supplemented with 25 µg/ml nalidixic acid to OD_{600}~0.3

S. enterica ssp. *enterica*, serotype Enteritidis (ATCC®13076™*nalR#6)
S. enterica ssp. *enterica*, serotype Typhimurium (ATCC®14028™*nalR#3)

→Contamination level: ~$1x10^3$-$1x10^4$ cfu/g food (intended)

→food matrix contaminated with 1:1 mixture of 2 *Salmonella* strains representing 2 different serotypes seryotypes (OD_{600}=0.001 (~$1.5x10^5$ cfu/ml) or OD_{600}=0.005 (~$7.5x10^5$ cfu/ml) in LB), 10 ml/kg application rate of bacterial solution)

→bacterial solution was added to whole egg and inter-mixed using a stirrer at 620 rpm for 1 min.

→contaminated whole egg was incubated for 30 min at RT and mixed upon 15 and 30 min. of incubation using a stirrer at 750 rpm for 1 min.

Salmocin treatment

→salmocin solutions used were purified protein powders in 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl

→carrier solution was 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, 100 mM NaCl

→treatments: Carrier
5 mg/kg SalE1b or 0.5 mg/kg SalE1b

→ Salmocin or carrier solution were added with a rate of 20 ml/kg to contaminated whole egg and inter-mixed using a stirrer at 750 rpm for 1 min.

→treated whole egg was incubated for 30 min at RT and mixed upon 15 and 30 min. of incubation using a stirrer at 750 rpm for 1 min.

Sample preparation and analysis for bacterial counts

→40 ml aliquots of whole egg solution were packed into bags and stored at 10°C

→total incubation time of meat at RT upon salmocin treatment: 2 h

→whole egg samples were supplemented with 4 vol. peptone water and homogenized in Bag Mixer

→serial dilutions of recovered bacterial solution were plated on XLD Agar supplemented with 25 µg/ml nalidixic acid

→plates were incubated at 37°C for 16-20h and cfu were counted

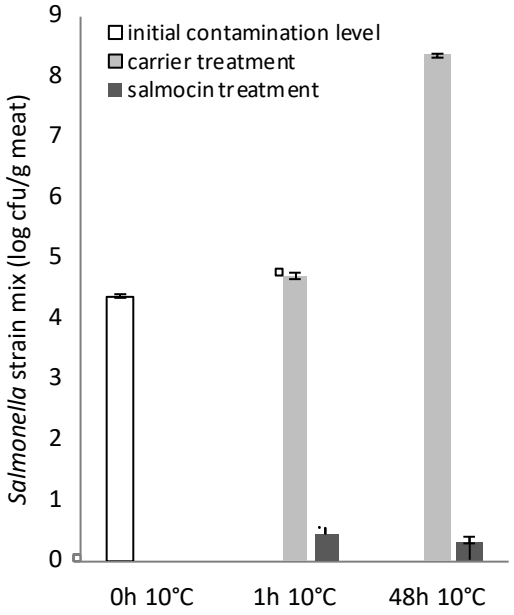
Efficacy – raw whole egg

Fig. 2-14

medium contamination level
intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

A 29-05-2018

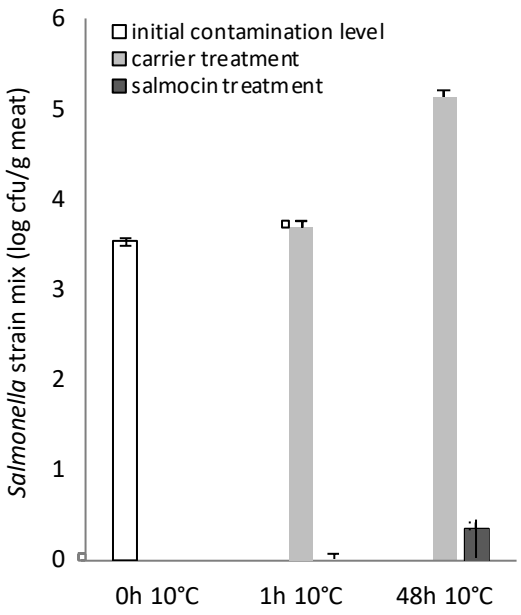
Effect of salmocin treatment on *Salmonella* contamination on raw whole egg



Inoculum:
S. enterica ssp. *enterica*, 2-strain mix, nalidixic acid resistant mutants of serotypes:
Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
(5.5x10^6 cfu/ml, 10 ml/kg)
Initial contamination: 2.43x10^4
Salmocin treatment: 5 mg/kg SalE1b (20 ml/kg)
Replicates: N=4

B 10-07-2018

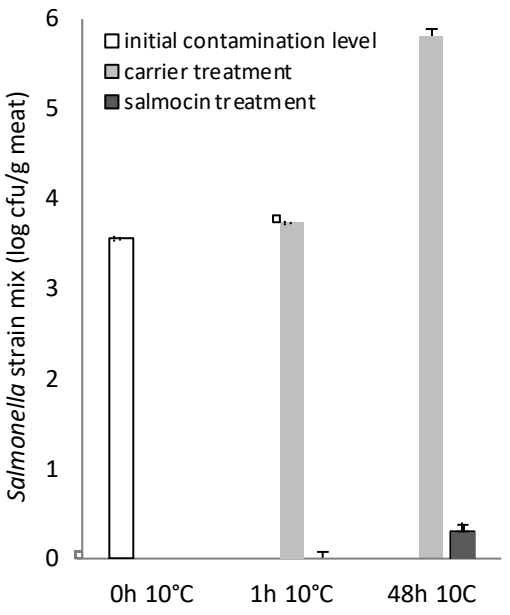
Effect of salmocin treatment on *Salmonella* contamination on raw whole egg



Inoculum:
S. enterica ssp. *enterica*, 2-strain mix, nalidixic acid resistant mutants of serotypes:
Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
(2.0x10^5 cfu/ml, 10 ml/kg)
Initial contamination: 3.36x10^3
Salmocin treatment: 0.5 mg/kg SalE1b (20 ml/kg)
Replicates: N=4

C 17-07-2018

Effect of salmocin treatment on *Salmonella* contamination on raw whole egg



Inoculum:
S. enterica ssp. *enterica*, 2-strain mix, nalidixic acid resistant mutants of serotypes:
Enteritidis (ATCC®13076™*nalR#6)
Typhimurium (ATCC®14028™*nalR#3)
(4.7x10^5 cfu/ml, 10 ml/kg)
Initial contamination: 3.59x10^3
Salmocin treatment: 0.5 mg/kg SalE1b (20 ml/kg)
Replicates: N=4

Efficacy – raw whole egg

Fig. 2-14 A

29-05-2018

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

Evaluation of data – contamination of whole egg with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

A

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	yes	46133 50800 61333 51600	4.66 4.71 4.79 4.71	Carrier	N=4	5.2x10^4	5.2x10^4	4.3	99.99	Yes (0.6150) No (0.0012)	Yes (<0.0001)	Yes (0.1113)	Yes (0.0286)	Yes (<0.0001)
1 h	yes	4 4 4 2	0.60 0.60 0.60 0.30	Salmocin SalE1b 5 mg/kg	N=4	3.5x10^0								
48 h	yes	242000000 212000000 238000000 210000000	8.38 8.33 8.38 8.32	Carrier	N=4	2.3x10^8	2.3x10^8	8.1	100.00	Yes (0.1270) No (0.00239)	Yes (<0.0001)	No (0.0208)	Yes (0.0286)	Yes (<0.0001)
48 h	yes	2 4 4 2	0.30 0.60 0.60 0.30	Salmocin SalE1b 5 mg/kg	N=4	3.0x10^0								

Raw data = 0 were set to LOQ/2

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment)

Efficacy (re-growth) – raw whole egg

Fig. 2-14 A

29-05-2018

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

Evaluation of data – contamination of whole egg with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

A

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	46133 50800 61333 51600	4.66 4.71 4.79 4.71	Carrier	N=4	5.2x10^4	2.3x10^8	3.6	429697	Yes (<0.0001)	Yes (0.4690)	Yes (0.0286)	Yes (<0.0001)
48 h	242000000 212000000 238000000 210000000	8.38 8.33 8.38 8.32	Carrier	N=4	2.3x10^8							
1 h	4 4 4 2	0.60 0.60 0.60 0.30	Salmocin SalE1b 5 mg/kg	N=4	3.5x10^0	0.5x10^0	-0.3	-14	No (0.5370)	Yes (0.8187)	No (>0.9999)	No (0.5374)
48 h	2 4 4 2	0.30 0.60 0.60 0.30	Salmocin SalE1b 5 mg/kg	N=4	3.0x10^0							

Raw data = 0 were set to LOQ/2

→no statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 48 h

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

Efficacy – raw whole egg

Fig. 2-14 B

10-07-2018

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

Evaluation of data – contamination of whole egg with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

B

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test
1 h	yes	3840 4620 6060 4480	3.58 3.66 3.78 3.65	Carrier	N=4	4.8x10^3	4.8x10^3	3.7	99.98	Yes (0.6572) No (0.0012)	Yes (<0.0001)	Yes (0.3487)	Yes (0.0286)
1 h	yes	2 2 2 4	0.30 0.30 0.30 0.60	Salmocin SalE1b 0.5 mg/kg	N=4	2.5x10^0							
48 h	yes	80000 284000 144000 44000	4.90 5.45 5.16 4.64	Carrier	N=4	1.4x10^5	1.4x10^5	4.8	100.00	Yes (0.9794) No (0.0239)	Yes (<0.0001)	Yes (0.2862)	Yes (0.0286)
48 h	yes	2 4 4 2	0.30 0.60 0.60 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	3x10^0							

Raw data = 0 were set to LOQ/2

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment)

Efficacy (re-growth) – raw whole egg

Fig. 2-14 B

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

10-07-2018

Evaluation of data – contamination of whole egg with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

B

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	3840 4620 6060 4480	3.58 3.66 3.78 3.65	Carrier	N=4	4.8x10^3	1.3x10^5	1.5	2805	Yes (0.0003)	No (0.0413)	Yes (0.0286)	Yes (0.0031)
48 h	80000 284000 144000 44000	4.90 5.45 5.16 4.64	Carrier	N=4	1.4x10^5							
1 h	2 2 2 4	0.30 0.30 0.30 0.60	Salmocin SalE1b 0.5 mg/kg	N=4	2.5x10^0	0.5x10^0	-0.3	20	No (0.5370)	Yes (0.8187)	No (>0.9999)	No (0.5574)
48 h	2 4 4 2	0.30 0.60 0.60 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	3x10^0							

Raw data = 0 were set to LOQ/2

→no statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 48 h

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

Efficacy – raw whole egg

Fig. 2-14 C

17-07-2018

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

Evaluation of data – contamination of whole egg with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

C

analysis of efficacy of salmocin treatment in reduction of *Salmonella* cell numbers of contaminated skin-on chicken breast during storage at 10°C

period of storage at 10°C	contamination with *Salmonella*	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent reduction	Analysis of statistical significance log_{10} values: Shapiro-Wilk normality test (p-value)	Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	yes	5560 5280 5413 5200	3.75 3.72 3.73 3.72	Carrier	N=4	5.4x10^3	5.4x10^3	3.7	99.98	Yes (0.8620) No (0.0012)	Yes (<0.0001)	No (0.0020)	Yes (0.0286)	Yes (<0.0001)
1 h	yes	4 2 2 2	0.60 0.30 0.30 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	2.5x10^0								
48 h	yes	566000 582000 870000 652000	5.75 5.76 5.94 5.81	Carrier	N=4	6.7x10^5	6.7x10^5	5.5	100.00	Yes (0.8531) No (0.0239)	Yes (<0.0001)	Yes (0.2731)	Yes (0.0286)	Yes (<0.0001)
48 h	yes	4 2 2 4	0.60 0.30 0.30 0.60	Salmocin SalE1b 0.5 mg/kg	N=4	3x10^0								

Raw data = 0 were set to LOQ/2

Statistical analysis performed using GraphPad Prism v. 6.01 comparing the treatments at one timepoint.

→efficacy of salmocins verified: statistically significant reduction of *Salmonella* contamination upon salmocin treatment (compared to carrier treatment)

Efficacy (re-growth) – raw whole egg

Fig. 2-14 C

17-07-2018

medium contamination level

intended bacterial load: 0.1-1x10^4 cfu/g (3-4 logs/g)

Evaluation of data – contamination of whole egg with a 2-strain mixture of *S. enterica* ssp. *enterica* serovars

Numerical representation of efficacy results

C

analysis of efficacy of re-growth of *Salmonella* cells on contaminated skin-on chicken breast fillet upon salmocin treatment during storage at 10°C

period of storage at 10°C	Cfu/g Raw values	Cfu/g log_{10} values	treatment comparison	repli-cates	mean cfu/g	mean difference cfu/g	mean log_{10} cfu/g reduction	mean percent increase in cell number	Analysis of statistical significance log_{10} values: Unpaired parametric t-test (p-value)	F-test for equality of variances (p-value)	Non-parametric Mann-Whitney test	Unpaired parametric t-test with Welch´s correction (p-value)
1 h	5560 5280 5413 5200	3.75 3.72 3.73 3.72	Carrier	N=4	5.4x10^3	6.6x10^5	2.1	12346	Yes (<0.0001)	No (0.0109)	Yes (0.0286)	Yes (<0.0001)
48 h	566000 582000 870000 652000	5.75 5.76 5.94 5.81	Carrier	N=4	6.7x10^5							
1 h	4 2 2 2	0.60 0.30 0.30 0.30	Salmocin SalE1b 0.5 mg/kg	N=4	2.5x10^0	0.5x10^0	-0.3	20	No (0.5370)	Yes (0.8187)	No (>0.9999)	No (0.5374)
48 h	4 2 2 4	0.60 0.30 0.30 0.60	Salmocin SalE1b 0.5 mg/kg	N=4	3x10^0							

Raw data = 0 were set to LOQ/2

→no statistically significant regrowth of residual *Salmonella* contamination upon salmocin treatment within 48 h

Statistical analysis performed using GraphPad Prism v. 6.01 comparing cell numbers for one treatment at two timepoints.

End of Appendix 1 – 24 April 2019

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Appendix 2: GRN 824 Statistical Power Calculations

24 April 2019

All statistical evaluations were performed with unpaired parametric two-tailed t-test using GraphPad Prism v. 6.01 and other tests, as appropriate, as described in our narrative responses and as shown in **Appendix 1**. To address FSIS's inquiry, the raw data of each on-matrix study were re-analyzed to confirm statistical differences in bactericidal efficacy between Salmocin- and control vehicle-treated groups. New statistical analyses applied to address the Agency's questions resulted in different p-values compared to the p-values entered in our original Notice for GRN 824. Nevertheless, and importantly, all statistical differences supporting our conclusions for studies on all food matrices were confirmed.

All experiments were designed to produce data of suitable quality for analysis. Pilot studies yielded expected differences in *Salmonella* cell numbers and guided the design of detailed on-matrix exposure studies. We expected contamination between carrier control groups and Salmocin treated groups to be at least 1,500 at early time points and >10,000 at later time points under prolonged storage. To allow for analysis of different treatments in parallel in the same experiment and to enable for a statistical power of 0.8, 4 replicates of each sample were analyzed (N per group).

Outputs of pilot studies were used for sample size/power analysis using the application **GraphPad StatMate 2.00.** Standard deviations of different groups were found not to be equal in these pilot studies; therefore, the average SD value of carrier treatment and one Salmocin treatment group at the earliest time point of analysis was used as recommended for pre-analysis.

The analysis with GraphPad StatMate 2.00 showed that experiments performed according to our assumptions produced data with power of 0.8 or better; the **output is shown below**:

Calculation of sample size using GraphPad StatMate 2.00

Your choices

Test chosen: Sample size for unpaired *t* test
Expected SD of each group = 500

Significance level (alpha) = 0.05 (two-tailed)

Detailed explanation

You requested a detailed explanation for N = 4 and power = 80%.

Assume that the true difference between means is 1170.28. Now imagine that you perform many experiments, with N = 4 per group in each experiment. Due to random sampling, you won't find that the difference between means equals 1170.28 in every experiment. Instead, you'll find that the difference between means will be greater than 1170.28 in about half the experiments, and less than 1170.28 in the other half.

In 80% (the power) of those experiments, the P value will be less than 0.05 (two-tailed) so the results will be deemed "statistically significant". In the remaining 20% of the experiments, the difference between means will be deemed "not statistically significant", so you will have made a Type II (beta) error.

Summary: A sample size of 4 in each group has a 80% power to detect a difference between means of 1170.28 with a significance level (alpha) of 0.05 (two-tailed).

Alternative explanation using confidence intervals

If you perform many experiments with N = 4 in each group, you expect that in 80% of these experiments (the power), the width of the 95% confidence interval for the difference between means will extend 1170.28 or less in each direction. In the remaining 20% of the experiments, you will expect the 95% confidence interval to be wider than that.

Table of tradeoffs

For any combination of sample size (N) and power, this table shows the difference between means that can be detected.

	Power				
N per group	**99%**	**95%**	**90%**	**80%**	**50%**
3	2226.27	1872.31	1683.61	1455.12	1017.99
4	1790.48	1505.81	1354.05	**1170.28**	818.72
5	1539.96	1295.11	1164.59	1006.53	704.16
6	1372.10	1153.94	1037.64	896.82	627.41
7	1249.52	1050.86	944.95	816.70	571.36
8	1154.93	971.30	873.41	754.88	528.10
9	1079.05	907.49	816.03	705.28	493.41
10	1016.43	854.83	768.68	664.35	464.78
12	918.25	772.25	694.42	600.18	419.88
14	843.96	709.78	638.25	551.63	385.91
16	785.22	660.38	593.82	513.23	359.05
18	737.27	620.05	557.56	481.89	337.12
20	697.15	586.31	527.22	455.66	318.78
25	619.93	521.36	468.82	405.19	283.47
30	563.75	474.12	426.34	368.47	257.78
35	520.52	437.76	393.64	340.22	238.01
40	485.92	408.66	367.47	317.60	222.19
50	433.39	364.49	327.75	283.27	198.17
60	394.90	332.11	298.64	258.11	180.57

70	365.12	307.07	276.12	238.65	166.95
80	341.20	286.95	258.03	223.01	156.02
90	321.44	270.33	243.09	210.10	146.98
100	304.76	256.30	230.47	199.19	139.35
150	248.37	208.88	187.83	162.34	113.57
200	214.90	180.73	162.52	140.46	98.27
300	175.31	147.43	132.58	114.58	80.16
400	151.75	127.62	114.76	99.19	69.39
500	135.69	114.12	102.62	88.69	62.05
1000	95.90	80.65	72.52	62.68	43.85

If you want to use unequal N

Instead of using 4 subjects in each group, you can use unequal N. Substitute any of the following experimental designs, without losing any statistical power. Note that total sample size increases if you use unequal N (you must increase N for Group B more than you decrease N for group A). This can make sense if treatment A "costs" more (considering expense, hassle and risk) than treatment B. Even though the total sample size goes up, choosing unequal N may reduce the total cost (or risk) of the experiment.

Sample size				
Group A	**Group B**	**Ratio**	**Total**	**When to choose**
4	4	1.000	8	If the "cost" of treatment A is 1.0 times the "cost" of treatment B.
4	5	1.250	9	If the "cost" of treatment A is 1.6 times the "cost" of treatment B.
4	6	1.500	10	If the "cost" of treatment A is 2.3 times the "cost" of treatment B.
3	6	2.000	9	If the "cost" of treatment A is 4.0 times the "cost" of treatment B.
3	9	3.000	12	If the "cost" of treatment A is 9.0 times the "cost" of treatment B.
3	12	4.000	15	If the "cost" of treatment A is 16.0 times the "cost" of treatment B.
3	15	5.000	18	If the "cost" of treatment A is 25.0 times the "cost" of treatment B.

You cannot reduce the sample size of Group A to fewer than half the number needed if you choose equal sample size (without losing statistical power).

Furthermore, in order to verify expected power of at least 0.8 for completed experiments, data shown in **Figures 2-8** and **Figure 2-9** of the original Notice were analyzed as examples, as shown below.

Determination of power of completed (sample) experiments using GraphPad StatMate 2.00

Analysis of data chosen from experiment shown in **Figure 2-8** as an example: Group 1: carrier treatment, Group 2: Salmocin treatment at 3 mg/kg, both at 1 h of storage; mean difference Group1-Group2=5075; for all other comparisons even higher mean difference was obtained. Results of the test shown below indicate that a power of 95% was valid for this experiment.

Your choices

Test chosen: Power of a "not significant" unpaired *t* test

	N	SD
Group 1	4	1418.12
Group 2	4	27.78

Significance level (alpha) = 0.05 (two-tailed)

Explanation for 95% power

Assume that the true difference between means is 3020.52. Now imagine that you perform many experiments, with the same sample size used in the completed experiment. Due to random sampling, you won't find that the difference between means equals 3020.52 in every experiment. Instead, you'll find that the difference between means will be greater than 3020.52 in about half the experiments, and less than 3020.52 in the other half.

In 95% (the power) of those experiments, the P value will be less than 0.05 (two-tailed) so the results will be deemed "statistically significant". In the remaining 5% of the experiments, the P value will be greater than 0.05 (two-tailed) so the results will be deemed "not statistically significant" and you will have made a Type II (beta) error.

Summary: Your experiment had a 95% power to detect a difference between means of 3020.52 with a significance level (alpha) of 0.05 (two-tailed).

Table of tradeoffs

For any power you choose, this table shows the difference between means that can be detected.

Delta	Power (%)	Delta	Power (%)
3591.55	99	1642.28	50
3020.52	95	1429.99	40
2716.10	90	1202.88	30
2510.71	85	937.07	20
2347.48	80	568.45	10
2207.44	75		
2081.68	70		
1854.56	60		

Analysis of data chosen from experiment shown in **Figure 2-9** as an example**:** Group 1: carrier treatment, Group 2: Salmocin treatment at 3 mg/kg, both at 1 h of storage; mean difference Group1-Group2=4244 (minimum mean difference in this experiment = 3781). Results of the test shown below indicate that a power of 95% was valid for this experiment. The same test is repeated with log-transformed standard deviation: a mean difference of 0.65 log is detected with 95% power.

Your choices

Test chosen: Power of a "not significant" unpaired *t* test

	N	SD
Group 1	4	0.111234
Group 2	4	0.298877

Significance level (alpha) = 0.05 (two-tailed)

Explanation for 95% power

Assume that the true difference between means is 0.68. Now imagine that you perform many experiments, with the same sample size used in the completed experiment. Due to random sampling, you won't find that the difference between means equals 0.68 in every experiment. Instead, you'll find that the difference between means will be greater than 0.68 in about half the experiments, and less than 0.68 in the other half.

In 95% (the power) of those experiments, the P value will be less than 0.05 (two-tailed) so the results will be deemed "statistically significant". In the remaining 5% of the experiments, the P value will be greater than 0.05 (two-tailed) so the results will be deemed "not statistically significant" and you will have made a Type II (beta) error.

Summary: Your experiment had a 95% power to detect a difference between means of 0.68 with a significance level (alpha) of 0.05 (two-tailed).

Table of tradeoffs

For any power you choose, this table shows the difference between means that can be detected.

Delta	Power (%)	Delta	Power (%)
0.81	99	0.42	60
0.68	95	0.37	50
0.61	90	0.32	40
0.56	85	0.27	30
0.53	80	0.21	20
0.50	75	0.13	10
0.47	70		

Your choices

Test chosen: Power of a "not significant" unpaired *t* test

	N	SD
Group 1	4	904.14
Group 2	4	19.18

Significance level (alpha) = 0.05 (two-tailed)

Explanation for 95% power

Assume that the true difference between means is 1925.83. Now imagine that you perform many experiments, with the same sample size used in the completed experiment. Due to random sampling, you won't find that the difference between means equals 1925.83 in every experiment. Instead, you'll find that the difference between means will be greater than 1925.83 in about half the experiments, and less than 1925.83 in the other half.

In 95% (the power) of those experiments, the P value will be less than 0.05 (two-tailed) so the results will be deemed "statistically significant". In the remaining 5% of the experiments, the P value will be greater than 0.05 (two-tailed) so the results will be deemed "not statistically significant" and you will have made a Type II (beta) error.

Summary: Your experiment had a 95% power to detect a difference between means of 1925.83 with a significance level (alpha) of 0.05 (two-tailed).

Table of tradeoffs

For any power you choose, this table shows the difference between means that can be detected.

Delta	Power (%)
2289.91	99
1925.83	**95**
1731.74	90
1600.79	85
1496.71	80
1407.43	75
1327.24	70
1182.44	60
1047.09	50
911.74	40
766.93	30
597.46	20
362.44	10

End of Appendix 2

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24 April 2019

Patrick Cournoyer, Ph.D.
Consumer Safety Officer
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

Dear Dr. Cournoyer,

In response to your communication of 5 April 2019, in which you forwarded questions from FSIS reviewers regarding GRN 824 (SALMOCIN), we provide here the following comments with the goal of clarifying certain sections of our Notice. For convenience, we show the original FSIS questions by category in *italics*, followed by our responses. The FDA-specific question on terminology was answered via the emails of April 09, 11 and 16, 2019.

Creating a food safety concern

The requested application rate is .1-3 mg of total Salmocins per kilogram of food product. They indicated on pages 9 and 13 of the submission that Salmocin can be applied as either a spray or a dip. For a dip, they indicated that the product could be dipped in a solution of 5-150 mg of Salmocin per liter. Without knowing the product's weight, it is unclear how dipping the product in a 5-150 mg/l solution ensures a contact rate of .1-3 mg of Salmocin/kg of food. We request additional information concerning how the dip application method ensures the range of Salmocin application concentration is met.

Notifier's response. We assumed that the weight of food products (e.g., meat) can be estimated prior to Salmocin treatment. We also assumed that bacterial contamination of food would occur at the exposed surfaces of a matrix. A solution containing 5-150 mg of total salmocin protein applied as a spray at 20 mL solution per kg food (page 13 of the Notice) exposes the matrix to 0.1-3 mg salmocins, which is a minimum effective range for bacterial control. A kg of meat cuts dipped in a liter of Salmocin solution containing 5-150 mg salmocins would expose the matrix to $\geq$0.1-3 mg salmocin protein, thereby also ensuring exposure of the food to an effective concentration of the bactericide.

We acknowledge that these application rates per unit weight of food will yield different exposures depending on the density and the surface area of the food treated, which in turn vary with food cut/size (surface-to-volume ratios) and matrix surface properties. Hence, to accommodate as many food matrices and treatment modalities as possible, we specified the 0.1-3 mg/kg range of Salmocin application rates based on antibacterial activity of $\geq$1 $\log_{10}$ CFU/g food relative to controls in a number of scenarios presented in the Notice. As is evident from the results of our studies, considerably greater $\Delta\log_{10}$CFU/g food were obtained in Salmocin-treated food products relative to carrier vehicle controls.

The spray and dip application options were included in the Notice to accommodate treatment of a wide variety of foods regulated by FDA or USDA that are susceptible to *Salmonella* contamination. FDA-regulated products such as fruits and some vegetables can be dipped or washed in a Salmocin-containing stream. In contrast, USDA-regulated products are more likely to be treated by the spray method (e.g., most meats) or by direct addition and mixing (e.g., egg products). Additional studies on optimal application modes and rates with various food types (e.g., red meat, skin-on poultry cuts, liquid/semi-solid foods, etc.) are in progress and application instructions will be provided in the product label.

Pose a human health risk

Very often substances that reduce microbial levels are sprayed on beef primals and subprimals (i.e., large volume beef products). After treatment, the outer surface of the primals and subprimals may be trimmed and the remaining products are cut to customer specification into roasts and steaks. The trim that was removed from the primal/subprimal can be used in the formation of other beef products such as ground beef. The dose of Salmocin (.1-3 mg/kg) to be applied is calculated by using the weight of the primal/subprimal. However, after trimming, the trim (outer layer) to which the Salmocin was applied, would not have the same weight, potentially resulting in a much higher effective rate of Salmocin/kg on the trim when ingested than the requested .1-3mg/kg rate. What effect would the effective higher rate on trim have upon public's health?

Notifier's response. We agree that depending on when and where the Salmocin product is applied (i.e., upstream, large meat cuts prior to trimming vs. downstream, smaller cuts) could result in variations in on-matrix concentration of salmocin proteins. However, from the standpoint of public health, which is the focus of the Agency's question, we believe that the risk to consumers due to variable concentrations of applied Salmocin is nearly non-existent. The application rate for Salmocin of 0.1-3 mg/kg food is based on the economics of using the product plus achieving adequate bactericidal control of *Salmonella*, as determined in efficacy studies on various matrices (presented in the Notice). The upper limit of the application rate range is not defined by Salmocin's toxicity.

The reasons we can project a high safety margin for Salmocin when used at the levels intended, or even at higher concentrations, include:

- After application, Salmocin will act on target bacteria on the food matrix, if present, and will be immediately susceptible to degradation by food-borne proteases on the matrix;
- Salmocin is a Food Processing Aid and salmocin proteins retain on-matrix bactericidal activity for only 1-48 hr (72 hr maximally) post application on most foods. Salmocin would not be present by the time treated foods reach a retail outlet;
- Salmocin proteins will be completely degraded by cooking (thermal denaturation);
- Should residual amounts of salmocin proteins remain on foods that are consumed uncooked (e.g., tuna sashimi, steak tartare, raw egg products), salmocins will be rapidly denatured in the low pH of the stomach, and will be degraded enzymatically in the upper GI tract by gastroduodenal enzymes (data provided in the Notice). Even in this scenario, the consumer would essentially be ingesting a very small amount of inactive extra protein in the diet.

Inhalation of Salmocin aerosol

The document discusses the acceptability of consuming Salmocin when applied at .1-3 mg/kg. However, we did not see a discussion concerning how breathing air from a spray with Salmocin over extended periods of time does or does not create a human health concern. In addition, the submission is unclear on the required personal protective equipment and application methods needed when Salmocin is being

mixed or applied as a spray. Pages 41 and 60 indicates that only minimal precautions need to be taken. Yet, in that same paragraph on page 60, it goes on to say that the aerosols should be minimized by a spray cabinet. Page 69 implies that masks or respirators should be used. Is a spray cabinet required for the application of Salmocin and what personal protective equipment should FSIS inspectors use when exposed to aerosols that may contain Salmocin?

Notifier's response. Consumers are unlikely to be exposed to Salmocin from treated food and even if they were the health concern would be minimal, as addressed in our response to the previous question. This response addresses the occupational risk from Salmocin preparation, application and disposal.

Salmocin contains no substances with occupational exposure limit values. Salmocin proteins are not toxic to humans because they have no targets in mammalian cells or tissues; they are strictly bactericidal against selected strains of *Salmonella*. Salmocins are foreign (non-human) proteins and like all foreign proteins there is a risk of developing an allergy or hypersensitivity upon repeated exposure for extended periods. All salmocin proteins have low allergenicity and immunogenicity potential, as determined in amino acid sequence searches (pp 54-58 of the Notice). Nevertheless, there are no empirical data on allergenic risk to humans. As such, and for an abundance of caution, our Notice suggested that minimal personnel protection might be warranted if in-plant personnel (applicators or inspectors) might become repeatedly exposed to aerosols or dusts of Salmocin during product preparation, application or disposal.

Based on current information available to Notifier, spray cabinets do not need to be used to apply Salmocin, but their use would be at the discretion of plant operators per their individual HACCT plans. General industrial hygiene practices when using salmocins are recommended. When aerosols or product dust are anticipated, goggles or other face protection devices to protect eyes are recommended, as well as ready access to eye/face flushing equipment. A lab coat and/or gloves plus long-sleeved attire should suffice to minimize skin contact, although there is no evidence that salmocins are skin sensitizers. Respiratory protection programs are specified in 29 CFR 1910.134. In our view, a NIOSH-approved N95 type face mask/respirator with or without an exhalation valve should effectively minimize respiratory contact to droplet aerosols and dust and essentially eliminate allergenic risk from exposure of mucous membranes. Notifier expects to learn more about potential risks of Salmocin exposure to applicators and inspectors in the occupational environment and final guidance will be provided in the product label.

Effectiveness

The figures used to show a reduction in Salmonella serovars use log_{10} values. However, it is unclear if the statistical tests were performed on the raw values or the transformed log values. FSIS requests additional information on what data were used for the statistical test. In addition, they stated that they used a parametric two-tailed t-test on the data (pages 91 and 100), but we cannot evaluate whether this is appropriate or not as:

- *There was no information concerning whether or not they checked the data to see if it met the assumptions of the test (and the results of those checks), and*
- *The power associated with the sample size and testing procedures was not included.*

We request additional information concerning the above data and statistical evaluation of that data.

Notifier's response. Upon review of the methods described in our original Notice, we found that we had incorrectly described some of the statistical methodology that we had used. Specifically, in GRN 824 Section C.14 Statistical Analyses (page 91) and in Section D9.10 Statistical Analysis (page 100), the two-tailed unpaired parametric t-test, the two-tailed unpaired parametric t-test with Welch's correction, and the non-parametric Mann-Whitney test were used. Mention of "one-way ANOVA (Tukey's multiple comparisons test)" as it appeared in the text was incorrect as that method was not used in this Notice.

To address FSIS's concerns, we recalculated our results and performed updated statistical comparisons. We provide below a detailed response to the Salmocin effectiveness questions, with specific focus on the statistical analyses used to determine the significance of bactericidal activity. For completeness, accompanying the narrative is **Appendix 1** that includes tabular results of analyses of effectiveness on the various food matrices described in the Notice. **Appendix 1 is for FDA/USDA internal review only**.

1. Statistical Methodology

As a starting point, all statistical analyses were performed with unpaired parametric two-tailed t-test using GraphPad Prism v. 6.01. To ensure suitability of statistical analysis procedures, data were analyzed for conformance with the following assumptions:

1) The data are continuous;
2) The different samples are independent;
3) There is no relationship between the individuals in one sample compared to the others;
4) Replicates are simple random samples from their respective populations;
5) Each individual in the population has an equal probability of being selected in the sample;
6) The data follow a normal probability distribution; and
7) The variances of the distribution of the different samples are equal.

Starting with statistical analysis, in a first step, normal probability distribution was ensured using the Shapiro-Wilk normality test (using GraphPad Prism v. 6.01) showing that there was no statistically significant difference between the data of samples and normal distribution, hence it can be assumed that data are normally distributed.

In a second step, the F-test was used (in GraphPad Prism v. 6.01) in order to assess whether different sample groups showed equal variances. Using raw data, this test detected unequal variances; therefore, for all analyses of statistical significance, all data used were log-transformed prior to being tested for equal variances by the F-test.

In cases of normal probability distribution and equal variances of sample groups, statistical analysis was performed with unpaired parametric two-tailed t-test using GraphPad Prism v. 6.01. In cases where data did not pass normality tests, statistical analysis was additionally done using non-parametric Mann-Whitney test. In cases of unequal variances of sample groups, statistical analysis was additionally performed by unpaired parametric t-test with Welch´s correction.

The approach of statistical analysis of log-transformed data using unpaired parametric two-tailed t-test or one of the other tests described above started with comparison of the carrier control group (i.e., "vehicle" control with no Salmocin) and the Salmocin treatment group with the highest Salmocin concentration, at the earliest time point of analysis for detection of a statistically significant reduction of *Salmonella* contamination in the Salmocin treated sample. If a statistically significant difference was detected, the carrier control group and all other Salmocin treatment groups in order of decreasing Salmocin concentration were subsequently compared. If the first comparison gave a positive result, data of all other time points were analyzed.

Lastly, regrowth of surviving residual *Salmonella* in Salmocin-treated samples was analyzed to show that the technical effect is temporary, hence supporting Salmocin's classification as a Food Processing Aid. Employing the unpaired parametric two-tailed t-test, the first statistically significant detectable growth was ensured by comparing samples of the carrier control treatment group of the earliest time point of analysis with those of longer storage periods. If this analysis was positive (i.e., significant regrowth occurred), samples of Salmocin-treated groups were compared at different time points of analysis (i.e., early vs. late) for determination of statistically significant increases in bacterial regrowth over time.

2. Test power

Experiments were designed to yield data that were suitable for analysis as proposed above. Pre-test (pilot) experiments indicated an expected difference in *Salmonella* cell numbers between carrier control group and Salmocin treatment groups of at least 1,500 at early time points of analysis and ≥10,000 at later time points under prolonged storage.

In order to allow for analysis of different treatments in parallel in the same experiment and to enable for a statistical power of at least 0.8, 4 replicates (N per group) of each sample were analyzed.

Results of pilot studies were used to determine sample size/power analysis using the program GraphPad StatMate 2.00. The standard deviations of different groups were found not to be equal in these pilot studies; therefore, the average standard deviation (SD) values of carrier treatment and one Salmocin treatment group at the earliest time point of analysis were used.

Examples of how the statistical power of each test was determined in this application are shown in **Appendix 2**. As with Appendix 1, **Appendix 2 is also for FDA/USDA internal review only**.

3. Verification of statistical significance and conclusions

Using the multi-step process of data analysis described above, the following conclusions were verified with respect to the statistical significance of Salmocin's efficacy on various food matrices. Key comments and conclusions, including the tests used, for each bactericidal activity figure included in our original Notice (i.e., **Figure 2-8 through Figure 2-14**) are summarized below. See accompanying **Appendix 1** for additional detail on statistical analyses in studies with each food matrix.

Figure 2-8 (GRN 824, page 27) – Efficacy on Skinless Breast Filets. Statistically significant reductions in *Salmonella* contamination by Salmocin treatment relative to carrier treatment were observed. The p-values for comparisons at 1h, 24h, 48h and 72h post treatment were <0.0001 (two-tailed unpaired parametric t-test) when the carrier only was compared to either product tested: SalE1a only (3 mg/kg) or SalE1a+SalE1b+SalE2+SalE7 (3+1+1+1 mg/kg). Regrowth assessment revealed statistically significant regrowth of surviving residual *Salmonella* within 72h of Salmocin treatment (already detected at 24h of incubation for the SalE1a 3 mg/kg application rate). The different kinetics of regrowth in Salmocin-treated vs. carrier-treated samples are most likely due to the different levels of residual bacterial contamination post treatment in each group.

Figure 2-9 (GRN 824, page 27) – Efficacy on Skinless Breast filets. Statistically significant reductions in *Salmonella* contamination by Salmocin treatment relative to carrier treatment were observed. The p-values for comparison at 1h, 24h, 48h and 72h post treatment were from <0.0009 to <0.0001 (two-tailed unpaired parametric t-test) when the carrier only was compared to any of the three products tested: SalE1a (3 mg/kg), SalE1a+SalE1b+SalE2+SalE7 (3+1+1+1 mg/kg), or SalE1a+SalE1b+SalE2+SalE7 (0.3+0.1+0.1+0.1 mg/kg). Statistically significant regrowth of surviving residual *Salmonella* after Salmocin treatment was detected at 72h and even at 24h post exposure (p-values from 0.0009 to <0.0001).

Figure 2-10 (GRN 824, page 29) – Efficacy on Skin-on Breast Filets. Statistically significant reductions in *Salmonella* contamination by Salmocin treatment relative to carrier treatment were observed for the three application rates of SalE1b. Although in these experiments most datasets displayed normal distribution and equal variances, there were also datasets with non-normal distribution and with different variances (Slides 15 and 16 in Appendix 1). Results of (1) two-tailed unpaired parametric t-test, (2) two-tailed unpaired parametric t-test with Welch's correction and (3) non-parametric Mann-Whitney test are shown in Slides 15 and 16 in Appendix 1. The t-test was statistically significant, p=0.1550 to <0.0001; the t-test with Welch's correction was also significant, p=0.0151 to <0.0001.

The non-parametric Mann-Whitney test is likely the most appropriate for this case, and showed that reductions in CFU/g meat for Salmocin relative to control vehicle solution were all statistically significant at all time points sampled (P=0.0286). Regrowth assessment verified temporally limited technical effect of Salmocin by detecting statistically significant regrowth of surviving residual *Salmonella* within 72h post treatment (Slides 17 and 18 in Appendix 1).

Figure 2-11 left panel (GRN 824, page 29) – **Efficacy on Skin-on Breast Filets.** Statistically significant reductions in CFU/g meat for Salmocin relative to control vehicle solution (bactericidal efficacy) were found at all four application rates and at all time points sampled (p=0.0049 to <0.0001, using the two-tailed unpaired parametric t-test). Regrowth assessment using unpaired parametric t-test with Welch´s correction showed statistically significant (p-values <0.0030) increase of surviving residual *Salmonella* within 72h post treatment.

Figure 2-11, right panel (GRN 824, page 29) – Efficacy on Skin-on Breast Filets. Statistically significant reductions in *Salmonella* contamination by Salmocin treatment relative to carrier treatment were observed for the four application rates of SalE1b evaluated. In this experiment also, there were datasets with non-normal distribution and with different variances (Slides 26-28 in Appendix 1). All three tests confirmed that the differences between treatments at all time points were statistically significant with p-value <0.05. Using the Mann-Whitney test, p=0.0286 was found for all comparisons. The t-test showed p= 0.01550 and p<0.0001, and the t-test with Welch´s correction showed p=0.0166 to p<0.0001. Regrowth assessment using unpaired parametric t-test with Welch´s correction or non-parametric Mann-Whitney test showed statistically significant increase of surviving residual *Salmonella* within 72h post treatment.

Figure 2-12 A, B, C (GRN 824, page 31) – Efficacy on Beef (two levels of contamination). In all comparisons, statistically significant reductions in *Salmonella* contamination in Salmocin-treated versus carrier-treated samples were found for the two application rates of SalE1b evaluated (p=0.0286; non-parametric Mann-Whitney test). Regrowth assessment showed a statistically significant increase of surviving residual *Salmonella* within 48h post Salmocin treatment in only one of the two studies conducted. This result is most likely due to high efficacy of Salmocin in reducing bacterial cell numbers under the conditions described and insufficient regrowth of survivors at the 48h post-treatment sampling point.

Figure 2-13, A (GRN 824, page 32) – Efficacy on Sample Seafood (Raw Tuna; high contamination level). Statistically significant reductions in *Salmonella* contamination by Salmocin treatment relative to carrier treatment were observed for the two application rates of SalE1b, with values of p=0.0007 and p=0.0206 (unpaired parametric t-test with Welch´s correction). No statistically significant regrowth was detected within 48h post Salmocin treatment, most likely due to high efficacy of Salmocin in reducing bacterial cell numbers under the conditions described and insufficient regrowth of survivors at the 48h post-treatment sampling point.

Figure 2-13, B (GRN 824, page 32) – Efficacy on Sample Seafood (Raw Tuna; moderate contamination level). Statistically significant reductions in *Salmonella* contamination by Salmocin treatment relative to carrier treatment were observed for the two application rates of SalE1b, with values of p=0.0003 and p=0.0016 (unpaired parametric t-test with Welch´s correction). No statistically significant regrowth was detected within 48h post Salmocin treatment, most likely due to high efficacy of Salmocin in reducing bacterial cell numbers under the conditions described and insufficient regrowth of survivors at the 48h post-treatment sampling point.

Figure 2-14, A, B, C (GRN 824, page 34) – Efficacy on Raw Whole Eggs (two levels of contamination). Statistically significant reductions in *Salmonella* contamination by Salmocin treatment relative to carrier treatment were observed for the two application rates of SalE1b, with a value of p=0.0286 (non-parametric Mann-Whitney test). No statistically significant regrowth was detected within 48h post Salmocin treatment, most likely due to high efficacy of Salmocin in reducing bacterial cell numbers under the conditions described and insufficient regrowth of survivors at the 48h post-treatment sampling point.

For added clarity with respect to our product's technical effect, we note that in these studies with salmocins as well as in Notifier's prior GRNs describing other bactericidal proteins for food safety, surviving bacteria are not likely to be resistant or tolerant to salmocins or other bacteriocin-class proteins. Bacterial viability and regrowth, when observed, are likely the result of some cells not having come in contact with the bactericide; this phenomenon applies to other antibacterials regardless of chemical class. The receptor-mediated nature behind bacteriocins' technical effect(s) ensures that a cell will not survive once it is exposed to a threshold concentration of protein. Induction of immunity proteins against bacteriocins, which some enteric bacteria are capable of doing, is an energy intensive trait that is not constitutively sustainable. Notifier's earlier studies with colicins, bacteriocins similar to salmocins but acting on *E. coli* Big Seven pathogens, showed that surviving cells are susceptible to the bactericide and repeated serial exposures of survivors to one or more colicins did not lead to resistance (e.g., GRN 593 COLICIN food antimicrobial; pp 64-66).

We thank the Agencies for their careful review of our Notice. We believe that we have adequately addressed the questions raised by FSIS; however, if needed we can readily respond to any further questions or provide additional clarification to these responses.

Sincerely,

(b) (6)

Yuri Gleba, Ph.D.
Chief Executive Officer
Nomad Bioscience GmbH

Additional materials provided in support of Notifier's response:

Appendix 1: GRN 824 Statistical Analyses – On-Matrix Studies
Appendix 2: GRN 824 Statistical Power Calculations

cc. K.O. Smedley, Center for Regulatory Services, Inc. – Consultant to Nomad Bioscience GmbH
D. Tusé, DT/Consulting Group – Consultant to Nomad Bioscience GmbH

Cournoyer, Patrick

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Wednesday, April 24, 2019 4:48 PM
To: Cournoyer, Patrick
Cc: 'DANIEL TUSE'
Subject: RE: FSIS questions for GRN 824
Attachments: GRN 824 Appendix 1 Statistical Analyses On Matrix.pdf; GRN 824 Appendix 2 Statistical Power Calculations.pdf; GRN 824 Notifier Responses to FSIS Questions.pdf

Dr. Cournoyer:

Attached is the response to the questions raised by FSIS on NOMADD's Salmocin GRN 824. (Note we had addressed the FDA concern on the subject of the GRN in an exchange of earlier emails (culminating in your email of April 16)).

Our narrative response addresses each question raised by FSIS individually, and the statistical response cites two appendices that include information that may be helpful during the FSIS review.

We appreciate the opportunity to address these questions. Should FSIS have any additional questions, or need clarification, please contact us and we will immediately respond.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell 703-786-7674
Fax 703-580-8637

From: Cournoyer, Patrick [mailto:Patrick.Cournoyer@fda.hhs.gov]
Sent: Friday, April 05, 2019 2:10 PM
To: 'smedley@cfr-services.com'
Subject: FSIS questions for GRN 824

Dear Dr. Smedley,

FSIS raised the questions below regarding GRN 824. The only question FDA has at this time is to ask that you acknowledge that we will refer to the subject of the notice as "bacteriocins from *Salmonella*" in our documentation. We would appreciate your response to these questions within approximately 10 business days.

Creating a food safety concern

The requested application rate is .1-3mg of total Salmocins per kilogram of food product. They indicated on pages 9 and 13 of the submission that Salmoncin can be applied as either a spray or a dip. For a dip, they indicated that the product could be dipped in a solution of 5-150 mg of Salmoncin per liter. Without knowing the product's weight, it is unclear how dipping the product in a 5-150mg/l solution ensures a contact rate of .1-

Cournoyer, Patrick

From: DANIEL TUSE <daniel@dt-cg.com>
Sent: Friday, May 03, 2019 5:28 AM
To: Cournoyer, Patrick
Cc: Trout, Bryan - FSIS; Kristi Smedley; DANIEL TUSE
Subject: Re: FSIS questions for GRN 824

Importance: High

Dear Patrick,

Kristi and I have discussed FSIS's question with NOMAD Bioscience's management.

NOMAD Bioscience's intention in its GRAS determination for SALMOCIN was to include uses of the product on all fish. Raw tuna filet was used as a model matrix to determine efficacy against *Salmonella* contamination; results and their statistical significance were provided in the Notice and in our subsequent communications. However, the product has not yet been assessed for efficacy on-matrix in other seafood, including on samples of fish of the order Siluriformes.

Please let us know if further clarification is needed.

With best regards,

Daniel Tusé, Ph.D.
Owner and Managing Director
DT/Consulting Group
2695 13th Street
Sacramento, CA 95818, USA
eMail daniel@dt-cg.com
Tel +1 707 290 9528

From: "Cournoyer, Patrick" <Patrick.Cournoyer@fda.hhs.gov>
Date: Thursday, May 2, 2019 at 1:35 PM
To: Kristi Smedley <smedley@cfr-services.com>
Cc: 'DANIEL TUSE' <daniel@dt-cg.com>, "Trout, Bryan - FSIS" <Bryan.Trout@fsis.usda.gov>
Subject: RE: FSIS questions for GRN 824

Dear Kristi and Daniel,

USDA/FSIS has asked whether NOMAD Bioscience intends for the GRAS determination to include uses with fish of the order Siluriformes.

Best regards,

Patrick Cournoyer, Ph.D.
Consumer Safety Officer

Cournoyer, Patrick

From: DANIEL TUSE <daniel@dt-cg.com>
Sent: Wednesday, September 25, 2019 9:07 PM
To: Cournoyer, Patrick
Cc: Trout, Bryan - FSIS; 'smedley@cfr-services.com'; DANIEL TUSE
Subject: Re: Additional question, GRN 824
Attachments: GRN 824 Nomad Bioscience Reply to FDA Sep 25 2019.pdf

Importance: High

Dear Dr. Cournoyer,

The attached Memorandum is being provided on behalf of Nomad Bioscience GmbH (Notifier) in response to the questions you raised in your September 13, 2019 inquiry regarding GRN 824 (bacteriocins specific for *Salmonella*).

We hope that these responses adequately address the Agency's concerns about Nomad's developmental studies with salmocins. We will be happy to provide additional information or clarification if needed to assist FDA in completing its review of GRN 824.

We look forward to your reply.

Best regards,

Daniel Tusé, Ph.D.
Consultant to Nomad Bioscience GmbH
Owner and Managing Director
DT/Consulting Group
2695 13th Street
Sacramento, CA 95818, USA
eMail daniel@dt-cg.com
Tel 707 290 9528

From: DANIEL TUSE <daniel@dt-cg.com>
Date: Monday, September 16, 2019 at 1:04 PM
To: "Cournoyer, Patrick" <Patrick.Cournoyer@fda.hhs.gov>
Cc: "Trout, Bryan - FSIS" <Bryan.Trout@fsis.usda.gov>, Kristi Smedley <smedley@cfr-services.com>, DANIEL TUSE <daniel@dt-cg.com>
Subject: Re: Additional question, GRN 824

Dear Dr. Cournoyer,

Dr. Smedley is traveling most of this week and hence I am replying on her and Nomad Bioscience's behalf. Thanks for the questions and issues raised regarding Nomad's SALMOCIN product that is the

MEMORANDUM

Re: GRN 824 – SALMOCIN (bacteriocins specific for *Salmonella*)

Subject: Nomad Bioscience GmbH (Notifier) responses to FDA questions

Date: 25 September 2019

On 13 September 2019, CFSAN requested that Notifier provide clarification on the levels of alkaloid impurities in *Nicotiana benthamiana*-produced salmocin proteins for control of *Salmonella*. FDA's questions referred to (1) the process used to produce bacteriocins, including colicins and salmocins, and the resultant levels of pyridine alkaloid impurities in each class of protein, and (2) Notifier's ability to control the manufacturing process to limit alkaloid content in salmocins to meet the product's release criteria (Specification).

The Agency's requests for clarification are reproduced below for convenience and shown in *italics*, followed by Notifier's responses.

FDA Request 1

- *The specifications for nicotine and anabasine in GRN775 (colicin preparations) are 75 and 15 ppm, respectively. In this notice, the specifications are 400 and 100 ppm. The notice states that the method of manufacturing for both products is the same. Please explain why the specification differs despite no change to the method of manufacturing.*

Notifier's Response

The methods for gene expression, extraction and purification of bacteriocins from *Nicotiana benthamiana* plant biomass are generally the same for colicins and salmocins, as defined in GRN 775 (colicins from *N. benthamiana*) and GRN 824 (salmocins from *N. benthamiana*). However, the physicochemical properties of bacteriocins differ within and between classes. Colicins, some of which share high amino acid homologies, do not express with the same yield in the host plant, and their purification efficiencies differ. This is also true for salmocins. Not surprisingly, the affinity of each protein for impurities such as heavy metals and alkaloids will differ as well.

Although the bacteriocin purification process is controlled and yields consistent protein recovery and potency results from batch to batch (e.g., GRN 824 Tables C-4 and C-5; page 87), the levels of co-extracted impurities will also show batch-to-batch consistencies. Our experience in bacteriocin purification shows that alkaloids tend to co-extract with salmocins at higher levels than they do with colicins. This effect might be due to a higher affinity of alkaloids for salmocins relative to colicins, but we have not mechanistically studied this possibility.

Hence, based on multi-batch studies to date, we set our target colicin specification for nicotine and anabasine at 75 and 15 ppm, respectively, because we can reliably stay below those levels. In contrast, we found it necessary to raise the salmocin specification for each alkaloid to 400 and 100 ppm due to the higher level of co-extracted alkaloids. Salmocins are related to colicins, yet they are different proteins with different properties, even though both classes of bacteriocins are obtained using the same production process.

We continuously strive to make process improvements to obtain higher-potency, higher-purity products. As we optimize the process for commercial production, we expect that additional improvements in purification will yield products with lower levels of impurities.

It is important to note that the levels of alkaloids in the current salmocin Specification should not pose undue risk to consumers at the projected product application rate and at the estimated per-capita daily consumption of salmocin-treated foods (GRN 824 Section 6.2; pp 50-52).

FDA Request 2

- *The notice does not give individual batch data showing that the product can be manufactured to meet the specifications for nicotine and anabasine. While the notice gives levels for averages of 3 batches, individual batch data would be more informative. Further, the 3-batch average for nicotine in SalE1a is 477 ppm, which exceeds the specification. Thus, we cannot confirm that the product can be manufactured to meet the given specifications at this time. Please respond, addressing these points.*

Notifier's Response

We thank the Agency for bringing this issue to our attention. For clarity and perspective, our full response is provided in sections (points 1-5 below).

1. The commercial SALMOCIN product will be a blend of salmocin proteins

The product SALMOCIN consists of a **blend** of selected salmocin proteins. Although in GRN 824 we had considered using either individual salmocins or blends of salmocins, the large number of *S. enterica* pathovars and the unpredictability of which of them could contaminate various foods, strongly argued in favor of salmocin blends to achieve the widest possible antibacterial spectrum of control. Our product Specification (Table 2-2, page 17; repeated as Table B-1, page 78) is therefore based on criteria for a blend and not on individual values for each component. In **Table 6-1** on page 51 of GRN 824, we show the 3-batch average (Lot) nicotine content for SalE1a as 477 ng/mg protein, thereby appearing to exceed the target specification of 400 ppm nicotine in a final product. Table 6-1 is *not* the Specification. That table shows the average alkaloid value of a putative 1:1:1 blend of lead salmocins SalE1a, SalE1b and SalE7 from which we determined the total alkaloid content possible (nicotine + anabasine) from analyses of multiple batches solely with the goal of establishing the Specification.

2. Lead and secondary salmocins as candidates for a final blended SALMOCIN product

In GRN 824, Section 2.4.1 "Biological activity of SALMOCIN on target pathogenic *S. enterica* serotypes" (pp 18-37), we present results of numerous studies demonstrating the range of activity and potency of several individual salmocins and mixtures of salmocins, both under defined conditions *in vitro* as well as on a number of food matrices (e.g., poultry meat skin-off and skin-on; raw beef cuts; raw tuna; raw whole eggs). Although initially better pathogen control on food matrices appeared to be achieved with multi-salmocin blends relative to individual salmocins (Fig 2-8 and Fig 2-9; page 27), the effect may have been largely due to the higher dose of antimicrobial (i.e., application rate of 3 ppm for SalE1a or SalE1b alone vs. 6 ppm for a blend of SalE1a+SalE1b+SalE2+SalE7). However, in subsequent studies we also show potent and broad antimicrobial activity of SalE1b acting singly, with an ability to control the majority of tested pathovars *in vitro* and effective at $\leq$3 ppm ($\leq$3 mg/kg food) on chicken, beef, tuna and egg products (Figures 2-10 through 2-14; pp 29-34).

These studies allowed us to identify SalE1a, SalE1b and SalE7 as the lead candidates. The Agency will note that we also evaluated other salmocins, including SalE2 and SalE3 and included them in our discussion for completeness, but discontinued development of these proteins because they offered no production or suitability advantages over the three lead candidates. Furthermore, among these 3 leads, **SalE1b** and **SalE7** showed potent complementary activity and had sufficiently low impurity levels to meet the criteria for a blended dry product.

3. Specification for SALMOCIN (blended salmocins) product

Importantly, we note that the values in the **Specification** in GRN 824 (Table 2-2, page 17; repeated as Table B-1, page 78) are for a 1:1 mix of **SalE1b and SalE7** – a blend of salmocins that best represents the **lead commercial product**. SalE1b provides high potency and very broad spectrum activity, while SalE7 exhibits lower potency but completes the activity spectrum for full pathovar control.

With respect to the limits for alkaloids, **SalE1a** was *not* included in the blend for the Specification, in part because (a) the activity spectra and potencies for SalE1a and SalE1b are very similar, making these proteins interchangeable, (b) the production yield of SalE1a is lower than that of SalE1b, and (c) at the time we had not succeeded in consistently reducing the level of alkaloid impurities in SalE1a to our satisfaction, but we had achieved higher purity more consistently with SalE1b. Our purification process is still undergoing scale-up and optimization with the goal of achieving higher protein recoveries with lower levels of impurities.

4. Batch production records for alkaloid content

To expand on GRN 824 Table 6-1, individual results of alkaloid levels in 3 Batches of SalE1b and SalE7 together with Lot averages (blended batches) are provided in the table below (values in ng alkaloid/mg protein; ppm). Also provided in the table are 3-Batch results for SalE1a.

Salmocin	**SalE1b**		**SalE7**		**SalE1a**	
Batch number	Nicotine	Anabasine	Nicotine	Anabasine	Nicotine	Anabasine
1	178	26.67	119.7	18.03	657	78.57
2	242	46.6	102.8	22.64	589	26.32
3	640	217	144.0	26.25	187.5	15.6
Average for 3-Batch Lots	353.3±250.3	96.76±104.6	122.17±20.7	22.31±4.12	477.83±253.72	40.16±33.69

By blending Batches into Lots, we have been able to meet the final blended product Specification. It is common in industry to blend Batches to produce Lots that meet the overall criteria of individual components, to produce a uniform final blended product meeting the Specification.

Blending 1:1 not only provides greater antimicrobial functionality, it also dilutes the impurity levels in half for each component on the basis of total protein. To illustrate, using SalE1b and SalE7 from their respective Batch 3 (the batches with the **highest** nicotine levels of 640 and 144 ppm, respectively) and blending them 1:1 yields a mixture with 392 ppm nicotine, a value still within the 400 ppm limit. Using **average** values for Lots from all 3 Batches of SalE1b and SalE7 yields a mix with 237.7 ppm nicotine, well below the limit in the Specification. A similar calculation with 1:1 mixtures of SalE1a and SalE7 using either maximum or average values will also yield nicotine levels at or below the 400 ppm limit. Similar conclusions apply for anabasine. These fluctuations in impurity levels appear related to the small batch sizes we have been using in our developmental studies. More efficient impurity removal and higher consistency in manufacturing batches are expected as we scale up purification with the assistance of a CRO. The marketed SALMOCIN product will meet the stated Specification.

5. SALMOCIN Specification and product life cycle

We stand by the current entries for alkaloid limits in the Specification of a putative commercial blended salmocin product (i.e., SalE1b+SalE7) because we have shown in GRN 824 using scientific procedures that such levels should not pose undue risk to consumers. Although we will continue attempts to further purify SalE1a, that particular protein is expendable as we can achieve product quality and suitability attributes with SalE1b and SalE7. In addition, since submission of GRN 824 we have cloned and expressed additional compositions of salmocins, and after full characterization some of these newly found proteins might be integrated in future blended products, provided they meet the required suitability, quality and safety criteria.

Process improvements will continue to be made during scale-up towards commercial production of salmocins, and we assert that regardless of the salmocin blend selected for commercial use, no final product will be released unless it meets all criteria in the Specification.