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

consultants to the regulated industry

#802

July 9, 2018

Dr. Antonio Mattia
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. Mattia:

SUBJECT: Transmittal of the NOMAD BIOSCIENCE GmbH –
GRAS Notice for ENDOLYSIN use as antimicrobial agent
In Cooked Foods

Enclosed you will find the GRAS notice for ENDOLYSIN to be used as an antimicrobial for controlling *Clostridium perfringens*, as submitted by NOMAD BIOSCIENCE GmbH. ENDOLYSIN is intended for use in cooked foods which may undergo temperature abuse and permit the growth of *Clostridium perfringens* . The manufacture of ENDOLYSIN is through plant hosts as described in GRAS notices previously filed by NOMAD (GRN 593, 676, 738, and 775).

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)
ENDOLYSIN 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

Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER 000842	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

RECEIVED JUL 12 2018 OFFICE OF FOOD ADDITIVE SAFETY

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, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I – 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)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: 2017/12/05

3b. 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)*: _____

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position
Yuri Gleba, Ph.D.	Chief Executive Officer

Company *(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
49 345 555 9887	49 345 1314 2601	gleba@nomadbioscience.com

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

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

Company *(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

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance
ENDOLYSIN

2. Submission Format: *(Check appropriate box(es))*
☐ Electronic Submission Gateway
☐ Paper
☒ Electronic files on physical media with paper signature page
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 FDA's files by reference? *(Check one)*
☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates by reference 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)* _____

6. Statutory basis for determination of GRAS status *(Check one)*
☒ Scientific Procedures *(21 CFR 170.30(b))* ☐ Experience based on common use in food *(21 CFR 170.30(c))*

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?
☐ Yes *(Proceed to Item 8)*
☒ No *(Proceed to Part IV)*

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, see attached Designation of Confidential Information
☐ 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

PART IV – INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population)*.

The product ENDOLYSIN is a food antimicrobial that acts specifically to control the bacterial pathogen *Clostridium perfringens* (*C. perfringens*). ENDOLYSIN consists of bacteriophage-derived proteins manufactured in green plants that are applied to or admixed in cooked foods such as meat, poultry, gravies, sauces and other cooked or baked food products that may become temperature abused during preparation or prior to serving and therefore support growth of the pathogen *Clostridium perfringens*, leading to a food-safety risk. Although intended to control *C. perfringens* in meat and poultry food products, the Endolysin is not meant to be applied to raw meat or poultry or other raw or uncooked meat-containing products; usage is intended only in foods post cooking. The product can be applied on or admixed in cooked foods at a rate of 1-10 mg ENDOLYSIN per kg food (i.e. 1-10 ppm). All subpopulations that consume cooked meats, poultry, gravies or sauces treated with ENDOLYSIN may consume traces of the product. This product is not intended for use in infant formulas.

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*
☒ Yes ☐ No

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☒ Any additional information about identity not covered in Part V of this form
- ☒ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☒ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☒ Comprehensive discussion of the basis for the determination of GRAS status
- ☒ Bibliography

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

PART VII – SIGNATURE

1. The undersigned is informing FDA that NOMAD BIOSCIENCE GMBH
(name of notifier)

has concluded that the intended use*(s)* of ENDOLYSIN
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☐ _____ *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

_____ *(name of notifier)* 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.

Center for Regulatory Services Inc., 5200 Wolfe Run Shoals Rd, Woodbridge, VA 22192, USA
(address of notifier or other location)

_____ *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

☒ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. ----------
(GRAS Affirmation Petition No.)

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
(b) (6) [redacted]	Kristi O. Smedley, Ph.D.	07/09/2018

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☒ Any additional information about identity not covered in Part V of this form
- ☒ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☒ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
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- ☒ Bibliography

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

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Center for Regulatory Services Inc., 5200 Wolfe Run Shoals Rd, Woodbridge, VA 22192, USA
(address of notifier or other location)

_____ *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

☒ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. ----------
(GRAS Affirmation Petition No.)

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
	Kristi O. Smedley, Ph.D.	

PART VIII – 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)
	GRN for ENDOLYSIN as a food antimicrobial to control Clostridium perfringens in cooked foods that are susceptible to temperature abuse (PDF of Notification)	Submission
	ENDOLYSIN GRN References - For FDA Internal Review Only (PDFs of All Cited References - Not for Republication)	Submission

OMB Statement: Public reporting burden for this collection of information is estimated to average 150 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, 1350 Piccard Drive, Room 400, Rockville, MD 20850. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

NOMAD BIOSCIENCE GmbH
Biozentrum Halle
Weinbergweg 22
D-06120 Halle/Saale
Germany
Tel. 49 345 555 9887
Fax. 49 345 1314 2601

Date: 9 July, 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 ENDOLYSIN Antimicrobial Against *Clostridium perfringens* in Cooked Meat, Poultry and Related Products

Dear Dr. Mattia,

Nomad Bioscience GmbH ("Nomad"; "Notifier") is submitting this GRAS Notice for its **ENDOLYSIN antimicrobial for controlling *Clostridium perfringens*** in cooked food products. NOMAD has concluded, under FDA's Final Rule pertaining to 21 CFR 170 (August 17, 2016), that the naturally occurring proteins comprising ENDOLYSIN are Generally Recognized as Safe (GRAS) for use as an antimicrobial treatment to reduce the levels of *Clostridium perfringens* bacteria (bactericidal) on cooked meat, poultry, gravy and other food products that may undergo temperature abuse and hence create a food safety risk for consumers.

Nomad previously submitted GRAS notices to 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). The manufacturing process for ENDOLYSIN and its use pattern and application rates are very similar to those described in prior notices; therefore, we make reference to those GRNs when appropriate for added perspective.

This Notice defines the use of ENDOLYSIN to prevent or reduce contamination of cooked food, especially meats, with *Clostridium perfringens* (*C. perfringens*). The anaerobic spore-forming bacterium *C. perfringens* is a source of one of the most common food-borne illnesses in the United States and Europe. *C. perfringens* gastroenteritis is caused by type A strains that produce the *C. perfringens* enterotoxin ("CPE"; Xiao (2012)). With a recent study potentially linking epsilon toxin from *C. perfringens* Types B and D strains to multiple sclerosis (MS), there may be an even greater need to control this pathogen in our food (Wagley 2018).

The CPE-mediated food poisoning outbreaks typically involve a large number of victims and are associated with cooked but temperature-abused meat or poultry dishes. Optimal conditions for food poisonings arise when food contaminated with CPE-positive *C. perfringens* spores is slowly cooled or held at a temperature range of 10-54 °C, allowing spore germination and rapid growth of *C. perfringens*.

Effective and safe antimicrobials to control food contamination by *C. perfringens* are needed. Currently, there are few interventions targeted toward the inactivation of bacteria in food (FDA 2018). Most of the available interventions involve time/temperature control or exposure to organic acids, but some of these treatments can adversely impact food taste and quality. In sharp contrast, Notifier's ENDOLYSIN product makes use of lytic enzymes employed by species- and strain-specific bacteriophages to burst bacterial cells towards the end of the phages' life cycle. ENDOLYSIN does not contain phages, but rather the enzymes encoded by the phages to attack their bacterial hosts. Multiple natural phage-derived endolysins can be produced in green plants for safety and ease of scale-up. The endolysins can be used singly or combined to adjust the target range of the product as well as its potency. The product ENDOLYSIN therefore can consist of a single endolysin protein or a mixture of two or more endolysins.

ENDOLYSIN can be applied to cooked meat, poultry and other products at a rate of 1-10 mg ENDOLYSIN per kg treated food to achieve effective control of *C. perfringens*. The endolysin proteins are naturally found in bacteriophages to which we are routinely exposed. Using scientific procedures, Nomad has concluded that ENDOLYSIN 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 ENDOLYSIN 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 ENDOLYSIN during food preparation, including the methodology used for such assessments.

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 ENDOLYSIN antimicrobial
2. GRN for ENDOLYSIN antimicrobial (Parts 1-7), which includes:
   APPENDIX A: ENDOLYSIN Safety Data Sheet
   APPENDIX B: ENDOLYSIN Manufacturing Process Summary
   APPENDIX C: Methodology
   APPENDIX D: Standard Operating Procedure for ENDOLYSIN Efficacy Determination
3. Copies of references cited in the GRN

Our submission documents have been bookmarked and hyperlinked for ease of navigation on-screen.

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
Nomad Bioscience GmbH

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1 General Introduction and Claim of Exemption from Premarket Approval Requirements for ENDOLYSIN to Control *Clostridium perfringens* in Food

Nomad Bioscience GmbH ("Nomad"; Notifier) ENDOLYSIN product is produced recombinantly using a plant-based manufacturing process to match the amino acid sequence of naturally occurring endolysin-family antimicrobial proteins. Bacteriophage endolysins (endolysins are also referred to herein as "lysins") destroy bacterial cells by hydrolyzing bacterial cell wall components. Lysins active against Gram-positive (Gram+) bacteria are generally modular monomeric proteins of 250-400 amino acid residues in length, with an N-terminal enzymatic (catalytic) domain and a C-terminal binding (targeting) domain.

Bacteriophages and their components, including endolysins, are ubiquitous in nature and we are constantly exposed to them from our environment (Barr 2017), including from the food we eat (Sillankorva 2012). Numerous in-depth studies including those by Drulis-Kawa (2015), Fischetti (2010), Hermoso (2007), Jun (2013), Loeffler (2003), Nakonieczna (2015), Rios (2016), Roach (2015), Schmelcher (2012), Yang (2014) and others provide a comprehensive picture of phage and lysin exposure and safety. Phages have been used medicinally to target bacterial pathogens (Nelson 2001; 2012; Roach 2015) and several food antibacterials consisting of phage mixtures are listed as GRAS (e.g. GRNs 218, 528, 435, 672 and others) and are currently on the market. Peer-reviewed studies have established an extensive record of safety for phages and by extension phage-encoded components, especially when used as food antimicrobials.

Notifier's ENDOLYSIN product consists of endolysin proteins without the phage virion. The product is applied to meats and other foods **post cooking** at a rate of 1 – 10 mg ENDOLYSIN/kg food to prevent the growth of or kill contaminating *C. perfringens*. This Notice provides exposure estimates and a risk assessment regarding consumption of cooked food products treated with ENDOLYSIN. Notifier concludes that under the conditions of use described herein, ENDOLYSIN 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 various meats, methods used to assess suitability as well as residual technical effect, and the results obtained. ENDOLYSIN is not intended for use in infant formulas.

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: 49 345 555 9887
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

ENDOLYSIN

1.4 Conditions of Use

ENDOLYSIN is comprised of one or more hydrolytic proteins natively encoded by bacterial lytic phages. By selecting which lysins are included in a formulation at optimized ratios, the host-specificity of the product and its potency can be controlled. This Notice concerns lysins and lysin mixtures designed to control pathogenic *Clostridium perfringens* in foods post cooking.

The product ENDOLYSIN can be formulated to contain one or a mixture of two or more individual lysin proteins, depending on the breadth of application needed in various food products. Details on endolysins, 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 endolysins' safety attributes when used as intended, including the source of supporting information, is provided in Table 7-1.

Cooking food does not necessarily kill *C. perfringens* spores; hence, ENDOLYSIN is intended to be used as a post-cooking food antimicrobial to control vegetative growth of *C. perfringens*. Specifically, the intended use of ENDOLYSIN is as a spray to be applied on food, or as a dry powder or a solution to be added to food by admixture (other means of application not described in this Notice are contemplated) to control *C. perfringens* on cooked red meats, poultry, sauces and gravy, or other cooked or baked foods, at an application rate of 1-10 mg ENDOLYSIN per kg of treated food (approximately 0.5-5 mg/lb).

The **subpopulations** potentially exposed to ENDOLYSIN are comprised of individuals of all ages who consume cooked meats and related products treated with Notifier's antimicrobial. The product is not intended for use in infant formulas.

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 ENDOLYSIN is generally recognized as safe when used to minimize contamination of cooked food products by *Clostridium perfringens* at an application rate of 1-10 mg ENDOLYSIN per kg of treated food.

1.6 Not Subject to Preclearance

Notifier has concluded that ENDOLYSIN 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 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 ENDOLYSIN 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 components of ENDOLYSIN product that may be used singly or in combination to achieve the desired antibacterial suitability in food. All lysins evaluated in this Notice are derived from natural phages that attack Gram+ bacteria from the genus *Clostridium* and have high selectivity for *C. perfringens*. The table lists each component endolysin by common name (column 1), together with its collection accession number(s), type of enzymatic activity by which it exerts antibacterial effects, the bacterial host/isolate origin, and the published reference in which the lysin is described in detail.

Table 2-1. Active components of ENDOLYSIN product formulation

Lysin	Accession No.	Activity	Origin	Reference
PlyCP26F	YP_007004008	N-acetylmuramoyl-L-alanine amidase	*Clostridium* phage CP26FNC_019496.1	(Simmons 2010)
PlyCP39O	YP_002265435	N-acetylmuramoyl-L-alanine amidase	*Clostridium* phage CP39-ONC_011318.1	(Simmons 2010)
psm	ZP_02640173	GH25_Lyc-like and SH3b domain-containing protein	*Clostridium* phage SM101NC_008265.1	(Nariya 2011)
CP25L	YP_008058948	N-acetylmuramoyl-L-alanine amidase	*Clostridium* phage vB_CpeS-CP51NC_021325.1	(Gervasi 2014)
ZP173	WP_003469359 (ZP_02640173)	GH25_Lyc-like and SH3b domain-containing protein	*C. perfringens* CPE str. F4969 NZ_ABDX01000023.1	(Schmitz 2011)
ZP278	WP_003469445 (ZP_02640278)	GH25_Lyc-like domain-containing protein	*C. perfringens* CPE str. F4969 NZ_ABDX01000024.1	(Schmitz 2011)

Structural Information on ENDOLYSIN Components

The structure of phage endolysins differs between enzymes targeting Gram+ and Gram-negative (Gram-) bacteria; a difference that is no doubt related to the different cell wall structures between the bacterial groups. As reviewed by Schmelcher (2012), lysins that target Gram+ bacteria display a modular design in which catalytic activity and substrate recognition are separated into two types of functional domains: Cell wall Binding Domains (CBDs) and Enzymatically Active Domains (EADs) (Fischetti 2010; Loessner 1995; 2005; Lopez 2004). While the EAD confers the catalytic mechanism of the enzyme (i.e., cleaving specific bonds within the bacterial peptidoglycan), the CBD can target the protein to its substrate and keep it tightly bound to cell wall debris after cell lysis, thereby preventing diffusion and subsequent destruction of surrounding intact cells that have not yet been infected by the phage (Loessner 2002).

By contrast, because Gram- bacteria have outer membranes, lysins from phages infecting Gram- hosts are typically small single-domain globular proteins (MW 15-20 kDa), usually without a specific CBD (Briers 2007; Cheng 1994). Gram- lysins fulfill a more typical catalytic role of classical enzymes (aiding multiple catalytic reactions during cell lysis), as opposed to their Gram+ counterparts, which are proposed to bind to one site

and have a very low off-rate (Loessner 2002; Schmelcher 2010). This feature of Gram+ targeting endolysins may provide a further safety advantage when used in food, because chelation to the cell wall would act to restrict distribution of the enzyme and shorten the duration of technical effect after binding.

Individual Endolysin Amino Acid Sequences

Six (6) recombinant lysins are described as candidate components of the ENDOLYSIN product, to be used either singly or in combination. Additional lysins may be included in the product at a future date provided they meet specification. The amino acid sequence of each of the current lysins listed in Table 2-1 is provided below; numbers in parentheses following each named lysin are the number of amino acids (aa) in each protein.

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

PlyCP26F (212 aa)

MIIGSRYGHSENCRGAKGLRDEVDAMKPLHFEFKKIMEQYGHTIIDCCSNANTQNGELSEGARKANAQILDLFISWHGNKGGGQGCEA
WIANNSRAKPYAERMCKNFSSLGFKNRGVKYSDKYYEMRNINAPNIIFETLFLDSEKDISIWSPIPYEVMARYLANAIDPNIPLEKEQDYYR
VCVQRFTNKEDAEKAQQRISNELGYYCFAEKI

PlyCP39O (213 aa)

MKIALRGGHSPNCKGANVLRDEQSCMWALADEVEKVLTSHGHTVVRCETTLSNEREDVRQGAKKGYNCDMFISLHMNASDGRGNGT
EAWVARSARSSIKEIASRLCKNYATLGLQNRGVKEKNYWEMTDTNCPNIIFETMFCDDKHDIDIWASTSWDKLARLIANAIDPNIPLEKE
QDYYRVCVQRFTNKEDAEKAQQRISNELGYYCFAEKI

Psm (342 aa)

MQSRNNNNLKGIDVSNWKGNINFESVKNDGVEVVYIKATEGNYFKDKYAKQNYEGAKEQGLSVGFYHFFRANKGAKDQANFFIDYLN
EIGAVNYDCKLALDIETTEGVGVRDLTSMCIEFLEEVKRLTGKEVVVYTYTSFANNNLDSRLGNYPVWIAHYGVNTPGANNIWSSWVGF
QYSENGSVAGVNGGCDMNEFTEEIFIDSSNFNLDNATTKNVSTKLNIRAKGTTNSKIIGSIPAGETFKIKWVDEDYLGWYYVEYNGVVGY
VNADYVEKLQMATTYNVSTFLNVREEGSLNSRIVDKINSGDIFRIDWVDSDFIGWYRITTKNGKVGFVNAEFVKKL

CP25L (377 aa)

MYINQSNIKFNGLRYGNDPNKIIIHNADATSCSVYDIDRWHKGNGWSGIGYDYFIRKEGSVWTGRPENAIGAHTIGQNSSSIGICLEGAF
MREKPTRAQLNSLYELIADIRARRGNLPVYGHKDFNNTDCPGINFPLEQFKNNSYRPTGGEIVSDNGFYRSDEERTNATIVGEGNIEVLDK
NCKVIENRYISSLDRVFVLGIYPASKYIEIIYPAGNEKYHAYISIENYSRISFDYHMQYKNDNGVTYVWWDSEDVNVKEHNEELQANQKAS
PMYRVGKWLRVTFYRTDGTPSDGFVRYEGEQAVKFYEEEKIKEGIVKVNTYLNVRDSINGNIIGKVFNGEEVSIIWTKDGWYYIDYNTNH
GKKRGYVSSKYVEEV

ZP173 (335 aa)

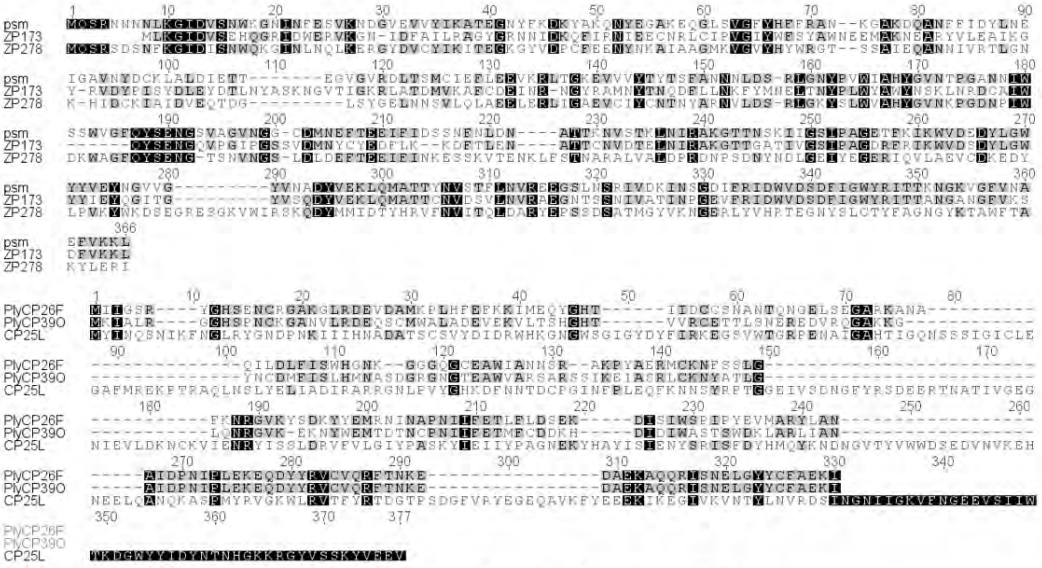
MLKGIDVSEHQGRIDWERVKGNIDFAILRAGYGRNNIDKQFIRNIEECNRLCIPVGIYWFSYAWNEEMAKNEARYVLEAIKGYRVDYPISY
DLEYDTLNYASKNGVTIGKRLATDMVKAFCDEINRNGYRAMNYTNQDFLLNKFYMNELTNYPLWYAWYNSKLNRDCAIWQYSENGQ
VPGIPGSSVDMNYCYEDFLKKDFTLENATTCNVDTELNIRAKGTTGATIVGSIPAGDRFRIKWVDSDYLGWYYIEYQGITGYVSQDYVEKL
QMATTCNVDSVLNVRAEGNTSSNIVATINPGEVFRIDWVDSDFIGWYRITTANGANGFVKSDFVKKL

ZP278 (351 aa)

MQSRSDSNFKGIDISNWQKGINLNQLKERGYDVCYIKITEGKGYVDPCFEENYNKAIAAGMKVGVYHYWRGTSSAIEQANNIVRTLGNK
HIDCKIAIDVEQTDGLSYGELNNSVLQLAEELERLIGAEVCIYCNTNYARNVLDSRLGKYSLWVAHYGVNKPGDNPIWDKWAGFQYSEN
GTSNVNGSLDLDEFTEEIFINKESSKVTENKLFSTNARALVALDPRDNPSDNYNDLGEIYEGERIQVLAEVCDKEDYLPVKYWKDSEGRES
GKVWIRSKQDYMMIDTYHRVFNVITQLDARYEPSSDSATMGYVKNGERLYVHRTEGNYSLCTYFAGNGYKTAWFTAKYLERI

Although these plant-expressed endolysins are distinct proteins, they exhibit partial domain similarities as indicated in the ClustalW primary amino acid sequence alignment shown in Figure 2-1.

Figure 2-1. Primary amino acid sequence alignment of plant-expressed endolysins



	psm	ZP173	PlyCP26F	PlyCP39O	ZP278	CP25L
psm		47%	10%	8%	32%	14%
ZP173	47%		8%	7%	17%	10%
PlyCP26F	10%	8%		56%	6%	7%
PlyCP39O	8%	7%	56%		6%	6%
ZP278	32%	17%	6%	6%		12%
CP25L	14%	10%	7%	6%	12%	

In Figure 2-1, the degree of sequence identity among lysins is indicated by shading (top) and as % homology in table (bottom).

Quantitative Composition

ENDOLYSIN 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 or addition to food. The dry formulation can be dissolved in water or other compatible aqueous medium (e.g. broth) for application to or addition to food.

The bulk product is formulated as a partially purified protein extract containing a minimum of 70 wt% lysin protein(s) relative to total protein. The lysins can be produced from edible expression host plants, such as spinach (*Spinacia oleracea*), chard (*Beta vulgaris* subsp. vulgaris) or beetroot (*Beta vulgaris*). They can also be produced in a non-food species host, for example *Nicotiana benthamiana*. If produced in the latter, the endolysin extract is further purified to reduce host impurities to non-toxic levels prior to product formulation. Information on lysin manufacturing is found in APPENDIX B; purification details are found in APPENDIX C.

ENDOLYSIN is dissolved/diluted in water/aqueous medium to a concentration of 0.5 – 2.5 mg/mL (500 – 2,500 mg/L) for spray application at a rate not to exceed 20 mL solution/kg (9 mL/lb) of food product. Alternatively, cooked meats or other foods can be dipped in a solution of ENDOLYSIN at a concentration of 0.5 – 2.5 mg/mL (500 – 2,500 mg/L). ENDOLYSIN can also be added directly to food during their preparation, or mixed in sauces or gravies, at a rate not to exceed 10 mg/kg or /L of treated food.

Endolysins can be prepared singly or in combination. For mixtures, each lysin protein is manufactured separately and then combined in defined ratios. Formulations are selected based on the food application and the strain or serotype of *C. perfringens* targeted for control. That is, an ENDOLYSIN formulation consisting of a single component comprises only the specified lysin as the active ingredient. An ENDOLYSIN formulation consisting of two or more different lysins comprises two or more active ingredients having synergistic or additive potency or expanded host range. Regardless, the total amount of lysin protein(s) formulated in the ENDOLYSIN product to be applied to food is ≤10 mg/kg or /L (≤~4.6 mg/lb).

Modes of Action

Environmentally resistant spores of *C. perfringens* can germinate and contaminate foods post cooking under a wide range of conditions (Albrecht 2017). Bacteriophages or their endolysins can be used to control vegetative cells in cooked foods that are at risk of temperature abuse. Endolysins can be considered food treatment enzymes restricted to controlling pathogenic *C. perfringens* without affecting the properties of the treated food, or the normal commensal intestinal microbiome. Lysins that target Gram+ bacteria such as *Clostridium spp* have a catalytic domain and a cell wall binding domain. Although the catalytic or enzymatically active domain (EAD) of some lysins shows specificity for cell wall components, much higher specificity, even at the serovar-level, is typically conferred through the cell wall binding domain (CBD). In some lysins, the binding affinity of the CBD to cell wall components is in the pico- to nano-molar range, which matches or exceeds the affinity of antibodies to their antigens (reviewed by Schmelcher (2012)). Currently there are at least four groups of lysins that can be formulated in ENDOLYSIN, and each can be characterized on the basis of the cleavage site of their catalytic domain that acts on bacterial cell wall structures. These include **lysozymes, glycosidases, amidases** and **endopeptidases**. Specifically:

1) N-acetylmuramidases (lysozymes) cleave the β-1-4-glycosidic bond between NAG-NAM;
2) N-acetyl-β-D-glucosaminidases (glycosidases) cleave β-1-4-glycosidic bond between NAM-NAG;
3) N-acetylmuramoyl-L-alanine amidases cleave the amide bond between sugar and peptide
4) L-alanoyl-D-glutamate endopeptidases and interpeptide bridge-specific endopeptidases cleave the peptide moiety of the cell wall peptidoglycan.

In the classification above, "NAG" stands for N-acetyl-glucosamine and "NAM" refers to N-acetyl-muramic acid; both are key structural components of bacterial cell walls that are attacked by lysins.

2.2 Method of Manufacture

Notifier uses a **plant-based manufacturing process** for producing ENDOLYSIN proteins; the method is an adaptation of the process used to manufacture biopharmaceuticals, which have been administered in 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, and meats, as described in GRN 593 and GRN 676, respectively.

The lysins are produced using recombinant technology to yield concentrated extracts at two different purities depending on the expression host plant. Favored host plants include the food species **spinach** (*Spinacia oleracea*), **red beet** (*Beta vulgaris*), **lettuce** (*Lactuca sativa*), and the non-food species ***Nicotiana benthamiana***, which is the most widely used host for plant-based production of biologics and biochemicals.

The lysin protein manufacturing process is described in APPENDIX B. The plant-derived biomass remaining after lysin protein extraction is treated and discarded (disposed) and is not used as a human food or animal feed product, additive or supplement.

2.3 Composition and Specification

Characteristic Properties

Notifier's ENDOLYSIN 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). Comparisons of maturation events between plant and bacterial phage lysins (when information for the latter was available) revealed that the mature plant- and phage-derived proteins are equivalent in amino acid sequence, while some plant-made proteins contain N-terminal acetylation. No truncations or deletions are evident from analyses.

Importantly, the full-length amino acid consensus sequences responsible for the structure, activity and safety of the endolysins have been retained in every case. Therefore, the proteins produced recombinantly in plants share the amino acid sequences of naturally occurring bacteriophage lysins, as reported in the literature and as verified from published gene and protein accession databases. Molecular characterization of plant-made lysins was accomplished by advanced mass spectrometry methods, described in APPENDIX C.

Lack of glycosylation

Native endolysin proteins naturally produced by bacteriophages are non-glycosylated. When produced in plants, none of the lysins have glycan addition sites along their backbone and therefore the plant-produced proteins are also non-glycosylated polypeptides.

Being simple polypeptides without a glycan coat or multiple disulphide bridges, endolysins 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 (Nelson 2012; Schmelcher 2012). Hence, very low residual levels of active lysins are expected from ingestion of treated foods.

Low immunogenicity

Being proteins, lysins are capable of inducing immune responses in mammals when administered systemically or mucosally. However, the pervasive exposure to phages and their subunits throughout evolution has yielded a very mild immune response in mammals including humans (Barr 2017). The formation of anti-lysin antibodies occurred in studies that administered lysins buccally or intravenously to observe their therapeutic antibacterial effects (Fischetti 2005, 2010; Hermoso 2007; Jado 2003; Loeffler 2003). Even when the lysins were administered at therapeutic doses, the anti-lysin antibodies did not reduce the efficacy of the therapy (i.e. the antibodies induced were not drug-neutralizing; Jado (2003), Loeffler (2003)).

The immune system can react differently to a protein when the potential immunogen is administered in one or a few high doses (acute and sub-acute exposure) versus lower doses administered over longer periods (chronic low doses). To comprehensively assess risk, Notifier studied at the molecular level the allergenic potential of candidate lysins for use in food and determined, from published information, that lysins have a low potential for inducing immune or allergic responses. Allergenic potential is discussed in Section 6.5.

Formulation

ENDOLYSIN is provided as a concentrated solution or as a dry powder at a purity of ≥70 wt% lysin protein/total protein.

The product is dissolved and/or diluted in water or other aqueous medium (e.g. broth) according to instructions and applied as a spray, dip, or directly mixed in food, gravy, sauces, etc. depending on the intended use.

Content of Potential Human Toxicants in ENDOLYSIN

None if endolysins are produced in food species plants. If produced in *N. benthamiana*, traces of host alkaloids should be monitored and controlled in the final product to ensure non-toxic levels in food.

Specification

The target Specification for ENDOLYSIN dry powder is presented in Table 2-2. The manufacturing process to produce a bulk product with this specification is described in APPENDIX B.

Table 2-2. Target Specification of ENDOLYSIN Product

Target Specification of ENDOLYSIN Product			
Parameter	**Method**	**Specification limit**	**Results of analyses***
Appearance	Visual	Powder, white to beige	Conforms
Specific Activity (endolysin protein basis)	Viability inhibition of *C. perfringens* strain ATCC13124 (NCTC8237) with lysins Psm, CP25L, ZP178, ZP278; strain NCTC11144 for lysins Ply26F, and Ply39O	≥2 $\Delta\log_{10}$ (lysins psm, CP25L, ZP173 and ZP278) ≥1 $\Delta\log_{10}$ (lysins PlyCP26F and PlyCP39O)	≥3 $\Delta\log_{10}$ (lysins psm, CP25L, ZP173 and ZP278) ≥1 $\Delta\log_{10}$ (lysins PlyCP26F, and PlyCP39O)
pH of a 1% solution	Potentiometric	6.5-8.5	7.5 ± 0.5
Heavy metals, total (sum of Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, Sn)	USP38<233> ICP-OES	≤ 30 ppm	< 1 ppm
Heavy metals: Lead	USP38<233> ICP-OES	≤ 5 ppm	< 1 ppm
Heavy metals: Cadmium	USP38<233> ICP-OES	≤ 5 ppm	< 1 ppm
Nicotine (per total lysin blend)**	HPLC/MS	≤ 90 ng/mg endolysin	Ave. 76 ng/mg
Anabasine (per total lysin blend)**	HPLC/MS	≤ 15 ng/mg endolysin	Ave. 2.1 ng/mg
Bioburden	USP32<61>	≤ 10 CFU/25 g sample	0 (absent)
Agrobacterium (vector) (CFU/10 g sample)	Selective plate-based assay	0 (absent)	0 (absent)

Undesirable microorganisms: *Escherichia coli, P. 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)	Specific activity at T_n vs. T_0; plate- based assay	> 6 months	In progress. Accelerated testing showed >9 month stability in solution

*Results of analyses for a dry ENDOLYSIN (mixed-endolysin) product are based on average results obtained from analyses of individual endolysin proteins blended at equal ratios (dwb). **Specifications for host alkaloids nicotine and anabasine are included only for endolysins produced in *Nicotiana benthamiana* and are omitted for endolysins produced in food plant species hosts that do not harbor these alkaloids.

2.4 Technical Effect and Suitability of Use

In this section, we describe the efficacy and suitability of ENDOLYSIN as an antimicrobial for cooked meats as examples of *C. perfringens*-susceptible products, as well as the duration of ENDOLYSIN's technical effect.

2.4.1 Biological activity of ENDOLYSIN on target pathogenic *C. perfringens* strains

Structure and mechanism of action underlying potency

Endolysins are a group of antimicrobial proteins produced by Gram-positive-tropic bacteriophages that specifically target bacteria from the Genus *Clostridium*, including pathogenic strains of *C. perfringens* that are associated with food-borne gastroenteritis. Endolysins (phage lysins) are phage-encoded peptidoglycan hydrolases (PGHs) employed by the majority of bacteriophages to enzymatically degrade the peptidoglycan (PG) layer of the host bacterium `from within' at the lytic or terminal phase of their multiplication cycle. PG functions as the major structural component of the bacterial cell, supporting an internal turgor pressure of 20–50 atmospheres in the case of Gram+ organisms (reviewed in-depth by Schmelcher (2012)).

Breach of the PG layer results in osmotic lysis and cell death of the bacterium, enabling liberation of progeny virions. Host cell lysis is strictly regulated and exactly timed by the virion. This process takes time. Importantly for the purpose of this Notice, endolysins are also capable of killing susceptible organisms when applied exogenously as recombinant proteins. For this reason, they have attracted increasing interest as potential antimicrobial agents, particularly in light of emerging and spreading resistance of bacteria against classical antibiotics.

Notifier's ENDOLYSIN product consists of one or more (blend) individually produced phage lysins that act alone or synergistically to destroy the cell wall of pathogenic strains of *C. perfringens*. Because simultaneous attack of multiple components within the Gram+ cell wall architecture lead to more rapid destruction, the potency of lysin mixtures against certain strains can be higher than the potency of individual lysins. Also, lysins have overlapping activity optima depending on pH, temperature and salinity; hence, mixtures of lysins can offer more consistent protection of food in multiple preparation and storage environments.

Range of activity of plant-produced endolysins against pathogenic strains of *C. perfringens*

Endolysin proteins were evaluated for bactericidal activity at the levels of purity expected for the commercial ENDOLYSIN product. Examples of biological activity in intended applications ("suitability") of plant-produced endolysins in controlling the growth of *C. perfringens* are provided.

Plant-produced endolysins were evaluated for activity against *C. perfringens* strains relevant to food contamination, using laboratory settings as well as treatment of intended foods such as cooked beef and poultry cuts. *Clostridium perfringens* can be found as a commensal member of the human intestinal microflora (Todar 2012). However, ingestion of Type A strains capable of producing enterotoxin (i.e. *C. perfringens* enterotoxin, or CPE) leads to pathology. Presumably, *C. perfringens* lytic phages and their lysins would be also found in the human gastrointestinal tract.

Clostridium perfringens strains used in these studies were chosen either by their characteristics (Type A, CPE+), or by clinical relevance (i.e. strains isolated from food or patient feces during food poisoning outbreaks). Strain characterization differed in some cases among culture collections where the test strains were obtained. For example, some strains attributed to Type A by NCTC are attributed to Type D by ATCC. Regardless, all strains evaluated in our studies are known human pathogens.

Table 2-3 lists the target test strains of *C. perfringens* evaluated.

Table 2-3. Human pathogenic strains of *C. perfringens* used to evaluate ENDOLYSIN efficacy

No.	Strain	Isolated from	Characteristics	Reference if available
1	NCTC8237 (ATCC 13124)	-	Type A. a-toxigenic, cpa and pfoA genes present. Type strain.	(Eastoe 1959)
2	NCTC8235 (ATCC1922)	Stew	Type A, cpa and cpe genes present.	(Hobbs 1953)
3	NCTC8239 (ATCC 12917)	Boiled salted beef	NCTC: Type A: a-toxin gene positive. Contains a fragment of the enterotoxin gene. ATCC: Type D: Heat resistance of spores. Agglutinating type 3. The presence of cpa, etx, and cpe genes was confirmed by PCR.	(Hobbs 1953)
4	NCTC8679 (ATCC 12920)	Human feces, food poisoning	Type A. a-toxin gene positive. Contains a fragment of the enterotoxin gene.	(Hobbs 1953)
5	NCTC9851 (ATCC 12925)	Braised heart	Type A. Agglutinating type 11 The presence of cpa and cpe genes was confirmed by PCR.	(Hobbs 1953)
6	NCTC11144	Beef, food poisoning outbreak	Type A.	(Hobbs 1953)
7	NCTC8359 (ATCC 12915)	Stewed steak	Type A. The presence of cpa and cpe genes was confirmed by PCR.	(Hobbs 1953)
8	NCTC8799 (ATCC 12924)	Roast meat	Type A. The presence of cpa and cpe genes was confirmed by PCR.	(Hobbs 1953)

No.	Strain	Isolated from	Characteristics	Reference if available
9	NCTC10613	Minced beef, food poisoning	Type A.	-
10	NCTC8238 (ATCC 12916)	Boiled, salted beef	Type A. The presence of cpa and cpe genes was confirmed by PCR.	(Hobbs 1953)
11	NCTC8449 (ATCC 12921)	Steamed lamb	Type A. a-toxin gene positive. This isolate also contains a fragment of the enterotoxin gene	(Hobbs 1953)
12	NCTC8797	Salted beef	Type A. a-toxin gene positive. This isolate also contains a fragment of the enterotoxin gene	(Hobbs 1953)
13	NCTC8798	Meat rissole, food poisoning outbreak in a school	Type A. a-toxin gene positive. This isolate contains a fragment of the enterotoxin gene	(Hobbs 1953)
14	NCTC10239 (ATCC 14809)	Rissoles	NCTC: Type A. a-toxin gene positive. This isolate also contains a fragment of the enterotoxin gene. ATCC: Type D. The presence of cpa, etx, and cpe genes was confirmed by PCR.	(Hobbs 1953)
15	NCTC10240 (ATCC 1481)	Chicken	Type A. a-toxin gene positive. This isolate also contains a fragment of the enterotoxin gene	(Hobbs 1953)
16	NCTC8678 (ATCC 12919)	Human feces, food poisoning	NCTC: Type A: Epsilon toxin – ve Agglutinating type 5 ATCC: Type D: The presence of cpa, etx, and cpe genes was confirmed by PCR.	(Hobbs 1953)
17	NCTC10614	Human feces, food poisoning case	Type A.	(Hobbs 1953)
18	NCTC10612	Human feces, food poisoning outbreak	Type A.	(Hobbs 1953)
19	NCTC10611	-	Type A.	(Hobbs 1953)
20	NCTC8247 (ATCC 12918)	Human feces	Type A. a-toxin gene positive. This isolate also contains a fragment of the enterotoxin gene	(Hobbs 1953)
21	NCTC10578	-	Type A.	-
22	NCTC2837	-	Type A. a-toxin gene positive.	-
23	NCTC6785 (ATCC 10873)	-	Type A. a-toxin gene positive.	(Epps 1945)

No.	Strain	Isolated from	Characteristics	Reference if available
24	DSM11778	Boulette (Hamburger)	Type A. beta haemolytic	-
25	DSM11779	Boulette (Hamburger)	Type A, beta haemolytic	-
26	DSM11780	Human feces	Type A, beta haemolytic	-
27	DSM11781	Boulette (Hamburger)	Type A, beta haemolytic	-
28	DSM11782	Human feces	Type A, beta haemolytic	-
29	DSM11783	Human feces	Type A, non-haemolytic	-
30	DSM2943 (ATCC 25768)	-	Lechithinase-negative The presence of the cpa gene was confirmed by PCR.	(Nakamura 1976)
31	DSM798 (ATCC 10543)	-	The presence of cpa, cpb2, and pfoA genes was confirmed by PCR.	(Boyd 1948)

A. Efficacy of semi-purified plant-made endolysins

Crude protein extracts of plant-expressed lysins were evaluated for antimicrobial activity first. Results of this evaluation demonstrate that plant-produced lysins could efficiently eradicate *C. perfringens in vitro*.

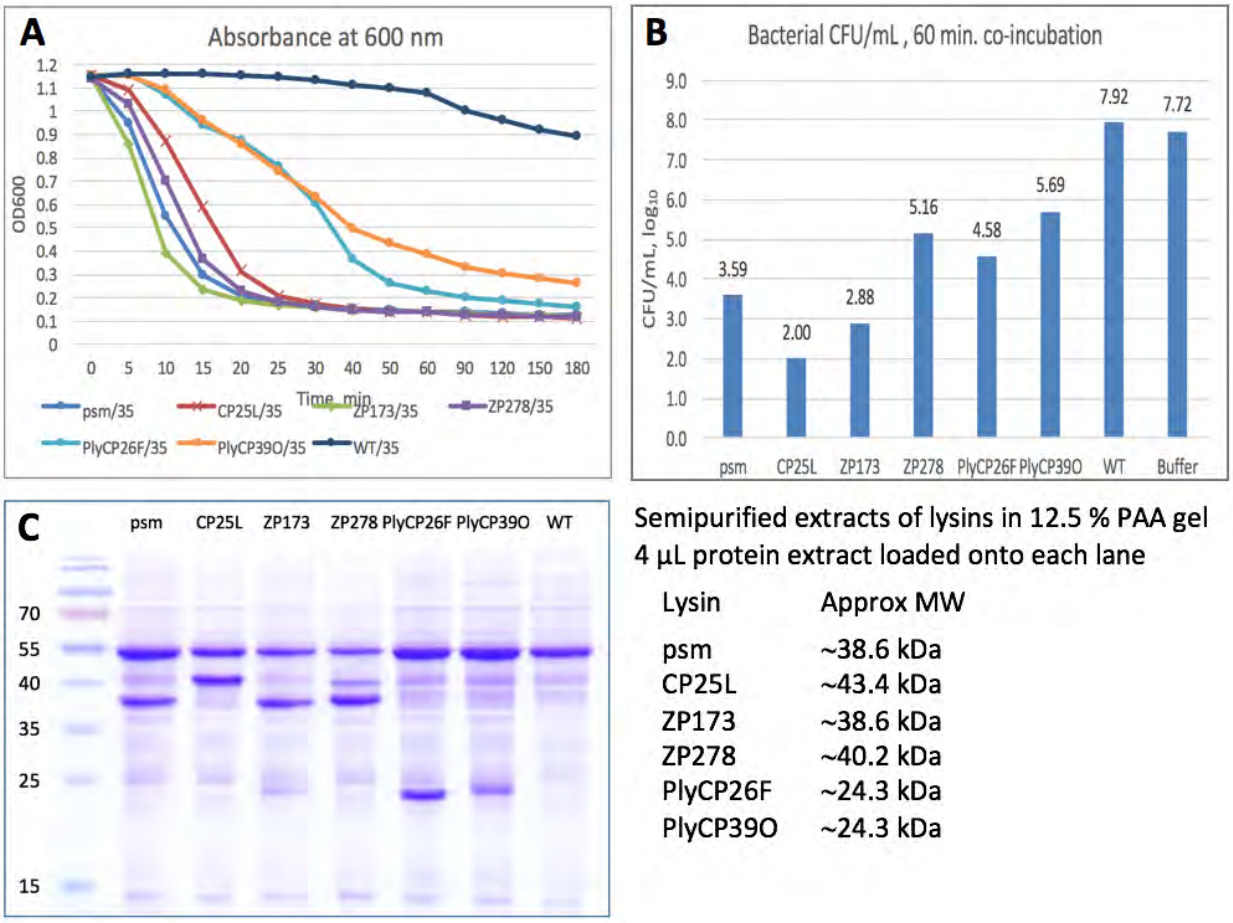
Antimicrobial activity of *N. benthamiana*-expressed lysins against *C. perfringens* NCTC8237. *Nicotiana benthamiana* was used as one of the expression hosts to produce lysin-containing plant extracts. The antimicrobial activity of cytosol-expressed lysins psm, CP25L, ZP173, ZP278, Ply26F and Ply39O was analyzed.

As described in APPENDIX B, protein extracts were prepared from agro-infiltrated or agro-sprayed *N. benthamiana* plants using buffer #35 (50 mM Na phosphate, 150 mM NaCl, 5 mM DTT, pH 7.5). *Clostridium perfringens* type strain NCT8237 was grown in TSB under anaerobic conditions to OD_{600}=0,803, resuspended in 1xPBS, pH 7.3. A volume of 950 µL of bacterial suspension mixed with 50 µL protein extract and incubated at RT.

Serial dilutions of analyzed samples were done in 1xPBS pH7.3 and 30 µL of each sample plated on TSA plates: Undiluted, and diluted 10, 10^2, 10^3, 10^4, 10^5 and 10^6 times. Plate images for scoring were taken following overnight incubation under anaerobic conditions at 37 °C.

Results are shown in Figure 2-2.

Figure 2-2. Efficacy of semi-purified plant-made endolysins against *C. perfringens* NCTC8237



In Figure 2-2, **Panel A** shows the change in turbidity of a suspension of *C. perfringens* type strain NCTC8237 as a function of incubation time in the presence of semi-purified plant extracts of individual lysins. Drops in turbidity indicate cell lysis (bactericidal effect). **Panel B** shows bacterial CFU/ml after 60 min incubation, relative to plant extracts with no lysins (WT) and buffer controls. **Panel C** shows the relative purity and expression levels of each of the lysins evaluated.

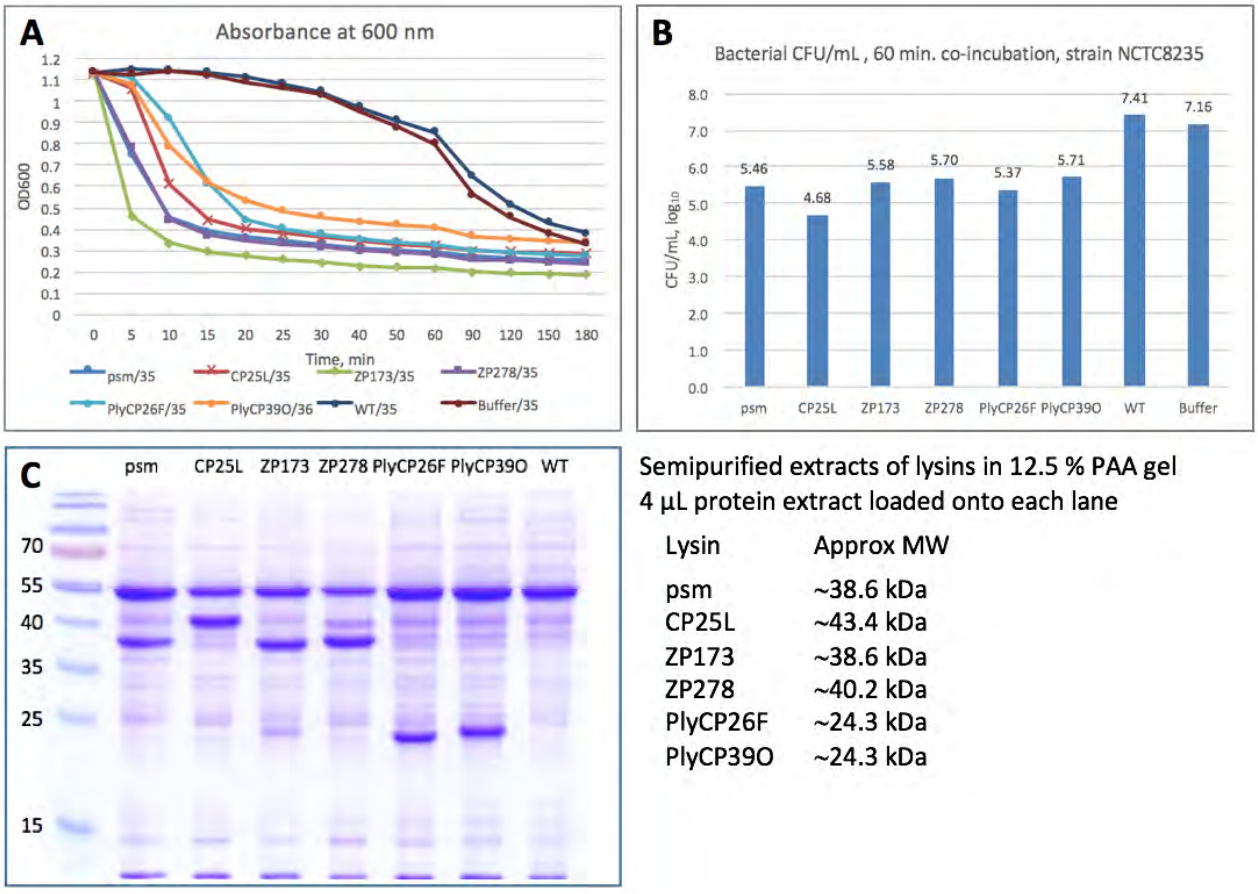
Antimicrobial activity of *N. benthamiana*-expressed lysins against *C. perfringens* NCTC8235. *Nicotiana benthamiana* was used as the expression host to produce semi-purified lysin extracts. The antimicrobial activity of cytosol-expressed *C. perfringens* lysins psm, CP25L, ZP173, ZP278, Ply26F and Ply39O was analyzed.

As described in APPENDIX B, protein extracts prepared from agro-infiltrated *N. benthamiana* plants using buffers #35 (50 mM Na phosphate, 150 mM NaCl, 5 mM DTT, pH 7.5) and #36 (50 mM Na phosphate, 200 mM NaCl, 5 mM DTT, pH 7.5). *C. perfringens* NCT8235 was grown in TSB under anaerobic conditions to OD_{600}=0,850, resuspended in 1xPBS, pH 7.3.

A volume of 950 µL of bacterial suspension mixed with 50 µL protein extract and incubated at RT. Serial dilutions of analyzed samples were done in 1xPBS pH 7.3 and 30 µL of each sample plated on TSA plates: Undiluted, and diluted 10, 10^2, 10^3, 10^4, 10^5 and 10^6 times. Plate images for scoring were taken following overnight incubation under anaerobic conditions at 37 °C.

Results are shown in Figure 2-3.

Figure 2-3. Efficacy of semi-purified plant-made endolysins against *C. perfringens* NCTC8235



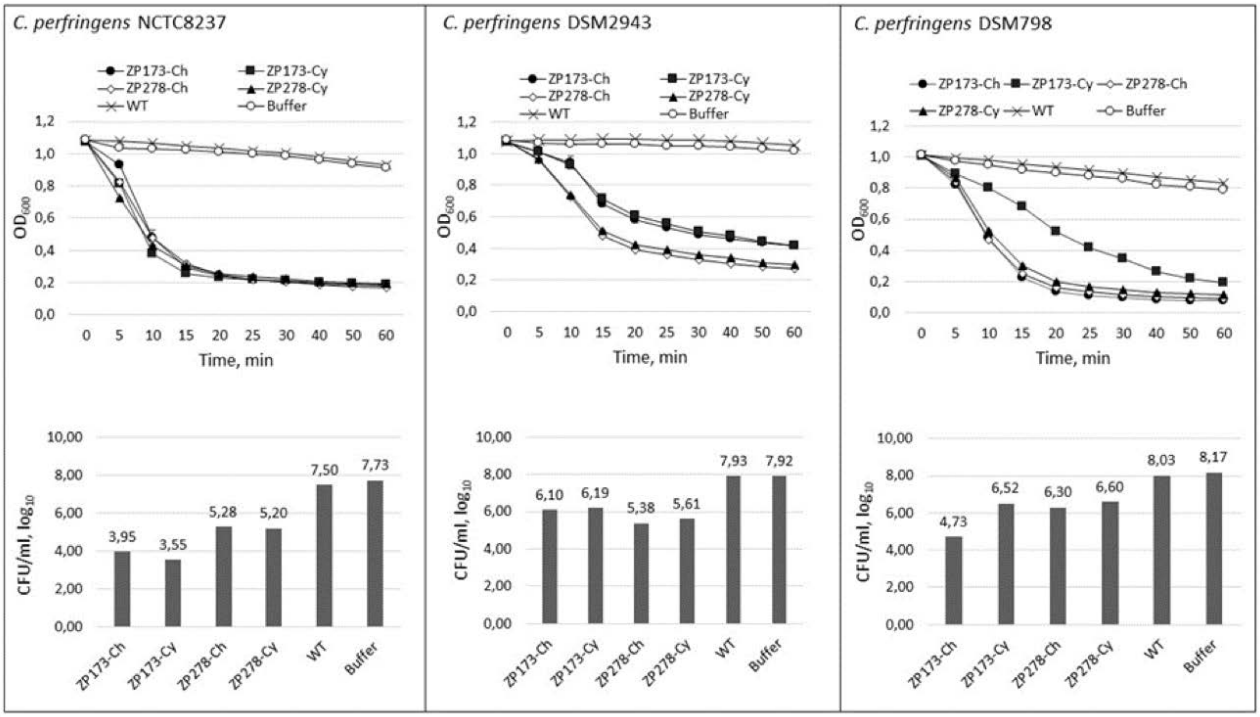
In Figure 2-3, **Panel A** shows the change in turbidity of a suspension of *C. perfringens* type strain NCTC8235 as a function of incubation time in the presence of semi-purified plant extracts of individual lysins. Drops in turbidity indicate cell lysis (bactericidal effect). **Panel B** shows bacterial CFU/ml after 60 min incubation, relative to plant extracts with no lysins (WT) and buffer controls. **Panel C** shows the relative purity and expression levels of each of the lysins evaluated.

Antimicrobial activity of spinach-expressed lysins against three *C. perfringens* strains. *Spinacia oleracea* (spinach) was used as the expression host to produce semi-purified lysin extracts. As described in APPENDIX B, protein extracts were prepared from agro-sprayed *S. oleracea* plants using standard buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 4.5) and diluted to ~1 µg/µL. Three type strains of *C. perfringens* were grown in TSB under anaerobic conditions to OD_{600} 0.7, and resuspended in 1xPBS, pH 7.3. A volume of 950 µL of bacterial suspension mixed with ~50 µL of protein extract was used for OD_{600} measurements and CFU enumeration. Serial dilutions for CFU determinations were done in 1xPBS pH 7.3 after 60 min. of co-incubation with the indicated lysins.

CFU were determined after overnight incubation under anaerobic conditions at 37 °C. Serial dilutions of analyzed samples were done in 1xPBS pH 7.3 and 30 µL of each sample plated on TSA plates: Undiluted, and diluted 10, 10^2, 10^3, 10^4, and 10^5 times. Plate images for scoring were taken following overnight incubation under anaerobic conditions at 37 °C.

The results are shown in Figure 2-4.

Figure 2-4. Efficacy of semi-purified spinach extracts of endolysins ZP173 and ZP278



In Figure 2-4, the three panels show bactericidal activity of spinach-expressed (CH = chloroplast-targeted; CY = cytosolic), semi-purified extracts of lysins ZP173 and ZP278 against three type strains of *C. perfringens*. **Left panel**, microbicidal activity against strain NCTC8237. **Center panels**, activity against strain DSM2943. **Right panel**, activity against strain DSM798. Activities are shown against plant extracts with no lysins (WT) and buffer controls.

B. Efficacy of purified plant-made endolysins

Purified plant-made lysins were evaluated for antimicrobial activity. Results of this evaluation demonstrate that plant-produced lysins could efficiently eradicate *C. perfringens in vitro*.

The results also demonstrated the possibility of using purified plant extracts for *C. perfringens* for food antimicrobial applications.

Activity of purified lysins against different target strains of *C. perfringens*. Individual lysins were manufactured as described in APPENDIX B and purified from plant extracts as described in APPENDIX C. The purified lysins were evaluated for antimicrobial activity against multiple strains of *C. perfringens* to determine their relative potency and host range.

The results of this initial screen are shown in Figure 2-5, displayed as a summary of the relative activity of the lysins against all strains tested.

Figure 2-5. Summary: Activity of purified endolysins against different strains of *C. perfringens*

Strain	Psm	CP25L	ZP173	ZP278	PlyCP26F	PlyCP39O
NCTC8237	6,519	4,163	7,026	3,570	1,314	0,288
NCTC8235	**2,996**	2,170	**2,996**	2,163	0,653	0,280
NCTC8239	**3,227**	2,082	**3,224**	**3,503**	0,814	0,945
NCTC8679	1,285	1,523	0,905	**1,489**	0,411	0,214
NCTC9851	**3,484**	1,528	**3,181**	**3,524**	1,054	0,686
NCTC11144	**4,203**	2,128	**4,239**	1,872	1,706	1,242
DSM11779	**4,184**	1,312	**4,281**	0,334	0,455	0,252
DSM11780	**4,906**	2,188	**4,807**	1,662	1,010	0,611
DSM11781	**3,179**	2,107	**3,184**	**2,981**	1,061	0,429
DSM11782	**2,088**	**2,405**	1,745	1,769	0,514	0,107
DSM11783	**4,391**	2,482	**4,090**	3,112	0,878	0,057
NC02837	**3,897**	2,509	**3,783**	3,157	0,690	0,042
NC06785	**4,499**	2,380	**4,515**	2,881	0,985	0,312
NC08238	**2,685**	**2,497**	**2,640**	**3,015**	0,961	0,881
NC08247	**3,998**	**3,601**	**3,795**	**3,458**	1,897	0,351
NC08359	**2,779**	1,124	**2,399**	1,897	0,130	0,006
NC08449	**3,395**	2,010	**3,042**	**3,054**	0,575	0,058
NC08678	2,280	1,601	2,117	**3,257**	0,793	0,127
NC08797	**4,377**	2,209	**4,174**	**4,294**	0,185	0,040
NC08798	**1,228**	**1,614**	**1,379**	**1,463**	0,513	0,257
NC10239	**2,111**	**1,808**	**2,005**	**2,038**	1,031	0,386
NC10240	**2,704**	**2,511**	**2,617**	**2,768**	0,645	0,195
NC10611	**3,634**	**3,804**	**3,647**	**3,688**	-0,021	0,139
NC10612	0,696	0,603	0,581	**2,056**	-0,149	-0,065
NC10613	**2,684**	**3,101**	**3,001**	**2,934**	0,580	0,391
NC10614	**3,959**	**3,906**	**4,080**	**4,346**	1,354	0,812

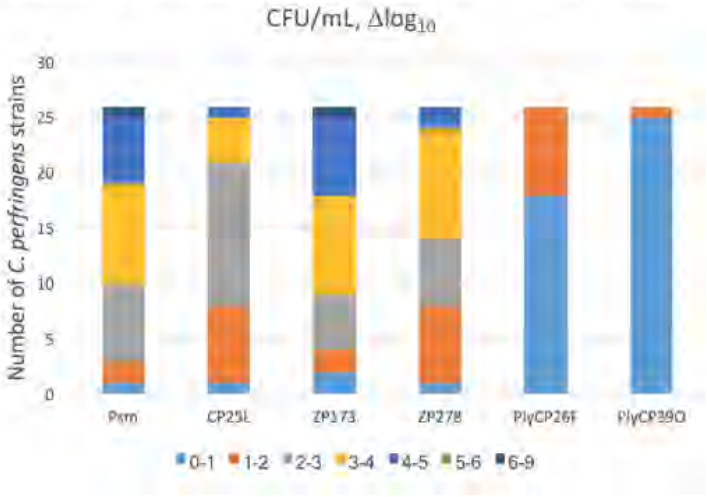
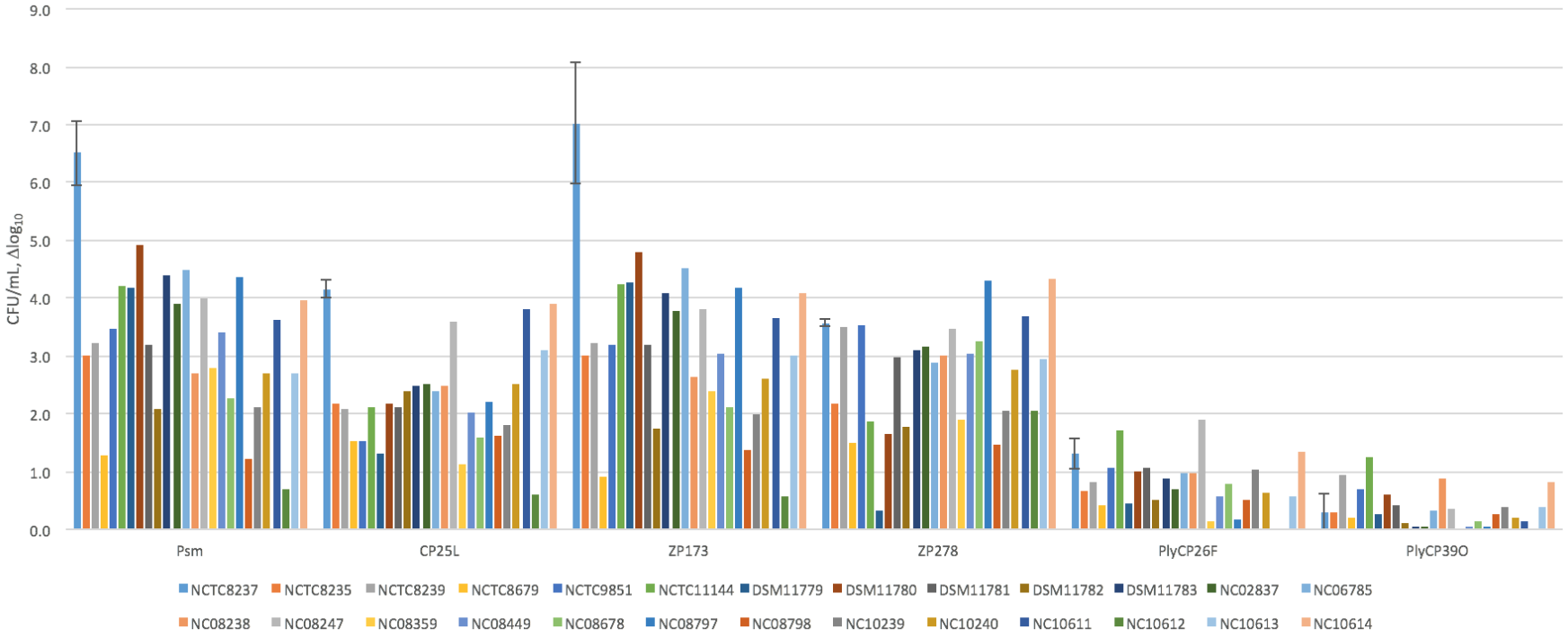


Figure 2-5 shows bactericidal activity of the purified plant-expressed lysins Psm, CP25L, ZP173 and ZP278. Results are averages of 3 independent experiments. Plant-made lysins are active against all 26 tested strains of *C. perfringens* shown in the table at top. The best performing lysins are highlighted in **bold** text. Activity as a function of host range is shown in the composite bar graph at bottom. *C. perfringens* strains were grown in TSB under anaerobic conditions to OD_{600}~ 0.8 and suspended in citrate-phosphate buffer, 50 mM NaCl, pH 5.5. Lysins were added to bacterial suspension at final concentration of 10 µg/ml. Serial dilutions for enumeration were done in PBS, pH 7.3 after 60 min. of co-incubation with lysins. All activities are expressed as $\Delta\log_{10}$ CFU/mL.

In this series, only purified lysins PlyCP26F and PlyCP390 showed low or no activity against the tested bacterial strains under conditions analyzed. All other lysins showed activity against multiple target strains.

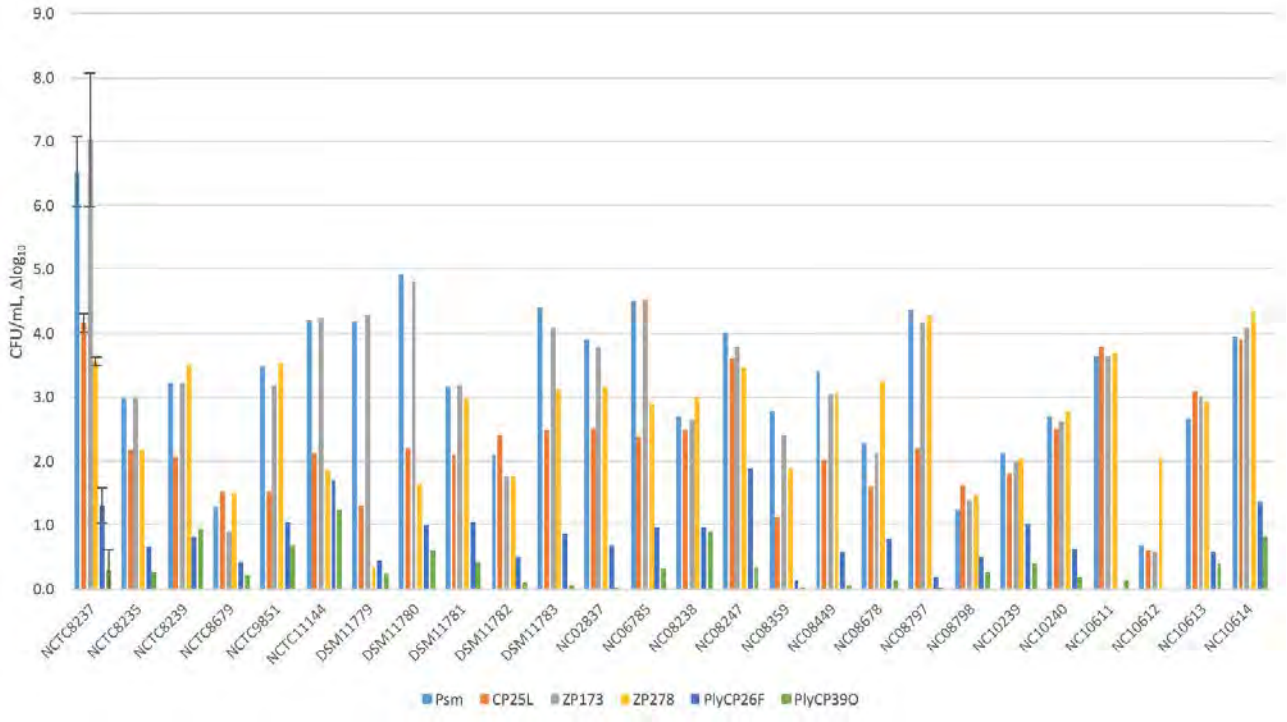
Activity and host range of purified endolysins against different target strains of *C. perfringens*. The deconvoluted results of antimicrobial activity against target strains of *C. perfringens* of each of the purified plant-made lysins are presented in Figure 2-6. Bars are $\Delta\log_{10}$ CFU relative to non-treated bacterial control; higher values mean higher potency.

Figure 2-6. Activity of purified plant-made endolysins against *C. perfringens* strains



Activity and host range of purified endolysins against different target strains of *C. perfringens*. The relative antimicrobial activity of six purified plant-produced lysins is shown against each of the indicated target strains of *C. perfringens*. Results are shown in Figure 2-7.

Figure 2-7. Relative efficacy *in vitro* of purified plant-made endolysins against *C. perfringens* strains



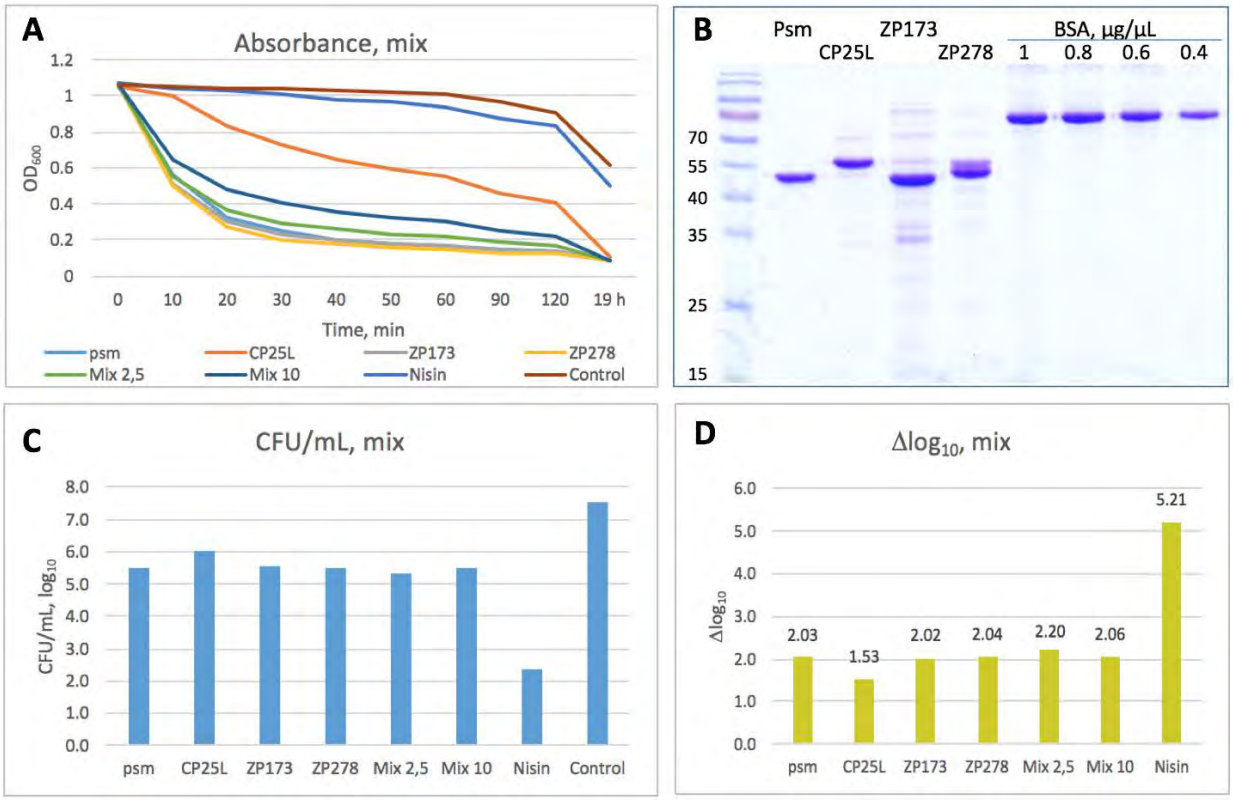
Activity of purified plant-made endolysins against a mixture of food-relevant *C. perfringens* strains. In this series, seven *C. perfringens* strains were grown separately in TSB. An equal amount of each strain was combined, centrifuged and resuspended in citrate-phosphate buffer with 256 mM (~1.5 %) NaCl, pH 5.5 to OD_{600}=1. A volume of 1 mL of bacterial suspension was combined with 10 µg purified lysin or 5 µg nisin (positive control) or a mix of Psm, CP25L, ZP173 and ZP278 lysins, 2.5 µg each or 10 µg each and incubated at RT. Nisin (Sigma Chemical) 5 mg/mL stock was prepared by dissolving powder in 0.02 N HCl.

Results are shown in Figure 2-8. In this first, non-optimized series, the indicated purified, plant-made lysins were evaluated against nisin as a positive control. Nisin is dependent on NaCl for enhanced activity. Hence, 1.5% NaCl (256 mM) was added to the test suspensions.

All lysins tested against *C. perfringens* individually and as lysin blends at 2.5 µg and 10 µg lysin protein were able to reduce the bacterial CFU/mL by approximately 2 logs. Nisin reduced bacterial CFU/mL by 5 logs. Note that although CFU values equilibrated by 19 hours, the speed of bactericidal effect was greater for lysin mixtures than it was for some individual lysins.

These results were used as a basis for optimization of mixtures of lysins and to study the effect of NaCl and other environmental variables, such as pH and temperature, on bacteriolytic activity of plant-made endolysins.

Figure 2-8. Lytic activity of plant-made individual endolysins and lysin mixtures against a mixture of 7 food-related *C. perfringens* strains



In Figure 2-8, **Panel A** shows the decrease in turbidity caused by cell lysis (bactericidal activity) over time for individual and blended lysins incubated with a suspension of *C. perfringens* comprised of equal amounts of 7 food contamination-relevant strains (NCTC No. 8235, 8239, 9851, 8449, 8797, 8798 and 10239). **Panel B** shows the relative purity of each lysin. **Panel C** shows bacterial growth of four lysins or mixtures of the four at two concentrations of protein, plus nisin (positive) and no-additive (vehicle) controls. The maximum differential ($\Delta\log_{10}$ CFU/mL; higher bars mean higher efficacy) growth inhibition for each group is shown in **Panel D**.

Efficacy of plant-made endolysins as a function of salinity. *C. perfringens* contaminates meats and savory dishes that typically have some level of salt. The pathogen is salt tolerant to a level of 5% NaCl (Albrecht 2017). The goal of this series was to assess efficacy and longevity of technical effect in the presence of NaCl. Defined media were used in this series to better control parameters.

Figure 2-9 summarizes the results of exposure of four plant-produced, purified lysins to varying concentrations of NaCl. The results show that several lysins (ZP173, ZP278, CP25L and PlyCP390) have higher antibacterial activity against *C. perfringens* in the presence of NaCl.

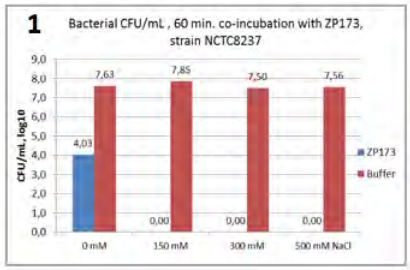
For ZP173, the presence of even 50 mM NaCl (0.09%) can increase potency by more than 2 logs, and that increasing concentrations of salt have no further impact on activity (either positively or negatively). For lysins ZP278 and CP25L, potency increases proportionately and peaks at 300 to 500 mM NaCl, with increases in activity of 3 logs (ZP278) to 6 logs (CP25L) over their respective non-lysin-treated bacterial growth controls. NaCl level did not have an appreciable effect on the activity of lysin PlyCP390.

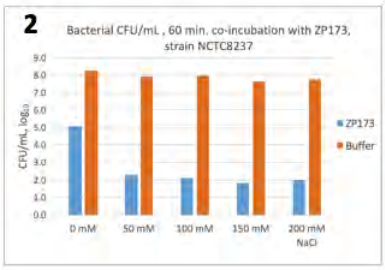
These results support the use of lysin mixtures with complementary salinity optima to ensure efficacy of the product when applied to various food matrices, especially with prepared foods.

Figure 2-9. Efficacy of plant-made endolysins as a function of salinity

Lysin ZP173

A

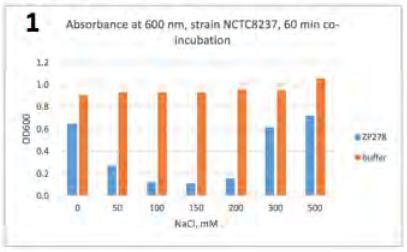


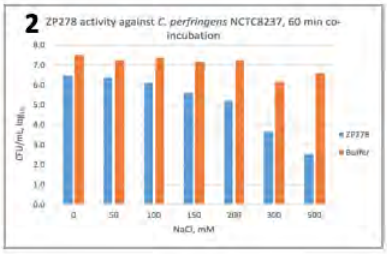


3 NaCl		Δlog vs buffer
Molarity	%	
0 mM	0	3.18
50 mM	0,.9	5.60
100 mM	0.58	5.84
150 mM	0.88	5.83
200 mM	1.17	5.78

Lysin ZP278

B

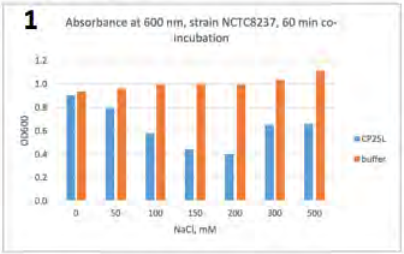


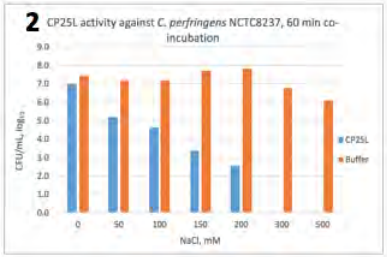


3 NaCl, mM	Δlog_{10}
0	1.03
50	0.85
100	1.26
150	1.57
200	2.02
300	2.52
500	4.09

Lysin CP25L

C

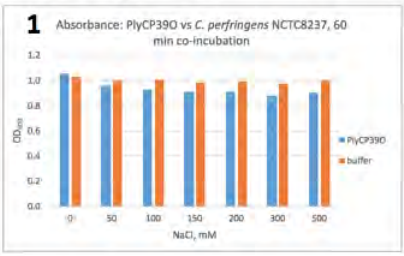


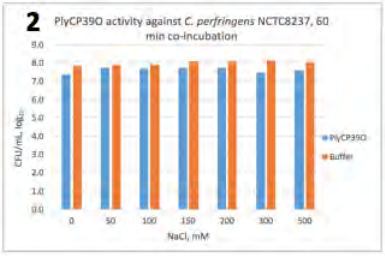


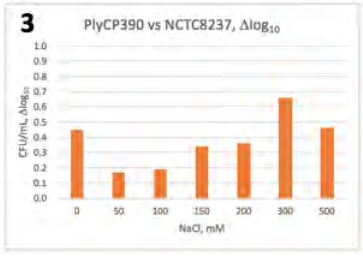
3 NaCl, mM	Δlog_{10}
0	0.47
50	2.01
100	2.55
150	4.31
200	5.27
300	6.75
500	6.06

Lysin PlyCP390

D



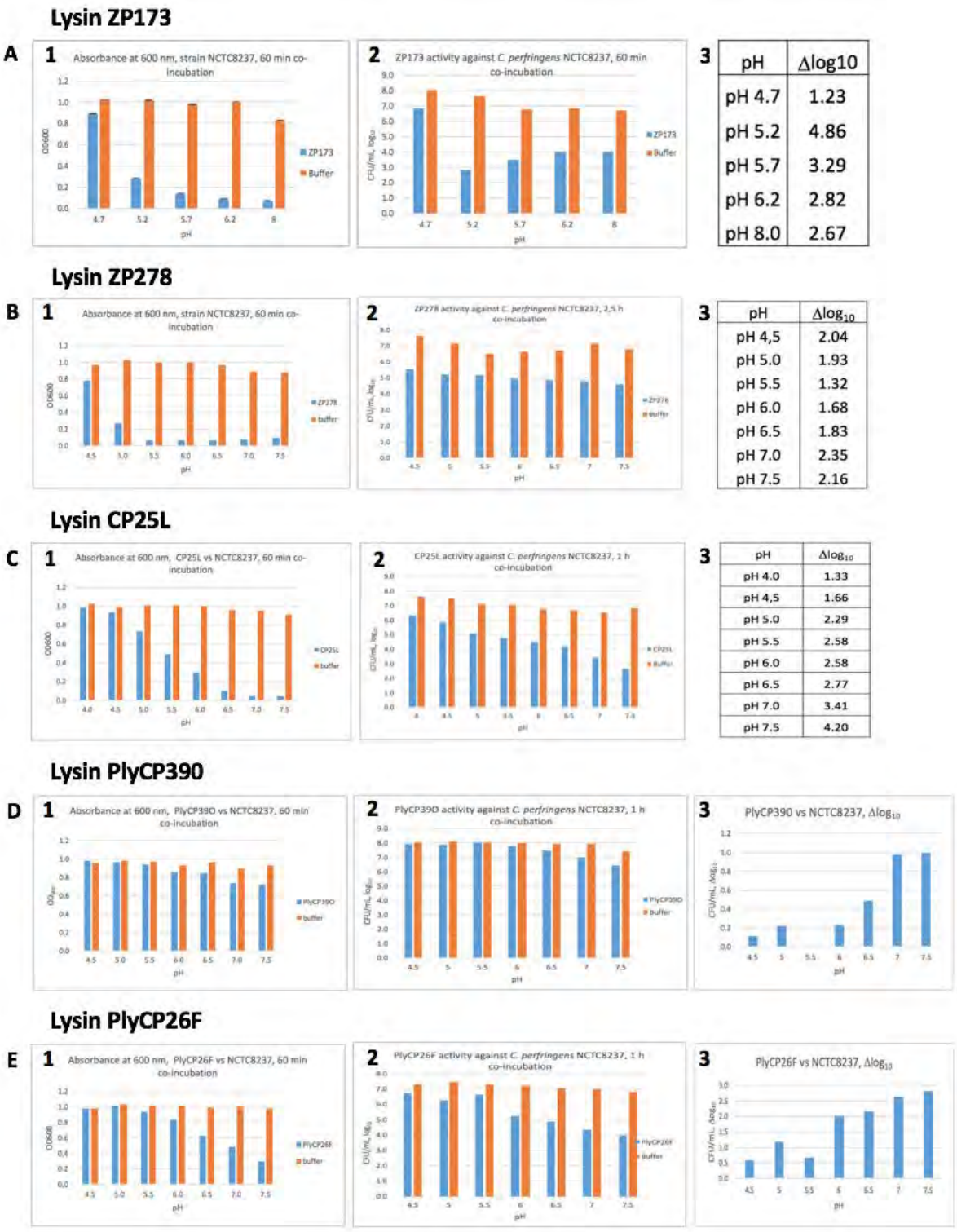




C. perfringens NCTC8237 type strain was grown in TSB anaerobically to approximately equal OD, centrifuged, resuspended in citrate-phosphate buffers of pH 5.5 supplemented with 0-500 mM NaCl. A volume of 1 ml bacteria was combined with 3 to 34 µg purified lysins (adjusted for potency) and incubated for 1 h at RT. Diluted, overnight cultures grown anaerobically at 37 °C were sampled for CFU/mL enumeration. **Rows A-D** show lysin evaluated. **Columns 1-3** show OD or CFU/mL, CFU/mL, and Δlog_{10} CFU/mL as a function of the indicated NaCl level, respectively.

Efficacy of plant-made endolysins as a function of pH. *C. perfringens* spores are resistant to environmental conditions, but the vegetative cells can grow at a pH range of 5-9. The antibacterial activity of lysins was assessed as a function of pH to determine efficacy in a food-relevant pH environment. Defined media were used in this series to better control parameters. Figure 2-10 summarizes these results.

Figure 2-10. Efficacy of plant-made endolysins as a function of pH



C. perfringens NCT8237 was grown in TSB under anaerobic conditions to predetermined OD_{600}, resuspended in citrate-Na phosphate buffers of pH 4.5-7.5 supplemented with 100 mM NaCl. Approximately 1 mL of bacterial suspension were mixed with the indicated lysins and incubated at room temperature for 60 min. Samples were serially diluted in 1xPBS pH7.3 and 30 µL of each sample plated on TSA plates, which were incubated at 37 °C overnight under anaerobic conditions prior to counting.

From the results summarized in Figure 2-10 it can be concluded that plant-made lysins show a pH range preference, as do most other enzymes. In the Figure, five lysins are evaluated as indicated in **Rows A-E** (namely, ZP173, ZP278, CP25L, PlyCP390 and PlyCP26F). Columns 1-3 show the respective efficacy: OD change as a result of net bacterial growth over lysis (**Column 1**), actual counts as log CFU/mL (**Column 2**), and net bactericidal activity as $\Delta\log_{10}$ CFU/mL relative to the group's bacterial growth control (**Column 3**).

These results show that ZP173 has a pH optimum of 5.2; ZP278 has highest activity at pH 4.5 with a relatively flat curve of activity from pH 5.0 to 7.5; and CP25L, PlyCP390 and PlyCP26F show highest activity towards neutral pH values (6.5 -7.5).

As with the salinity experiments, these results also underscore the value of using mixtures of lysins with complementary pH optima to ensure that there is effective bactericidal activity on various food matrices, especially with prepared foods.

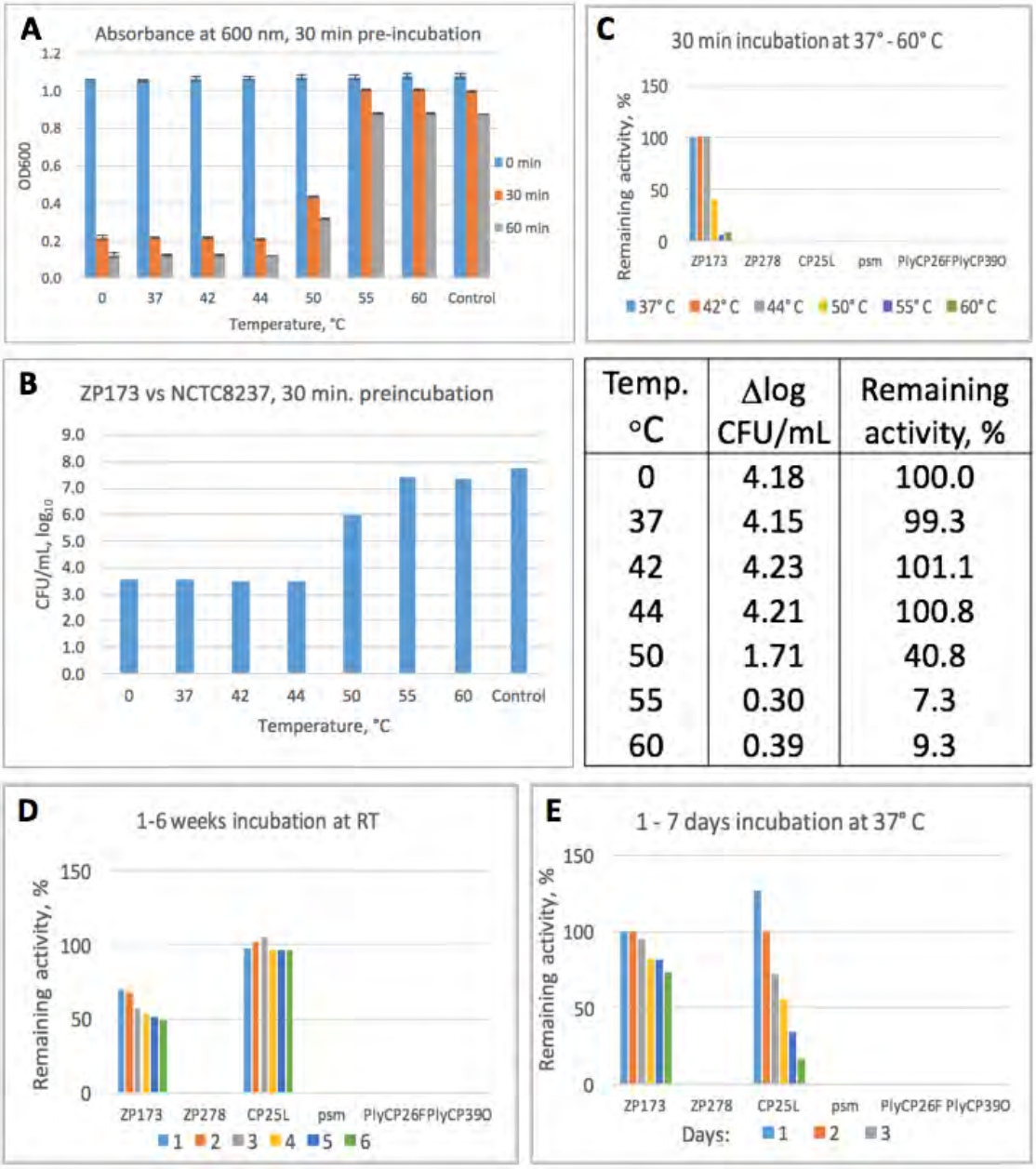
Impact of temperature on efficacy and stability of plant-made endolysins. *Clostridium perfringens* is a spore-forming anaerobic bacterium that can exist as a vegetative cell or as a dormant spore in food. Thorough cooking at a minimum of 60 °C (140 °F) will kill the vegetative cells, but spores may survive. At temperatures between 21 °C to 50 °C (~70 °F to ~120 °F), the spores can germinate into vegetative cells that produce enterotoxin. Germination of the spores and outgrowth into vegetative cells occurs in food inadequately refrigerated. Toxin production normally occurs in the intestinal tract. Although *C. perfringens* shows a remarkably wide temperature range for growth of between 15 °C and 55 °C (59 °F to 131°F), its optimum growth temperature is 43-47 °C (109-117°F) (Albrecht 2017).

In this series of experiments, various plant-made lysins were evaluated for antibacterial efficacy at different temperatures to assess compatibility with food preparation and storage practices. The focus was on thermal stability and retention of potency. Defined media were used in this series to better control parameters.

Two types of thermal stability and activity studies were conducted. The first evaluated **high-temperature** effects, to see if lysins could retain their activity if held at temperatures that would be found post-cooking and during food cooling, that could allow spore germination and vegetative growth of *C. perfringens*. For example, as contaminated food is heated during preparation (cooking) but is allowed to cool at room temperature, spores will germinate and the pathogen would propagate as a permissive temperature range is reached. The second type of study was designed to assess **moderate-temperature** effects and entailed maintaining lysins in the absence of their targets at either ambient temperature or 37 °C for various periods of time, followed by evaluation of efficacy against a type strain of *C. perfringens*.

Figure 2-11 shows the results of these studies. One of the plant-made lysins under development, ZP173, showed high thermal stability in spite of it being derived from a mesophilic phage. In a **high-temperature** test, ZP173 was incubated at various temperatures for 30 min prior to exposure to *C. perfringens* for 60 min at ambient temperature. After exposure, sampling and serial dilution was followed by overnight anaerobic incubation at 37 °C for CFU/mL enumeration. **Panel A** shows OD_{600} nm absorbance among the groups starting with an inoculum size of 3.5 $\log_{10}$ CFU/mL (lower bars = lysed cells = positive bactericidal activity). **Panel B and the table** show $\Delta\log_{10}$ CFU/mL, and **the table and Panel C** show that Lysin ZP173 retains 100 % of its initial activity after 30 min incubation at temperatures up to 44 °C, and 40 % of its initial activity after incubation at 50 °C. Notably, ZP173's activity dramatically declined at >50 °C, with less than 10 % of its original activity at 55 to 60 °C. This finding verifies that even the most thermostable lysins can be heat denatured and deactivated.

Figure 2-11. Impact of temperature on endolysin efficacy and stability



Temp. °C	Δlog CFU/mL	Remaining activity, %
0	4.18	100.0
37	4.15	99.3
42	4.23	101.1
44	4.21	100.8
50	1.71	40.8
55	0.30	7.3
60	0.39	9.3

Figure 2-11, **Panels A-C and Table – high-temperature stability**. Purified plant-made lysin ZP173 was aliquoted into PCR tubes and incubated at temperatures indicated for 30 min. *C. perfringens* NCT8237 was grown in TSB under anaerobic conditions to OD_{600}=0.894, resuspended in 1xPBS of pH 7.3. A volume of 998 µL of bacterial suspension was mixed with 2 µL (2 µg) lysin protein and incubated at room temperature. Absorbance was recorded following 30 min and 60 min co-incubation. Samples for CFU enumeration were taken after 1 h co-incubation. Serial dilutions of analyzed samples were done in 1xPBS pH 7.3 and 30 µL of each sample was plated on TSA plates: Undiluted, and diluted 10, 10^2, 10^3, 10^4, 10^5 and 10^6 times. CFU/mL were calculated from images of plates taken following incubation under anaerobic conditions at 37 °C.

Figure 2-11, **Panels D and E – moderate-temperature stability.** The indicated plant-made lysins were incubated in buffer without bacterial targets at either room temperature or 37 °C. Each was then co-incubated with *C. perfringens* NCTC8237 anaerobically at room temperature for 60 min, followed by sampling, serial dilution, overnight incubation at 37 °C, and CFU/mL enumeration.

The influence of incubating lysins in solutions at **moderate temperatures** for longer periods of time was also assessed and the results of multiple experiments are summarized also in Figure 2-11, **Panels D and E**. The indicated lysins were stored in buffered solution without the bacterial target at ambient temperature for up to 6 weeks, and at 37 °C for up to 7 days, then evaluated for antibacterial activity against *C. perfringens* NCTC8237, as described.

Lysin ZP173 was stable in solution at 37 °C for up to 3 days and conserved 50 % of its initial activity after 6 weeks storage at room temperature. Lysin CP25L was extremely stable at room temperature and after 6 weeks its activity remained practically unchanged. However, its activity started to decay after two days at 37 °C, and after 7 days of incubation at 37 °C CP25L retained only about 20% of its original activity.

There was no decline in the activity of any of lysins incubated at 4 °C; some, such as lysins ZP173 and CP25L, retained 100 % of their original activity after >9 months of storage at 4 °C. Our results support the fact that purified lysins are stable for up to several months in refrigerated conditions, even in solution, and that they can retain high activity after several days at 37 °C and several weeks of storage at ambient temperature, in the absence of their bacterial target. Additional stability information is presented in APPENDIX C.

These findings are analogous to what has been reported for whole bacteriophage mixtures, which are GRAS listed as food processing aids, in that the phages are inactive but stable in the absence of their bacterial target but become activated to infect and lyse the cells through endolysins once they encounter a susceptible host. Likewise, plant-made lysins are stable either in dry form or even in solution in the absence of susceptible bacterial cell walls but become bound (via targeting domain) and catalytically active upon contacting their target – in this case, specifically, *C. perfringens*.

Summary of results of antimicrobial effects of semi-purified and purified plant-produced endolysins on *C. perfringens*

The results presented herein illustrate that plant-produced endolysins show bactericidal activity against food contamination-relevant strains of *C. perfringens*. Efficacy is demonstrated for lysins produced and evaluated as semi-pure extracts of two representative plant expression hosts, namely, *Nicotiana benthamiana* and *Spinacia oleracea* (spinach), as well as for lysins purified to a high degree from these hosts.

In bactericidal studies *in vitro*, purified lysins PlyCP26F and PlyCP390 showed less activity against the tested bacterial strains under the conditions used. All other lysins showed a broad range of activity against all 26 pathogenic strains of *C. perfringens* evaluated.

Lysins had higher bactericidal activity in the presence of NaCl, which is expected to be present in the types of food (avian and mammalian meats, gravies, etc.) that are typically contaminated by *C. perfringens*. Therefore, we expect plant-made lysins to be compatible with food preparation practices and the salinity of the matrix.

Similarly, the pH of the environment had an impact on the activity of plant-made lysins, which is a common trait for food-processing and other enzymes. Our results showed that lysin ZP173 has a pH optimum of 5.2; ZP278 has highest activity at pH 4.5 with a relatively flat curve of activity from pH 5.0 to 7.5; and CP25L, PlyCP390 and PlyCP26F show highest activity towards neutral pH values (6.5-7.5). As with the salinity experiments, these results also underscore the value of using mixtures of lysins with complementary pH optima to ensure that there is effective bactericidal activity on various prepared food matrices.

Temperature also had an effect on lysin performance. Lysin ZP173, for example, was active from 37-44 °C, while other lysins worked best under ambient conditions. Hence, lysins operate under temperature conditions that can allow *C. perfringens* spores to germinate upon cooling of cooked, contaminated food, as the food cools towards room temperature.

Our results also show that purified lysins are stable in refrigerated conditions, even in solution, and that they can retain high activity after several days and weeks of storage at ambient temperature in the absence of their bacterial target. Once exposed to a susceptible strain of *C. perfringens*, the lysins bind to peptidoglycan to catalyze its degradation with measurable technical effect lasting from 0 to 43 h, with most of the efficacy evident between 2 and 18 h post application.

From Notifier's own scientific studies on plant-produced lysins and from similar studies in the published literature, we conclude that lysins can effectively reduce the viability of pathogenic strains of *C. perfringens* at concentrations, salinities, pH ranges, temperatures and exposure durations that are relevant in food contamination and intervention scenarios. Results of studies on the bactericidal efficacy of lysins on food matrices follow.

2.4.2 Suitability of ENDOLYSIN in temperature-abused meat products

Application of ENDOLYSIN on meats reduces the viability of pathogenic strains of *C. perfringens*

Plant-produced lysins were also tested for antibacterial activity on samples of cooked meat to determine the product's suitability as an antimicrobial on different target matrices. To illustrate suitability, **turkey** meat was selected because that meat matrix is highly susceptible to *C. perfringens* contamination. Samples were contaminated with reference strain NCTC8237 and incubated with and without lysin treatment under a variety of conditions, using nisin as a positive control and non-lysin-treated bacteria as negative (growth) control. Subsequently, the suitability of endolysins on **beef** cuts was also evaluated. The general methods used for efficacy determination are summarized here and in APPENDIX C, while the SOP for determining efficacy and duration of technical effect on meat matrices is found in APPENDIX D.

Briefly, to simulate actual use, samples of turkey meat were chopped into 10 g pieces and cooked for 30 min in a glass Falcon tube and stored at -70 C. For evaluation, the meat was defrosted at room temperature and 10 g meat was combined with 3 ml buffer, 100 µL diluted bacteria, and 25-50 µg lysin or 50 µg nisin (positive control) to a total volume of 10 ml. The mixture was transferred into 6-well plates and incubated at RT anaerobically. Subsequently, 50 µL samples for CFU enumeration were taken after 2 h, 18 h and 43 h of co-incubation at RT. Serial dilutions were done in citrate-phosphate buffer with 50 mM NaCl, pH 5.5 for low salt exposure, and in citrate-phosphate buffer with 256 mM NaCl, pH 5.5 for high salt exposure. A volume of 50 µL of diluted bacteria were plated onto TSA plates and grown anaerobically overnight.

The following series of figures illustrate results obtained on turkey meat under different exposure conditions. The results of this series of studies show that lysin addition significantly reduces *C. perfringens* viability on previously cooked, contaminated turkey meat matrices under a variety of expected use conditions. Depending on the concentrations used and the amount of NaCl present on the matrix, lysins showed higher potency than nisin in controlling *C. perfringens* contamination.

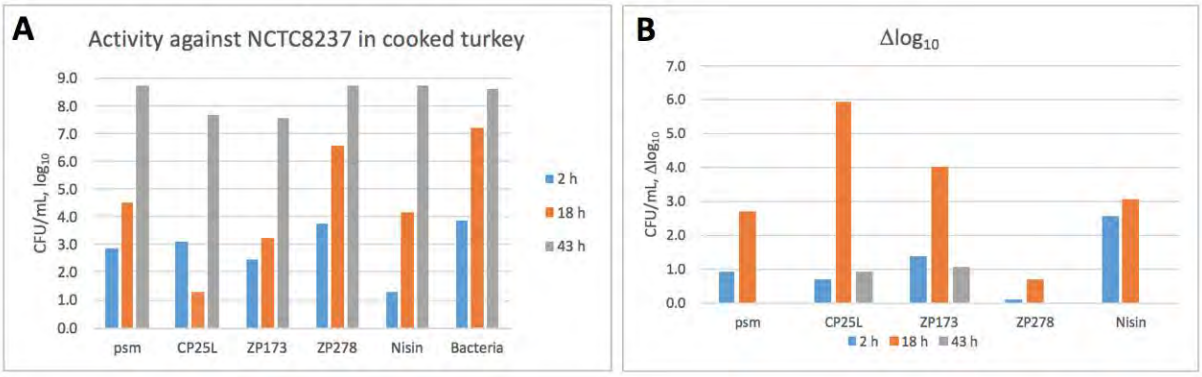
ENDOLYSIN activity against *C. perfringens* type strain NCTC8237 on turkey meat cooked and held at room temperature

The bactericidal activities of four (4) plant-produced, purified lysins (psm, CP25L, ZP173 and ZP278) and of nisin were evaluated as a function of incubation time on cooked turkey meat samples contaminated with *C.*

perfringens NCTC8237. Figure 2-12 summarizes the results obtained. The incubation medium was supplemented with 0.09% NaCl. Strong activities were observed for psm, CP25L, ZP173 and Nisin after 2 h and 18 h of co-incubation anaerobically at room temperature. Almost no antibacterial effects for either lysins or nisin were detected after 43 h at RT.

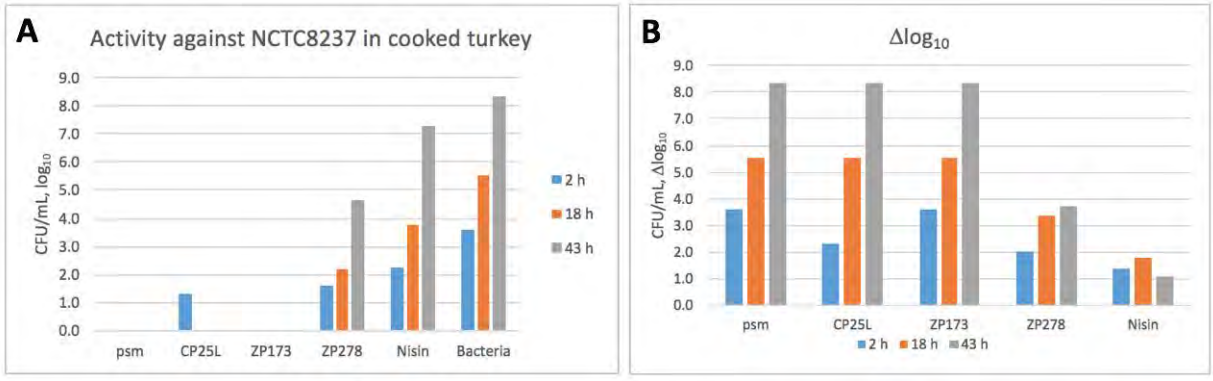
The activity of lysins was re-evaluated under the same conditions as in Figure 2-12 but with addition of a higher level of NaCl to the incubation medium. The bactericidal activities of four (4) plant-produced, purified lysins (psm, CP25L, ZP173 and ZP278) and of nisin were evaluated as a function of incubation time on cooked turkey meat samples contaminated with *C. perfringens* NCTC8237. The incubation medium was supplemented with **1.5% NaCl**. Figure 2-13 summarizes the results obtained. Strong activity of lysins psm, CP25L and ZP173 was detected after 2 h, 18 h and 43 h of co-incubation at RT. Nisin was found to be less active than lysins when meat is supplemented with 1.5 % NaCl under the conditions analyzed.

Figure 2-12. Lytic activity of plant-made endolysins against *C. perfringens* NCTC8237 with 0.09% NaCl



C. perfringens NCTC8237 was grown to OD_{600}=0.234 in TSB anaerobically. Cooked turkey breast meat (10 g) was combined with 100 µL of 10x diluted bacterial culture, 3 mL citrate-phosphate buffer with 50 mM NaCl, pH 5.5, 50 µg purified lysin or 50 µg Nisin (5 µg/mL lysin or Nisin) and incubated at room temperature anaerobically. Nisin (Sigma) 5 mg/mL stock was prepared by dissolving powder in 0.02 N HCl. CFU/mL enumeration was done at 2 h, 18 h and 43 h of co-incubation. **Panel A** shows CFU/mL (lower bars = higher lytic activity). **Panel B** shows differential counts relative to control "bacteria" samples without lysin or nisin treatment (higher bars = higher antibacterial effect).

Figure 2-13. Lytic activity of plant-made endolysins against *C. perfringens* NCTC8237 with 1.5% NaCl

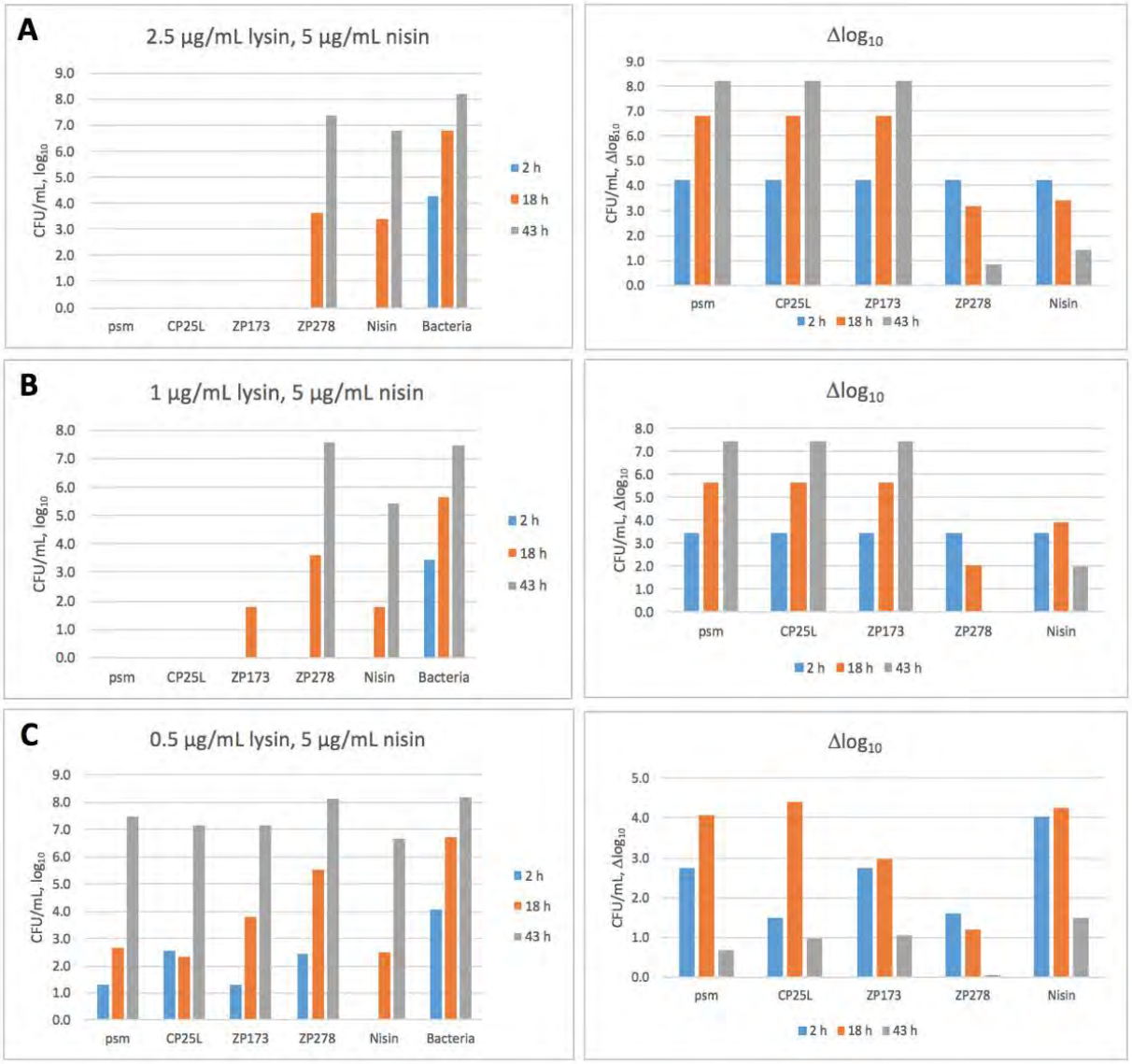


C. perfringens NCTC8237 was grown to OD_{600}=0.248 in TSB anaerobically. Cooked turkey breast meat (10 g) was combined with 100 µL of 10x diluted bacterial culture, 3 mL citrate-phosphate buffer with 856 mM NaCl, pH 5.5, 50 µg purified lysin or 50 µg nisin (5 µg/mL lysin or nisin) and incubated at room temperature anaerobically. Nisin (Sigma) 5 mg/mL stock was prepared by dissolving powder in 0.02 N HCl. CFU/mL enumeration was done at 2 h, 18 h and 43 h of co-incubation. **Panel A** shows CFU/mL (lower bars = higher lytic activity). **Panel B** shows differential counts relative to control "bacteria" samples without lysin or nisin treatment (higher bars = higher antibacterial effect).

Optimization of endolysins' antibacterial dose/response on cooked turkey meat

A dose/response study was conducted with lysins psm, CP25L, ZP173 and ZP278, with nisin and non-lysin-treated inoculum as positive and negative controls. Cooked turkey meat samples were contaminated with *C. perfringens* type strain NCTC8237 and efficacy at various application rates (dose) was assessed (response) as a function of incubation time. The incubation medium was supplemented with 1.5% NaCl. Figure 2-14 summarizes the results obtained in this series. Total CFU/mL are shown in left panels; differential CFU/mL relative to bacterial growth control at each time point are shown in right panels.

Figure 2-14. Antibacterial efficacy of plant-made endolysins against *C. perfringens* NCTC8237 on cooked turkey meat



In the dose/response series shown in Figure 2-14, *C. perfringens* type strain NCTC8237 was grown to OD_{600}=0,233 in TSB anaerobically. Cooked turkey breast meat (10 g) was combined with 100 µL of 10x diluted bacterial culture, 3 mL citrate-phosphate buffer with 856 mM NaCl, pH 5.5, and either 2.5 µg (Panel A), 1.0 µg (Panel B), or 0.5 µg (Panel C) of purified lysin or 50 µg nisin and incubated at room temperature anaerobically. Nisin (Sigma) 5 mg/mL stock was prepared by dissolving powder in 0.02 N HCl. CFU/mL enumerations were done following 2 h, 18 h and 43 h of co-incubation.

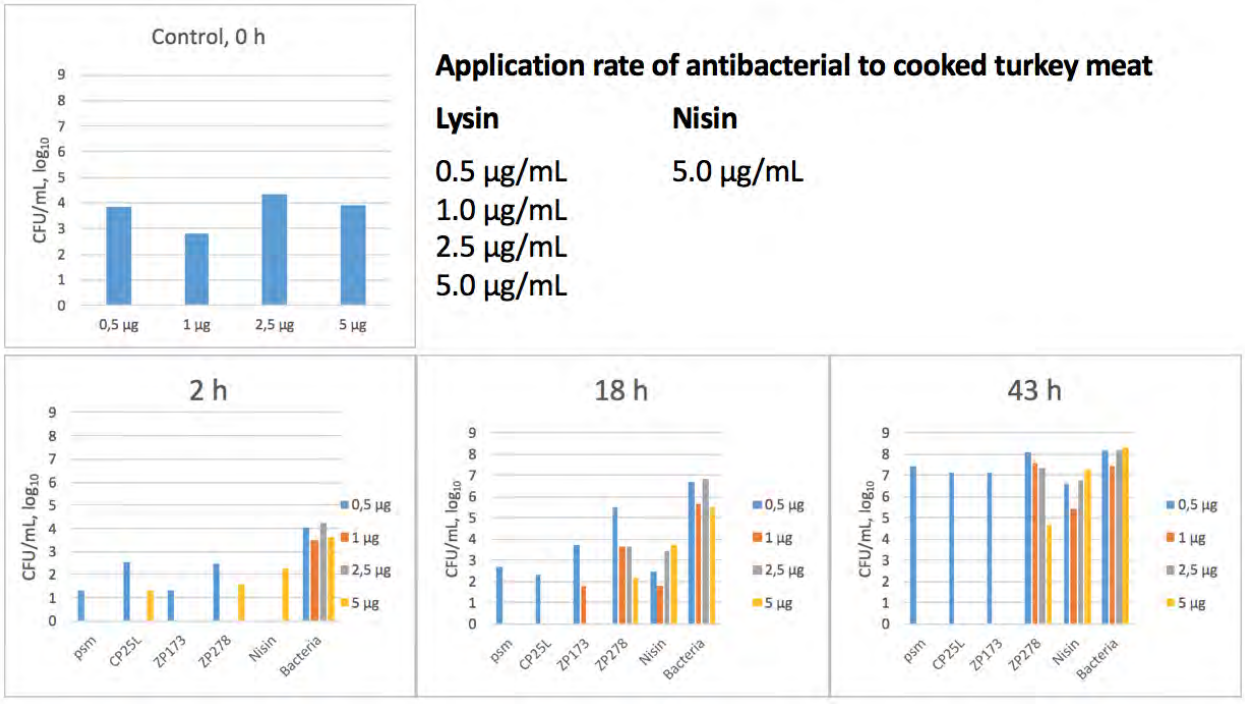
In Figure 2-14 **Panel A**, a dose of 2.5 µg/mL of Psm, CP25L or ZP173 stopped bacterial proliferation in meat completely (100%). The antibacterial activity of lysin ZP278 was similar to nisin's. **Panel B** shows results using a lower dose of lysins (1 µg/mL) while maintaining nisin's dose at 5 µg/mL. Strong activity of psm, CP25L and ZP173 was detected after 2 h, 18 h and 43 h co-incubation at RT. Nisin was found less active than lysins when the meat samples were supplemented with 1.5 % NaCl. Lastly, **Panel C** shows results using only 0.5 µg/mL lysins while maintaining nisin's dose at 5 µg/mL. Even at this lowest concentration, 0.5 µg/mL of the lysins psm, CP25L or ZP173 showed similar activity as 5 µg/mL nisin. "Bacteria" = no-treatment control.

Individual plant-made endolysins are as potent as Nisin in controlling *C. perfringens* on turkey meat cooked and held at room temperature

When evaluated on cooked turkey meat samples, the individual lysins psm, CP25L, ZP173 and ZP278 exhibited the same range of antibacterial activities as the nisin positive control at equal or lower concentrations. At 0.5 µg/mL, lysins psm, CP25L, ZP173 were approximately as potent as lysin ZP278 and nisin at 5 µg/mL. In fact, at 2 h of incubation, the shortest sampling time tested, lysins psm and ZP173 were more potent than nisin at only 1/10th the nisin application rate. Results of this dose/response summary are shown in Figure 2-15.

Most of the bactericidal activity was observed during the initial exposure periods, with loss of bactericidal activity observed for all agents with longer incubation times. Sterilizing effects of contaminated turkey meat were observed with some lysins at application rates of 1.0 to 2.5 µg/mL (1 to 2.5 ppm).

Figure 2-15. Relative antibacterial efficacy of individual plant-made endolysins and nisin against reference strain *C. perfringens* NCTC8237 on cooked turkey meat

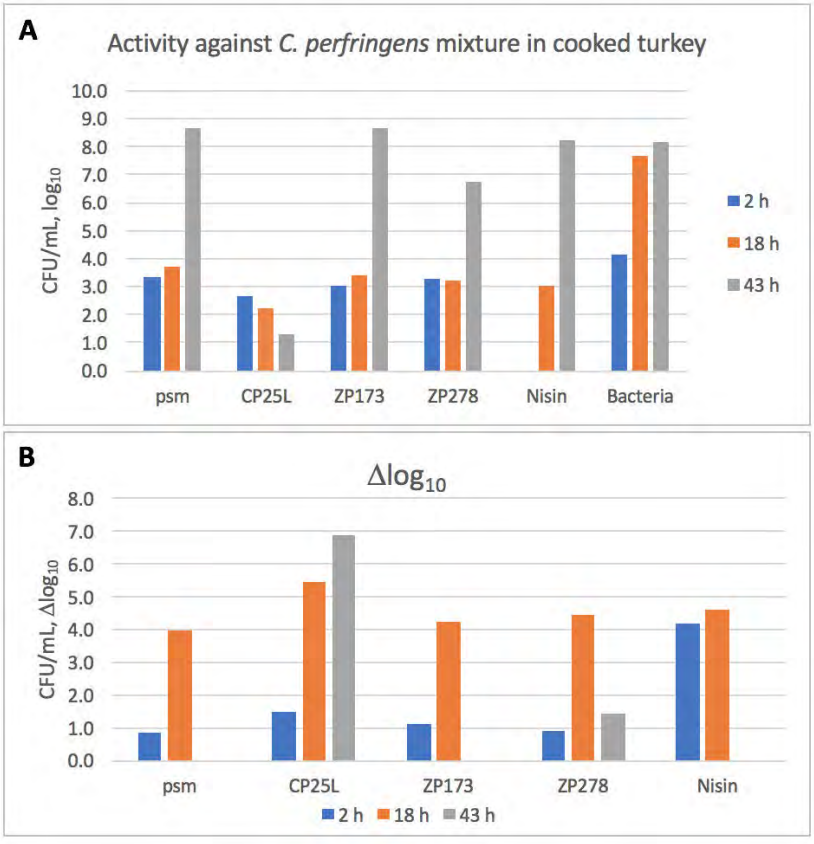


Reference strain *C. perfringens* NCTC8237 grown to OD_{600}=0.233 in TSB anaerobically. Cooked turkey breast meat samples (10 g) were combined with 100 µL of 10x diluted bacterial culture, 3 mL citrate-phosphate buffer with 856 mM (1.5%) NaCl, pH 5.5, to which were added the indicated concentrations of individual plant-made lysins or nisin to 5 µg/mL. The treated samples were incubated at room temperature anaerobically. Nisin (Sigma) 5 mg/mL stock was prepared by dissolving powder in 0.02 N HCl. Sampling and CFU/mL enumeration was done following 2 h, 18 h and 43 h of co-incubation. "Bacteria" = control samples.

Individual endolysins are bactericidal to a mixture of 5 food contamination-relevant strains of *C. perfringens* on turkey meat cooked and held at room temperature

Because food may become contaminated simultaneously with one or more strains of *C. perfringens*, the antimicrobial activity of ENDOLYSIN was evaluated against a mixture of five (5) *C. perfringens* strains isolated from contaminated foods and food-borne outbreaks. ***C. perfringens* strains NCTC No. 8239, 9851, 8449 and 8797 were mixed** in approximately equal amounts and used to contaminate cooked turkey meat. The mixtures were treated individually with either one of four different plant-made lysins, psm, CP25L, ZP173 or ZP278, nisin (positive control), or non-treated (negative control). Sampling for CFU/mL enumeration took place at 2 h, 18 h and 43 h of co-incubation. Samples were diluted, plated and grown anaerobically overnight at 37 °C for enumeration. Results of this series are shown in Figure 2-16.

Figure 2-16. Suitability of individual plant-made endolysins as antibacterials against a mixture of 5 food contamination-relevant strains of *C. perfringens* on cooked turkey meat



Five *C. perfringens* strains identified in the narrative above were grown to OD_{600} appr. 0.23 in TSB anaerobically. Each strain was diluted to OD_{600}=0.005 and mixed in equal amounts to get 1 mL of bacterial culture of OD_{600}=0.025. Cooked turkey breast meat samples (10 g) were combined with 100 µL of mixed diluted bacterial culture, 3 mL citrate-phosphate buffer with 856 mM NaCl, pH 5.5, 10 µg purified lysin (1 µg/mL) or 50 µg Nisin (5 µg/mL) and incubated anaerobically at room temperature. Nisin (Sigma) 5 mg/mL stock was prepared by dissolving powder in 0.02 N HCl. Sampling for CFU/mL enumeration was done following 2 h, 18 h and 43 h of co-incubation. **Panel A** shows total CFU for each exposure condition at the indicated sampling time. **Panel B** shows differential growth (Δlog_{10} CFU/mL) relative to bacterial growth control at the indicated time point.

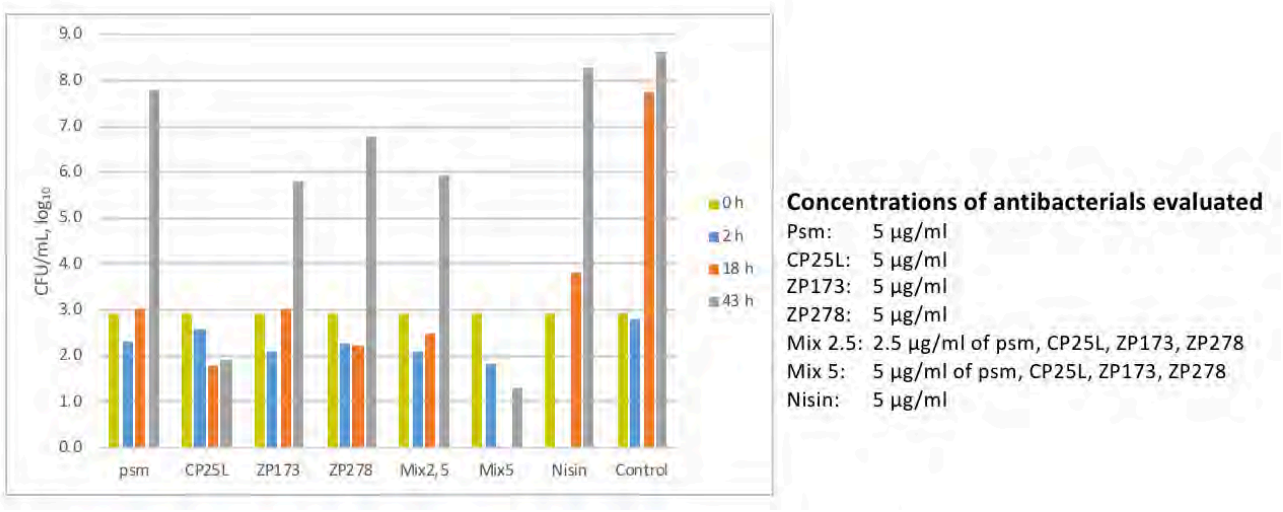
The results of this series indicate that all plant-made lysins evaluated against a mixture of food-contaminating strains of *C. perfringens* on cooked turkey meat were able to reduce bacterial growth from a minimum of ~1-1.5 log CFU/mL to as much as 7 log CFU/mL relative to uncontrolled bacterial growth

(Figure 2-16, **Panel B**). The highest inhibitory effects of the lysins were observed between application and 18 h; only CP25L and ZP278 showed continued bactericidal activity at the 43-h sampling time. Nisin at 5-times the concentration of any of the lysins showed approximately 4 $\Delta\log_{10}$ CFU/mL antibacterial activity relative to control, only from the time of application to the 18 h sampling time. These results verify the bactericidal activity of purified lysins against food-relevant *C. perfringens* contamination on a relevant meat matrix that is susceptible to contamination by multiple pathovars of this pathogen.

Suitability of ENDOLYSIN (lysin mixtures) on 5 food contamination-relevant strains of *C. perfringens* on turkey meat cooked and held at room temperature

The suitability of a mixture of purified, plant-made endolysins (ENDOLYSIN) was assessed on cooked turkey meat contaminated with a mixture of *C. perfringens* strains. Results are shown in Figure 2-17.

Figure 2-17. Suitability of ENDOLYSIN (lysin mixtures) in controlling five *C. perfringens* strains on minced cooked turkey meat



Five (5) *C. perfringens* strains (NCTC8235, NCTC8239, NCTC9851, NCTC8449, NCTC8797) were grown to OD_{600} appr. 0.23 in TSB anaerobically. Each strain was diluted to OD_{600}=0.0002 and mixed in equal amounts to get 1 ml of bacterial culture of OD_{600}=0.001. Ten grams (10 g) cooked minced turkey breast meat was combined with 3 mL citrate-phosphate buffer with 856 mM NaCl, pH 5.5, 100 µL of diluted mixed bacterial culture (~10^3 CFU/mL), 50 µg purified lysin or 50 µg nisin, or a mix of four lysins, and incubated at RT anaerobically for the indicated times. "Mix 2.5" consisted of four lysins, 25 µg each (2.5 µg/mL total lysin), and "Mix 5" consists of four lysins, 50 µg each (5 µg/mL total lysin). Nisin (Sigma) 5 mg/mL stock was prepared by dissolving powder in 0.02 N HCl. CFU/ml enumeration was done following 2 h, 18 h and 43 h of co-incubation. Final concentrations of test agents evaluated are shown in the legend next to the results graph.

As shown by the results in Figure 2-17, all lysins evaluated individually or as mixtures showed antibacterial effects at room temperature even at the shortest (2 h) incubation time point. After 18 h incubation, individual lysins CP25L, ZP278, and lysin mixtures at two concentrations (Mix 2.5 and Mix 5) showed antibacterial activity. After 43 h of incubation at RT, CP25L and Mix 5 (5 ppm lysin mix) showed residual effects. Mix 5 sterilized the contaminated meat matrix at 18 h of incubation. Nisin sterilized the meat matrix after 2 h and reduced bacterial counts at 18 h but showed no residual activity at the 43-h sampling point.

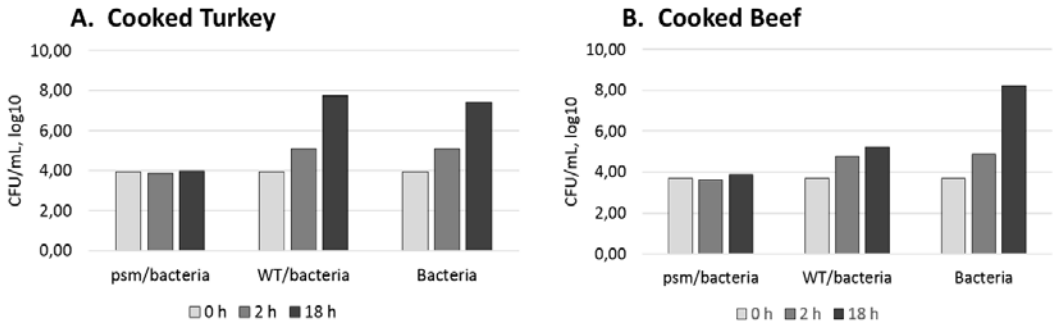
These results show that individual lysins and mixtures of lysins at the same concentration as nisin (dwb) are equivalent or superior to nisin in controlling a mixture of *C. perfringens* pathovars on cooked, temperature-abused minced turkey meat.

Antibacterial suitability of endolysins on multiple meat matrices at room temperature

Most evaluations of efficacy and suitability were conducted using cooked turkey meat, as poultry meat is susceptible to *C. perfringens* during a range of stages, showing pathology in live birds as well as food contamination in poultry meat. To assess the suitability of ENDOLYSIN in the control of *C. perfringens* on other susceptible meat, a comparative study was conducted using contaminated **turkey and beef** cuts. Following similar procedures described in studies represented by results in Figures 2-9 through 2-13, the plant-made endolysin psm was evaluated first as a model lysin to demonstrate antibacterial activity in cooked, minced meat samples. Figure 2-18 shows the results of this study. **Panel A** shows the $\log_{10}$ CFU/mL on **turkey meat** of psm-treated (left bars), non-lysin vehicle (middle bars) and bacterial growth control (right bars). **Panel B** shows the $\log_{10}$ CFU/mL on **beef meat** of psm-treated (left bars), non-lysin vehicle (middle bars) and bacterial growth control (right bars). Approximately 10^4 CFU/mL of *C. perfringens* were used to contaminate each meat sample (Time 0).

Lysin psm completely arrested *C. perfringens* growth up to the 18-h time exposure evaluated (left bars), whereas vehicle-treated (middle bars) and non-treated bacteria (right bars) showed exponential growth. The same results were found whether the contaminated meat was turkey (Panel A) or beef (Panel B).

Figure 2-18. Suitability of lysin psm in controlling *C. perfringens* growth in turkey and beef matrices

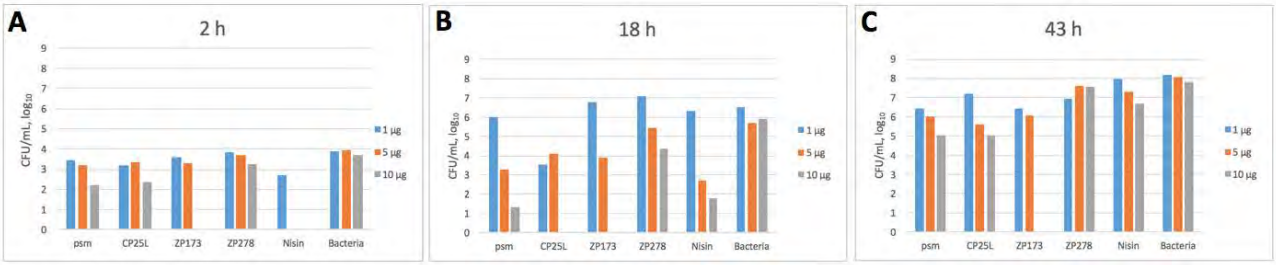


The activity of plant-made lysin **psm** against *C. perfringens* NCTC8237 in cooked and minced turkey and beef meat are summarized. Crude psm extract were prepared from agro-infiltrated *N. benthamiana* plants harvested 6 dpi following standard procedures. Protein extract was diluted to conc. ~0.44 µg/µL. *C. perfringens* NCTC8237 strain was grown in TSB under anaerobic conditions to OD_{600}=0.265. A volume of 100 µL of bacteria and 700 µL ≈ 300 µg of protein extract was added to 10 g of cooked meat and vortexed well. To equalize the volume of all samples, 700 µL of 1xPBS, pH7.3 were added to meat/bacteria samples. All the samples were placed into 6-well plates and incubated under anaerobic conditions at room temperature. Serial dilutions of analyzed samples were done in 1xPBS pH7.3, and 30 µL of each sample were plated on TSA plates to include undiluted, and diluted 10, 10^2, 10^3, 10^4 and 10^5 times. Enumeration of CFU/mL was done following overnight incubation under anaerobic conditions at 37 °C.

Activity of purified plant-made endolysins on beef contaminated with *C. perfringens* type strain NCTC8237 at room temperature

To expand on the initial study with lysin psm (above), multiple purified plant-made lysins were also evaluated for antibacterial activity against *C. perfringens* reference strain NCTC8237 on contaminated samples of beef meat. In this series, cooked beef samples were contaminated with 10^4 CFU/mL of *C. perfringens* suspension in buffer containing 1.5% NaCl at Time 0 and admixed with individual lysins at concentration of 1, 5 and 10 µg/mL. Nisin at varying concentrations was used as a positive control. The samples were incubated at room temperature anaerobically and sampled for enumeration of CFU/mL at 2, 18 and 43 h. Figure 2-19 shows the results of this study; **Panel A**, **Panel B** and **Panel C** show CFU/mL of residual viable bacteria at 2-h, 18-h and 43-h sampling points, respectively.

Figure 2-19. Suitability of endolysins in controlling *C. perfringens* growth in cooked beef



Cooked beef samples were contaminated with 10^4 CFU/mL of *C. perfringens* NCTC8237 suspension in buffer containing 1.5% NaCl at Time 0 and admixed with individual lysins (psm, CP25L, ZP173 or ZP278) at concentration of 1, 5 and 10 µg/mL. Nisin at equivalent concentrations was used as a positive control. Samples were incubated at room temperature anaerobically and sampled for enumeration of CFU/mL at 2, 18 and 43 h.

The results show that at 1 µg/mL (1 ppm) plant-made lysin CP25L arrested bacterial growth for up to 18 h. At an application rate of 5 µg/mL (5 ppm) plant-made lysins Psm and CP25L were able to stop bacterial proliferation for up to 18 h, whereas lysin ZP173 was able to stop bacterial proliferation for up to 43 h. At the highest level tested of 10 µg/mL (10 ppm), all lysins but ZP278 arrested bacterial growth by at least 1 log CFU/mL for up to 43 h. At the highest dose, lysin ZP173 showed sterilizing effects at all time points sampled. Nisin showed short-lived antibacterial activity with no growth recorded only at the 2-h sampling point; progressive loss of nisin activity was evident at the 18-h and 43-h time points.

Antibacterial suitability of endolysins when used in mixes or blends

It is clear from results of these collective studies that plant-made endolysins, whether used in semi-pure or purified form, are able to arrest the growth of *C. perfringens* strains of relevance to food-borne contamination. As shown in Figure 2-5, plant-made lysins showed antibacterial **activity against all 26 strains of *C. perfringens*** tested. While some lysins showed a narrower host range (e.g. PlyCP26F and PlyCP390), others showed high potency against a wide range of strains (e.g. psm, CP25L, ZP173 and ZP278), individually targeting as many as 9 different pathovars.

These results suggest that, while individual lysins could be used in food protection, there may be advantages to using mixtures or blends of lysins to ensure that any of the food contamination-relevant strains that may be present on food can be controlled efficiently. This approach was demonstrated successfully by Notifier in the control of enteropathogenic *E. coli* on various foods using a blend of the plant-made antibacterial proteins **colicins**, as described in GRN 593 and GRN 676. In a similar manner, one or more lysins active against any one of the targeted strains of *C. perfringens* may be used as mixtures or blends. Formulation may be done with different amounts of each lysin depending on the potency of each enzyme. Figure 2-8 shows results of individual lysins and of blends of 4 lysins (psm, CP25L, ZP173 and ZP278) at either 2.5 µg/mL or 10 µg/mL when applied to a mix of seven (7) *C. perfringens* strains. Even at the low concentration (2.5 µg/mL) the lysin mix exerted more rapid bactericidal effects than some individual lysins. Likewise, Figure 2-17 shows that 2.5 and 5.0 µg/mL lysin mixtures were superior bactericides to equal doses of individual lysins.

If two or more lysins are combined, it is preferred to combine lysins having different target specificities as determined by the binding domains of the lysins for achieving broad anti-*Clostridium* activity and/or for avoiding the remote potential for development of resistance to a lysin by mutation of the bacterium. Alternatively, or additionally, two or more lysins may be selected for use as blends on the basis of each

enzyme's type of catalytic activity (e.g. **lysozymes, glycosidases, amidases** and **endopeptidases)**. For example, a lysin having lysozyme activity may be combined with a lysin with amidase activity.

Blends of multiple lysins can therefore contain from one to four different and complementary cell wall-degradative activities against *C. perfringens*, hence increasing the potency of the ENDOLYSIN product. Another advantage of blending lysins it to make the best use of their overlapping optima for activity, and hence extend the technical effect of the product over broader ranges of pH, temperatures and salinities.

Summary of suitability of endolysins for controlling *C. perfringens* on turkey and beef matrices

Semi-purified, plant-expressed lysins were first tested against type strains of *C. perfringens* and found to be highly effective at controlling bacterial growth. Six different lysins were evaluated in those studies and all were candidates for use individually or as blends, depending on the conditions expected. Blends of semi-purified lysins could comprise the cruder product embodied by the ENDOLYSIN Concentrate concept, which could be used in more cost-constrained applications.

Subsequently, purified plant-produced lysins were individually evaluated for antibacterial activity against 26 strains of *C. perfringens* that have been associated with food-borne contamination and illness outbreaks. The results of this series of studies show that although lysins exhibit strain preferences, all strains were sensitive to and could be controlled by various application rates of any of the six lysins.

Overall, the lysins psm, ZP173 and ZP278 were the most potent in reducing *C. perfringens* viability, with net reductions of from 1 to 8 $\Delta\log_{10}$ CFU/mL relative to non-treated bacterial controls. Lysin CP25L exhibited net $\Delta\log_{10}$ CFU/mL reductions of from 1.5 to 4, and lysins PlyCP26F and PlyCP39O worked to a lesser extent (up to 1 $\Delta\log_{10}$), primarily due to the mildly acidic pH used in these specific studies relative to the alkaline pH optima for these two lysins.

The pH of the studies *in vitro* and subsequently of the cooked turkey and beef samples was selected as typical of what is encountered with these meats. Under such conditions, which clearly allowed rapid bacterial growth, the lysins psm, ZP173 and ZP278 showed the highest potency and host range. For use with more alkaline foods, the lysins CP25L, PlyCP26F and PlyCP39O can be considered. These results underscore the value of the concept of blending.

In sum, application of plant-made lysins to contaminated cooked turkey or beef matrices at rates up to 10 mg lysin/kg food (up to 10 ppm) effectively controlled the growth of *C. perfringens* strains relevant to food-contamination and illness, with 1 to 8 net $\Delta\log_{10}$ reductions in CFU/mL.

Application rate of endolysins to meat food products

Based on the results of these studies, ENDOLYSIN product formulations consisting of single plant-made lysins or blends thereof, may be applied to cooked poultry, beef or other food matrices that are susceptible to contamination with *C. perfringens* at **application rates ranging from 0.05 to 10 mg total lysin per kg treated food** (i.e., 0.05 to 10 ppm).

2.4.3 Duration of ENDOLYSIN's technical effect

The duration of ENDOLYSIN's technical (antibacterial) effect in buffer and after application to meat products was evaluated. The general method used and results of specific experiments are summarized. The Standard Operating Procedure for determining efficacy and duration of technical effect is found in APPENDIX D.

Definition of technical effect and duration of technical effect

The **technical effect** is defined as the bactericidal effect of ENDOLYSIN and is quantified by determining the population of target pathogens remaining in an experimentally contaminated food matrix (CFU pathogen/g meat product) after exposure to either ENDOLYSIN or a control (vehicle) solution. The **duration of technical effect** is the time after ENDOLYSIN treatment at which bacteria that have survived (or have germinated from spores during incubation) are quantified to begin normal growth relative to the control treatment.

Summary of methods

Defined media, as well as samples of turkey and beef meat, were contaminated with measured concentrations of pathogenic *C. perfringens* strains, exposed to lysins or a non-lysin containing control vehicle, and incubated at either room temperature (RT) or at 37 °C under anaerobic conditions. After treatment and storage, the suspensions/meat aliquots were sampled at 2 h, 18 h and 43 h for enumeration. Viability of bacteria over time was determined as a function of treatment condition.

Bacterial strains and contamination

Contamination of meats during food preparation, delayed serving and storage may include one or more strains of *C. perfringens*. Hence, these studies employed either a single indicator strains of *C. perfringens* or a mixture of five (5) *C. perfringens* strains, all of which have been associated with food-borne outbreaks. The bacterial suspensions contained approximately equal CFU of each strain and were applied to meat samples at rates of 3-4 log CFU per gram of meat (4-5 log CFU/10 g meat sample).

ENDOLYSIN application

The contaminated meat samples (cooked turkey and beef) were mixed with solutions of lysins or with control solution not-containing lysin (purified extract from host plant not inoculated with lysin expression vector). An additional non-treated bacterial growth control was used in each study for reference.

Exposure conditions

Various food- and food preparation-relevant environmental conditions were used to determine the longevity of technical effect of ENDOLYSIN. These included different salinities (i.e. added NaCl), pH, temperature, lysin dose and length of exposure.

Sampling

Samples of treated, contaminated meat were collected at 2 h, 18 h and 43 h. For microbiological analysis, the samples were homogenized for uniform recovery of viable bacteria, centrifuged, resuspended and serially diluted and plated on selective medium. CFU were determined per mL of isolate per 10 g of meat and the results extrapolated per volume or per kg food.

Results

The viability of bacterial samples on cooked turkey and beef treated with ENDOLYSIN or carrier solution as a function of exposure and storage condition were calculated. The data from these studies were summarized in **Figure 2-12 through Figure 2-18**. Activities of >1 to >8 $\Delta\log_{10}$ CFU/mL (reduction in viability of *C. perfringens* reference strain NCTC8237 or a mixture of 5 food contamination-relevant *C. perfringens* strains) were observed under all conditions evaluated for all plant-made lysins. The duration of activity was primarily observed from 2 h to 43 h under most conditions, and preferentially from 1-18 h post application

of the lysins at realistic application rates ranging from 0.05 to 10 µg lysin per 10 g meat sample (0.005 – 1 µg/g meat), or 0.005 – 1 ppm. To accommodate the host range of lysins with lower potencies, especially in a blend, Notifier intends to apply ENDOLYSIN at a maximum of 10 mg/kg food (≤10 ppm).

Conclusion

The duration of technical effect of single lysins or mixtures of lysins against individual or mixtures of pathogenic strains of *C. perfringens*, and under a variety of exposure conditions, ranges from 2 to 43 hours with most of the antibacterial effects observed from 1h to 18 h post application.

2.4.4 Susceptibility of endolysins to proteolytic degradation

Another factor influencing high potency/short duration kinetics of lysins, as well as their safety, 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. What we did not do then is scan the polypeptides for cleavage sites for the digestive enzymes pepsin, trypsin and chymotrypsin, as those enzymes were sequentially applied *in vitro* to verify colicin degradation.

Here we applied the above approach to project universal instability of all lysins comprising the product when exposed to gastroduodenal enzymes. The enzymes in the gastrointestinal tract cleave at the following amino acid residues (ExPASY (2017); accessed Nov 22, 2017):

Pepsin: X+Y, where X = aromatic a.a. such as Tyr, Phe or Try, or a hydrophobic aa, and Y = nonspecific;
Trypsin: X+Y, where X = Lys or Arg, and Y = nonspecific; and
Chymotrypsin: X+Y, where X = aromatic aa such as Tyr, Phe or Try, and Y = nonspecific (similar to pepsin).

Appling this information yielded the minimum number of theoretical cleavage sites by trypsin and pepsin and/or chymotrypsin for each of the endolysins under study. Table 2-4 summarizes these results.

Table 2-4. Endolysin cleavage susceptibility for digestive enzymes pepsin, trypsin and chymotrypsin

Endolysin	Target site (a.a.) and abundance	Pepsin and/or Chymotrypsin	Trypsin
PlyCP26F (212 aa)	**9F, 3W, >9Y; 15K, 12R**	21	27
PlyCP39O (213 aa)	**5F, 5W, >5Y; 13K, 12R**	15	25
Psm (342 aa)	**17F, 8W, >19Y; 23K, 10R**	44	33
CP25L (377 aa)	**13F, 7W, >26Y; 23K, 20R**	46	43
ZP173 (335 aa)	**13F, 10W, >24Y; 16K, 18R**	47	34
ZP278 (351 aa)	**9F, 8W, >23Y; 23K, 17R**	40	40

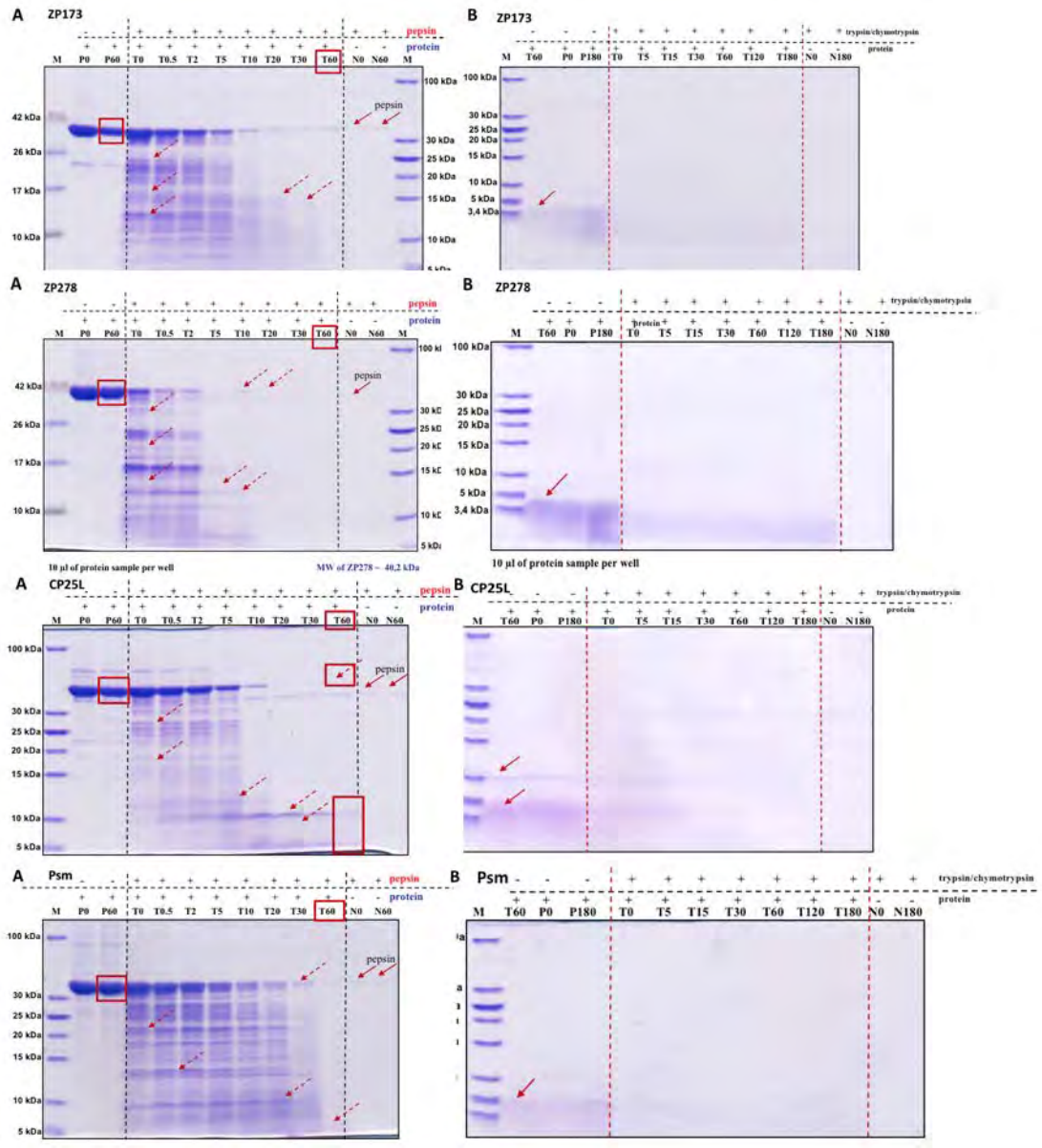
Endolysins (no. of amino acids) are listed in column 1, followed by the number and type of a.a. target cleavage sites (column 2), and the number of cleavage sites for each indicated gastrointestinal digestive enzyme (columns 3 and 4).

Although it is not expected that the gastrointestinal proteases will definitively cleave at all of the potential cleavage sites on the lysins' polypeptide backbone due to protein folding and molecular accessibility, even one or two cleavages could render the enzymes non-functional in either their peptidoglycan targeting domain, their enzymatic (catalytic) domain, or both.

Verification of endolysin digestibility in simulated gastrointestinal environment

To verify our predictions of digestibility, individual lysins were subjected to two stages of proteolytic digestion in **simulated gastric fluid** (SGF) followed by exposure to **simulated intestinal fluid** (SIF). The protocol for this series is shown in APPENDIX C. The results of digestibility studies for plant-made endolysins ZP173, ZP278, CP25L and psm are shown in Figure 2-20. Although some lysins (e.g. psm) were more resistant than others to peptic digestion, all lysins showed degradation fragments in SGF. Remaining products of peptic digestion were further degraded in SIF, as shown by degradation bands (arrows).

Figure 2-20. Digestibility of lysins ZP173, ZP278, CP25L and psm in simulated gastrointestinal fluids



Plant-made endolysins ZP173, ZP278, CP25L and psm were subjected to SGF buffer followed by exposure to pepsin to assess digestibility in simulated gastric environment (Panels **A** in left column). Degradation products of SGF exposure were subjected to trypsin/chymotrypsin digestion to assess further digestibility in simulated intestinal environment (Panels **B** in right column). The protocol used in this series of studies is described in APPENDIX C.

None of the lysins comprising the ENDOLYSIN product are glycoproteins or contain multiple disulfide bridges that could confer higher environmental stability, including stability to digestion. Supporting the gastrointestinal proteolytic lability of endolysins reported in this Notice are the multiple studies on the properties of lysins reviewed by (Fischetti 2005, 2010; Garcia 2008; Nelson 2012; Oliveira 2012; Schmelcher 2012), and the original work of Plotka (2014). There are no reports of lysins being resistant to proteases. Similarly, the same reports and reviews cited above verify the thermal instability of endolysins above their natural range of operating temperatures. Even one of the most heat-tolerant lysins (e.g. Ph2119 from *Thermus* spp.; Plotka (2014)) does not survive food cooking temperatures. Another observation added in proof of digestibility is the fact that we analyzed lysin sequences in part by tryptic digest peptide mapping. All lysins were readily cleaved by enzymatic digestion prior to MS analysis (see APPENDIX C).

In sum, all evidence suggests that the mesophilic lysins being developed for the ENDOLYSIN product are susceptible to digestion as are all other lysins reported in the public literature. Endolysins added to foods post cooking and baking that are ingested are therefore not expected to survive gastrointestinal passage or reach the colon where the majority of the commensal microbiome is found.

Summary and conclusion

Results of these experiments indicate that plant-produced ENDOLYSIN is antibacterial against pathogenic strains of *C. perfringens*, at bacterial contamination levels (3-4 log CFU/g meat) that could render the meat not fit for consumption due to organoleptic effects even if the contamination was due to non-pathogens. These experiments demonstrate that application of the plant-made lysins, singly or in combination over a wide range application rates of 0.005 to 10 mg lysin/kg food, results in rapid bactericidal control of pathogenic *C. perfringens* applied to samples of cooked turkey or beef meat, and that the technical effect observed is transient, lasting for approximately 2 to 18 h post application and perhaps up to 43 h (in the case of lysin ZP278), but not longer, depending on the storage conditions.

2.5 Overall Conclusion

Results of the studies reported herein indicate that plant-produced ENDOLYSIN is antibacterial against pathogenic strains of *C. perfringens*, at bacterial contamination levels (3-4 log CFU/g meat) that could render the meat questionable for consumption due to organoleptic effects even if the contamination was due to non-pathogens. The technical effect provided by ENDOLYSIN treatment was seen when contaminated meat samples were stored at either room temperature or 37 °C. The room temperature results mimic what would happen to food that was (a) inappropriately cooked and/or (b) inappropriately stored with poor/no refrigeration, and/or (c) cooked or refrigerated appropriately but allowed to stand at room temperature for too long, allowing germination of *C. perfringens* spores and contamination of the food matrix by growing pathogen cells. Ingestion of the vegetative cells leads to *C. perfringens* gastroenteritis and pathology.

With proper refrigeration, germination of *C. perfringens* spores, propagation of the pathogen in the food matrix and food-borne intoxication can be avoided. The ENDOLYSIN product effectively controls *C. perfringens* in situations when the food is not properly prepared or stored (i.e., temperature abused). The studies reported herein attempted to simulate improper temperature storage of susceptible foods.

Importantly, for most lysins evaluated at application rates ranging from 0.05 to 1.0 µg lysin/g of turkey or beef meat (0.05 to 1.0 mg/kg meat), the antibacterial technical effect was observable from the first post-application time point of T = 2 h until the 18 h sampling point, when the growth of lysin-surviving bacteria is clearly evident (Figure 2-12 to Figure 2-18). These experiments demonstrate that application of the plant-made lysins over a wide range of 0.05 to 10 mg lysin/kg food, results in rapid bactericidal control of

pathogenic *C. perfringens* applied to samples of cooked poultry or beef meat, and that the technical effect observed is transient, lasting for approximately 2 to 18 h post application and perhaps up to 43 h (in the case of lysin ZP278), but not longer, depending on the storage conditions. Control of *C. perfringens* in temperature-abused food would be most important during a relatively short duration, as growth of other microorganisms over longer storage times without refrigeration would lead to spoilage and preclude consumption. Our results suggest that addition of ENDOLYSIN to foods post cooking or prior to serving could reduce viable cells of the pathogen during the susceptible window when *C. perfringens* is likely to proliferate, including from germinating spores.

ENDOLYSIN application should not interfere with the organoleptic properties of cooked foods. The ENDOLYSIN formulation is applied at a very low application rate of $\leq$10 ppm initially and dissolves or diffuses in the food matrix post application. There is no color-masking of food after application, and solutions of ENDOLYSIN are generally clear and have no objectionable odor. No organoleptic evaluation was conducted for this Notice, but organoleptic assessment will be conducted later in product development.

No special handling procedures or protective measures are anticipated when preparing, applying or disposing of the ENDOLYSIN product, and none are suggested from the public literature. The proteins and excipients used in ENDOLYSIN formulations are either GRAS or food-grade. As a precaution, basic eye (goggles), respiratory (mask) and skin (gloves) protection could be implemented during handling of bulk powders or during preparation of bulk solutions of ENDOLYSIN, and during mixing, transfer or disposal of the product. A draft Safety Data Sheet (SDS) for ENDOLYSIN is shown in APPENDIX A and provides general guidance on safe handling practices.

3 Dietary Exposure

The application rate of ENDOLYSIN should not exceed 10 mg ENDOLYSIN per kg of treated food. Application rates of 1-10 mg ENDOLYSIN/kg meat product have been shown effective in controlling enteropathogenic strains of *C. perfringens* on cooked mammalian- and avian-sourced meat cuts, as detailed in Section 2.4.2.

3.1 Estimated Dietary Intake of Selected Meats

Meats and gravy are typical foods associated with *C. perfringens* gastroenteritis (CDC 2017). To assess consumption of foods susceptible to *C. perfringens* contamination and subsequently estimate the amount of lysin exposure of consumers from foods treated with ENDOLYSIN, 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 2014a). 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 2015) 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 2014b) 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 variation in reported in 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

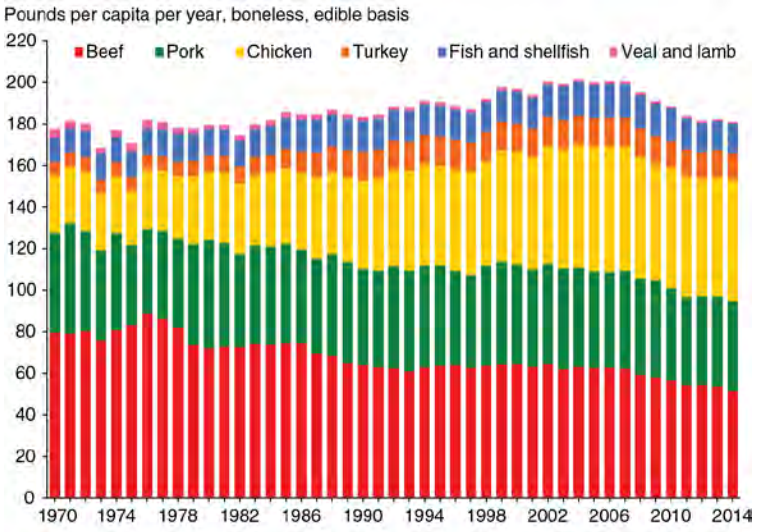
[3] NHANES extrapolates consumption from a single day survey of typical dietary intake

[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.

Gravy and meat sauces. It was more difficult to obtain data on per capita consumption of "gravy" or "non-preserved/non-pasteurized meat sauces" of the types that are susceptible to *C. perfringens* contamination. Gravy/meat sauce can be obtained in ready-to-eat form from retail stores, as dry mixes to be finished at home, or prepared by consumers from pan drippings during meat preparation at home. Because of this range of gravy preparation practices, no reliable data could be obtained on annual gravy consumption per capita. To compensate for this disparity in data sourcing relative to information on meat consumption, the per capita meat consumption figures for meat were increased by 5% by weight in an effort to approximate gravy consumption. If this is an overestimate, then the total consumption of meats and gravy, and hence total exposure estimates for lysins added to food, would be exaggerated rather than underestimated.

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. The graphic reproduced in Figure 3-1 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 ENDOLYSIN exposure calculations, we assumed a high red meat consumption value of 120 lbs/person-year or 5.3 oz/person-day (55 kg/person-year; 150 g/person-day), and a poultry consumption value of 75.1 lbs/person-year or 3.29 oz/person-day (34.1 kg/person-year or 93.4 g/person-day). This leads to an additive estimate of 195.1 lbs of all meats/person-year (8.59 oz/person-day), or 89.1 kg/person-year (243.4 g/person-day). To account for gravy estimates, as discussed above 5% w/w was added to the overall meat consumption values in an effort to approximate gravy consumed with meat-containing foods. In metric units, this yields an estimated total per capita consumption 255.6 g/person-day (i.e. 243.4 g total meat/person-day + 12.17 g (5 % of 243.4 g gravy estimate) = 255.6 g/person-day). In English units, this estimate translates to about 208 lbs/person-year, or 9.2 oz/person-day.

For convenience, the calculated estimate for all subpopulations was rounded upwards to **260 g/person-day**. Obviously, not every person (or age group) will consume the same quantities or ratios of red meat and poultry plus gravy on a given day, but the estimated consumption values allow for a calculation of average consumer exposure to applied lysins.

3.2 Dietary Intake of Endolysins (exposure) from ENDOLYSIN-Treated Food Products

The projected exposure of ENDOLYSIN from ingestion of treated meat and gravy products was calculated as follows using the per capita statistics summarized in Section 3.1 and the maximum (worst case) application rate of ENDOLYSIN (i.e. 10 mg/kg food; Section 3.3) to various cooked products during food preparation.

Total cooked meat + gravy consumed per day per person (rounded off to nearest whole unit):

$$\frac{208 \text{ lbs}}{\text{year}} \times \frac{\text{year}}{365 \text{ days}} \times \frac{1000 \text{ g}}{2.2 \text{ lb}} = \frac{260 \text{ g treated food}}{\text{person-day}}$$

At a projected maximum ENDOLYSIN application rate of 10 mg/kg treated food, the highest amount of ENDOLYSIN active ingredient consumed would be:

$$\frac{10 \text{ mg ENDOLYSIN (max)}}{1000 \text{ g food}} \times \frac{260 \text{ g treated food}}{\text{person-day}} = \frac{2.6 \text{ mg ENDOLYSIN consumed}}{\text{person-day}}$$

The total projected per capita maximum intake of ENDOLYSIN active ingredients from consumption of treated meat and gravy products would be about **2.6 mg per day (<1 g/year)**.

3.3 Dietary Exposure to Host- and Process-Derived Impurities

Food species of plants such as spinach, red beet or lettuce as well as non-food species can be used in the manufacture of endolysins. Residual host plant and process impurities in the ENDOLYSIN product when produced in food species present no safety concerns. The use of non-food plant species such as *N. benthamiana* as manufacturing hosts introduces the potential for new risks not found when endolysin production is done using food species. A detailed description of risk factors and their mitigation can be found in GRN 775, which describes Notifier's COLICIN food antimicrobial produced in *N. benthamiana* using essentially the same process described in APPENDIX B of this Notice.

On a protein basis, the ENDOLYSIN product consisting of a blend of endolysins produced from *N. benthamiana* has a target purity of ≥70%. The residual host-derived constituents of chromatographically purified endolysins are proteinaceous, and there are no unusual proteins in the host that would introduce risk, especially at such low levels (Leffingwell 1999). At application rates of ≤10 mg endolysin protein/kg food treated, proteinaceous host-derived impurities introduced to the treated food would be ≤3 mg/kg food (≤3 ppm). With a US meat consumption average of 0.26 kg/day, the **exposure to host-derived proteins would be ≤0.8 ppm/day**, which is an insignificant level and not expected to impact either safety or nutritional content.

The remaining salts derived from the buffer (citrate, phosphate, NaCl) are safe and allowed for food use. Hence, we assess that non-alkaloidal host- and process-derived impurities in *N. benthamiana*-produced ENDOLYSIN would pose little to no risk to consumers.

The main impurities of concern are nicotine and anabasine, given their presence in *N. benthamiana* and their toxicity profiles. The other alkaloids potentially present, namely nornicotine and anatabine, have similar activities but are present at very low levels in the plant and pose no significant risk (GRN 775). Hence, our focus has been on assessing risk from the two main alkaloids present. Using residual values for nicotine and anabasine defined in Section 6 and obtained from the process modification defined in Stephan (2017) and further described in APPENDIX B, and the dietary intakes of cooked meat products defined herein, we estimated the per capita daily exposure levels.

Endolysin blends do not need to contain all lysin candidates because these enzymes exhibit overlapping activities and functionalities. Further, each purified lysin has a different level of residual alkaloid due to slight differences in extraction buffers used. Hence, endolysin blends can be formulated to provide the needed suitability while maintaining safe levels of residual alkaloids. For example, a lysin blend may consist of 3 mg total lysin (e.g. 1 mg ZP173 + 1 or 2 mg CP25L) applied per kg food (or other lysins depending on the salinity and pH of the treated food). Our data show that effective control of *C. perfringens* can be achieved even at low application rates of individual endolysins and endolysin blends (i.e. 0.05 mg lysin protein/kg treated food). Using average values for residual nicotine (76 ng/mg lysin) and anabasine (2.1 ng/mg lysin) (Table 6-1), the potential of alkaloid intake was estimated to derive both average and maximum potential exposure values. To estimate a maximum alkaloid level of exposure, the maximum total endolysin application rate of 10 mg/kg food was used. Likewise, the average alkaloid exposure value was estimated from an average expected lysin application rate of 3 mg/kg food.

The **average** (at 3 mg/kg lysin application rate) and **maximum** (at 10 mg/kg lysin application rate) consumer exposure to residual alkaloids was calculated from the per capita daily consumption of cooked, ENDOLYSIN-treated meat, as shown in Table 3-2.

Table 3-2. Estimated alkaloid exposure from consumption of ENDOLYSIN-treated meat

	ENDOLYSIN Treated Meat
Daily food consumption	260 g meat/day
Nicotine in treated food	Ave 228 ng/kg Max 760 ng/kg
Daily nicotine intake	**Ave <60 ng/day** **Max <200 ng/day**
Anabasine in treated food	Ave 6.3 ng/kg Max 21 ng/kg
Daily anabasine intake	**Ave <1.7 ng/day** **Max <5.5 ng/day**
Total alkaloid intake	**Ave <62 ng/day** **Max <206 ng/day**

Daily per capita food consumption estimates were derived from multiple public sources, as detailed in Section 3.1 of this notice. Nicotine and anabasine estimates were derived from average levels measured by Notifier in 5 different purified endolysins (see Section 6). **Average** residue levels were calculated for a 2-3-endolysin mix at the ratios indicated in the text. **Maximum** residue levels were estimated for a maximum colicin application rate of 10 mg colicin/kg food, proportionately increasing the levels of each alkaloid. Multiplying the level of alkaloid applied per kg of food times the daily per capita consumption of cooked meat yields the estimated intake level of each alkaloid.

It bears mentioning that *N. benthamiana*-produced lysins can be blended with different lysins produced in food plant-species to further increase potency or host range; in such instances the level of *N. benthamiana* alkaloids in the final ENDOLYSIN product would be even lower due to dilution. For perspective, several studies have reported the average per capita daily food-borne exposure to nicotine from consumptions of common vegetables (e.g., tomato, eggplant, peppers) as 1,000 ng/day (i.e. 1 µg/person-day) (Andersson 2003; Davis 1991; Domino 1993; Liu 2013; Moldoveanu 2016; Nielsen 2013; Siegmund 1999). In a "worst case" scenario where all meats are treated with a maximum of 10 mg/kg *N. benthamiana*-produced ENDOLYSIN, and the product achieves 100% market penetration, exposure to solanaceous alkaloids from ENDOLYSIN would be only about 1/5 of what is currently, and safety, consumed in the average diet.

3.4 Additional, Natural Exposure to Endolysins (Intake Not Related to ENDOLYSIN Product)

Exposure of consumers to various endolysins (those controlling *C. perfringens* and other bacteria) would be expected from a variety of sources, including ingestion of foods and beverages, given the ubiquitous nature of bacteriophages.

Small amounts of endolysins could be present in foods that may be treated with bacteriophage mixtures applied in existing antibacterial products, and from chance environmental exposure. No studies were found in the literature that reported the exposure to lysins from other products or from environmental sources.

Given the instability of lysins in the environment, including food, absent a peptidoglycan or bacterial target, the expectation of routine consumer exposure to lysins is plausible albeit at very low levels. Sources of potential exposure other than from Notifier's product could include:

- Exposure from foods previously treated with bacteriophage mixtures
- Natural exposure to bacteriophages adsorbed to domestic animals, soil and water samples
- Natural exposure from lytic phage-susceptible bacteria naturally present in food

Table 3-3 summarizes exposure to lysins and estimates the intake by food source. A maximum application rate of 10 mg total lysins per kg food products (10 ppm; worst case) was used in the calculations.

A worst-case intake estimate for meats is provided and assumes exposure to ENDOLYSIN from consumption of appropriately treated cooked meats. Also provided are intake estimates from treated meats and other products that may be re-heated or re-cooked prior to consumption. Endolysins are destroyed by high-temperature heating (cooking/re-heating) and such practices would essentially reduce endolysin levels to zero, regardless of their source, as is also shown in the table. Endolysins are also susceptible to gastrointestinal degradation.

For the purpose of estimating risk, the exposure to lysins not derived from treatment of food with Notifier's product was assumed to be 15% of the level applied via ENDOLYSIN treatment, or **<0.4 mg/person-day**. In actuality, the level is likely much lower for the reasons stated above.

Table 3-3. Estimated human daily exposure to endolysins from all food sources

Source of exposure	**Estimated daily per capita lysin exposure**	
	From treated food post cooking or other sources	**From treated food that is re-heated/re-cooked**
ENDOLYSIN treatment, total of all meat and gravy products consumed (detailed in this GRN; derived from Table 3-1)	**2.6 mg**	**nil**
Bacteriophage antibacterial treatments; natural exposure from food and environmental sources	**0.4 mg**	**nil**
Total (estimated maximum, all food sources)	**3 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

None.

5 Experience Based on Common Use in Food Before 1958

None and not applicable. ENDOLYSIN has not been used in foods before.

6 Basis for Conclusion of ENDOLYSIN's GRAS Status

6.1 Overall Safety of Endolysins

Notifier has used scientific procedures to conclude that its ENDOLYSIN food antimicrobial product (comprised of one or more endolysin proteins) is GRAS under the conditions of intended use. Information supporting our determination of ENDOLYSIN as GRAS for use as an antimicrobial on meat and related cooked or baked food 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 meats, are described separately in this Notice, specifically in Section 2 and Section 3, respectively.

Historical and technical bases were used to support the conclusion that plant-made lysin proteins (singly and/or in combination in an ENDOLYSIN blend) are GRAS. Endolysins are the tools uses by bacteriophages to lyse their host cells, escape, and spread to other cells at the end of their replication cycle. It is widely accepted that humans have a very long history of exposure to bacteriophages from various natural sources, including exposure from human commensal and domestic animal microflora. Bacteriophages are the most abundant organisms on earth, with an estimated total population of 10^{31-32} (Schmelcher 2012). More than 100 million phage species are estimated to exist.

Since shortly after birth, humans are exposed to and colonized by bacteriophages throughout their lives. As reviewed by Barr (2017), phages colonize all body niches, including the skin, oral cavity, lungs, gut, and urinary tract. In the gastrointestinal tract alone, there is an estimated residency of 2 trillion ($2x10^{12}$) phages. Therefore, phages are an integral part of the human virome.

Phages are amply consumed in food; they have been isolated from drinking water and from a wide range of food products. As reported in multiple studies reviewed extensively by Sillankorva (2012), poultry products, processed foods like ground beef, sausages, fish, fruits and vegetables, cheese and other dairy products often contain more than 10^8 phages/g of food. Several studies have suggested that all or nearly all of the ground beef and chicken meat sold contain various levels of multiple bacteriophages. Phages also have been found in animal feed. Therefore, humans consume phages daily by drinking water and by eating unprocessed foods.

This perspective on consumer exposure to phages underscores the fact that each phage will make hundreds or thousands of copies of one or more endolysin during its replication cycle. Hence, it follows that commensal, food-borne, and environmental exposure to phages equates to pervasive exposure to endolysins.

Therefore, through evolution as well as from traditional practices used in food cultivation, preparation and consumption, humans have likely been chronically exposed to lysins from food and environmental sources for millenia. The published studies cited above suggest that the level of human exposure to lysins from

various foods is likely consistent (chronic) due to dietary and cultural practices. Unfortunately, no literature reports were found that estimated the natural level of endolysin exposure. Studies by Drulis-Kawa (2015), and older studies by Groman (1963) and Protass (1966) suggest that phage infection of their bacterial hosts is a tightly regulated, energy efficient process to prevent catastrophic collapse of susceptible host populations. Hence, a single infective virion may give rise to 10^2 to 10^3 viral particles per bacterial cell (Groman 1963), reproducing linearly. If each viral genome gave rise to 2-10-times their copy number in one or more endolysins, each cell could contain 10^3-10^4 endolysin molecules. Theoretically, at a bacterial density of only 10^3 CFU/g food consumed raw, ingestion of up to 10^4 endolysin molecules per gram of food is conceivable. Given that the average MW of a Gram-positive-tropic lysin is ~40 kDa, this would be an insignificant amount of lysin protein consumed per gram of food.

Higher levels lysins than those produced by phages naturally as "background" are expected to be consumed from multiple food protection interventions, including from ENDOLYSIN-treated meats and potentially also from meats pre-treated or co-treated with bacteriophage-containing antimicrobials, such as products described in several GRAS notices (e.g. Intralytix's GRN 528 ListShield®; GRN 435 SalmoFresh®; GRN 672 ShigaShield®, and others; and Micreos' GRN 218 for PhageGuard® products, and others).

Such phage-containing products are typically applied to food prior to cooking, yet their application could increase the total daily exposure to lysins. However, because properly cooking food would destroy phages and lysins, as heat denatures and destroys all protein from mesophilic organisms, and ENDOLYSIN would be added to foods post cooking, the principal exposure to endolysins is projected to be from Notifier's product.

Estimates of potential exposure from all sources are included in Table 3-3. These estimates take into account that the majority of exposure would be from properly applied ENDOLYSIN post cooking, and that most meat dishes would be consumed cooked, with a minority being consumed raw. Hence, because phages and their lysins are not thermostable to cooking temperatures, the anticipated intake of active endolysins (from all sources) is expected to be very low.

The estimates in Table 3-3 include a worst-case scenario where all the ENDOLYSIN in treated food survives to ingestion of the food, and an alternative scenario where the treated food is re-heated or re-cooked to proper temperature prior to serving, which would essentially destroy all lysins. The maximum daily intake per capita of lysins from ENDOLYSIN and other sources can be estimated to be ~3 mg.

Because there are no mammalian including human cellular or tissue targets for endolysins, the ingested protein would be processed (digested) as any other food protein and degraded in the acid environment of the stomach and through the action of gastroduodenal enzymatic activities (shown in Section 2.4.4). The amount of protein ingested from lysins applied to food would be insignificant; hence, use of the ENDOLYSIN product would not contribute measurably to dietary protein intake.

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

Importantly, the physicochemical properties of endolysins, notably their instability to heat (the most heat tolerant lysins studied, such as ZP173, are denatured at <55 °C) and their susceptibility to enzymatic degradation and clearance *in vivo* (Fischetti 2005, 2010; Garcia 2008; Loeffler 2003; Nelson 2012; Oliveira 2012; Resch 2011; Rios 2016; Schmelcher 2012) such as would occur in the gastric and upper intestinal environments, contribute significantly to their safety profile and support the use of lysins as food preservatives and food antimicrobials.

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

Multiple plant hosts can be used in the manufacture of endolysin 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 endolysins, often with more favorable economics 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 present potential safety concerns. These potential toxicants need to be removed or diluted to ensure that the final product is safe. *Nicotiana* species synthesize a number of bioactive substances, some of which are toxic in sufficiently 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 (e.g. tomato, pepper, eggplant, etc.) and from foods treated with Notifier's antimicrobials was included in GRN 775 (Section 6.1, pp 32-39). That document is publicly available and hence the same discussion is not repeated here; however, we included 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 endolysins applied to food these two alkaloids are not a risk. The 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 crude extracts and purified endolysins expressed in *N. benthamiana*. The levels reported in this table were used to calculate alkaloid intake levels on the basis of ENDOLYSIN application rates and per capita consumption of treated foods. Those estimates are presented in Section 3.3. In a "worst case" scenario where all cooked meats are treated with a maximum of 10 mg/kg *N. benthamiana*-produced ENDOLYSIN and the product achieves 100% market penetration, exposure to solanaceous alkaloids from ENDOLYSIN would be only about 1/5 of what is currently, and safety, consumed from vegetables in the average diet.

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

Sample	Sample Content (lysin, purity, and concentration)	Nicotine Calculated Concentration (µg/ml)	Anabasine Calculated Concentration (µg/ml)	Nicotine Calculated Concentration (ng/mg protein)	Anabasine Calculated Concentration (ng/mg protein)
1	ZP173 (Plant extract) 400µl, 0.55 mg/ml	73.000	26.60	132727	48363
2	ZP278 (Plant extract) 400µl 1.275 mg/ml	84.500	18.40	66274	14431
3	Psm (Plant extract) 400µl 0.55 mg/ml	183.500	51.30	333636	93273
4	CP25L (Plant extract) 400µl 0.925 mg/ml	174.500	24.90	188649	26919
5	Ply26F (Plant extract) 400µl 1.05 mg/ml	123.500	49.40	117619	47048
6	Ply390 (Plant extract) 400µl 0.825 mg/ml	200.000	40.90	242424	49576
95	ZP173 (purified) 200µl 0.7 mg/ml	0.014	< LOQ	20	< 1.4
99	ZP278 (purified) 200µl 0.9 mg/ml	0.182	< LOQ	202	< 1.1
112	psm (purified) 200µl 0.52 mg/ml	0.028	< LOQ	54	< 1.9
110	CP25L (purified) 200µl 0.31 mg/ml	0.014	< LOQ	45	< 3.2
136	Ply26F (purified) 200µl 0.32 mg/ml	0.029	< LOQ	91	< 3.1
72	Ply390 (purified) 200µl 0.5 mg/ml	0.022	< LOQ	44	< 2
	Average content from all purified lysins			**76**	**< 2.1**

Table 6-1 shows the residual content of nicotine and anabasine in crude extracts (top rows) and chromatographically purified endolysins (bottom rows) expressed in *N. benthamiana*. The alkaloids were analyzed by HPLC-MS/MS (APPENDIX C). The method has lower limits of quantitation (LOQs) of 0.005 µg/ml for nicotine (5 parts per billion) and 0.001 µg/ml for anabasine (1 part per billion). Because the various lysins shown in the table can be blended in different ratios, the average content of nicotine and anabasine was quantified for all purified lysins (shaded area) and listed as average values (bottom row).

6.3 Low Safety Risk from Consumption of Process Impurities in Endolysins

The process modification in the manufacture of antimicrobial proteins from *N. benthamiana* is described in Stephan (2017) and in APPENDIX B. The same gene expression options are available with *N. benthamiana* as with food species hosts (Schulz (2015); GRN 593, GRN 676 and GRN 775). Biosynthesis of endolysin 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 endolysins from *N. benthamiana* are very similar to those used with food species, and all are commonly used in food processing. Hence, there are no additional biologic or abiotic risks introduced in the manufacturing the final product when using *N. benthamiana* as the host.

6.4 Low Potential for Development of Bacterial Resistance to Endolysins

We have also considered the potential for development of resistance to endolysins by exposed but surviving *C. perfringens* and its implications on safety. Phage endolysins are not known to be environmentally persistent, and are degraded by cooking and by gastrointestinal processes (Nelson 2012; Schmelcher 2012). In extensive reviews on the properties of endolysins, the case is made for natural

selection of endolysins' short half-lives: The molecules are not persistent; hence it is more difficult for bacterial resistance to develop (Loeffler 2003; Nelson 2012; Resch 2011). In nature, bacteriophages only produce lysins during the terminal steps in their replication cycle, and these steps are known to be tightly controlled and regulated (Pohane 2015; Young 1992).

In addition, lysins that target Gram+ bacteria such as *C. perfringens* and other clostridia have binding domains to peptidoglycan motifs that adhere the enzymatic domains tightly to cell wall components; hence, it is estimated that very small quantities of lysins exist free of their cell-wall targets in natural environments (Schmelcher 2012). This would act to reduce exposure of the pathogen to subtoxic doses of lysins; a scenario that might encourage development of resistance.

Similarly, adding lysins to foods exogenously would lead to dilution and eventual degradation of the lysins unless they encounter a susceptible target, namely, the *C. perfringens* cell wall. In that event, the lysins would adhere to the cell wall through their binding domain and proceed to degrade the wall through the enzymatic (catalytic) domain. Once the target is degraded, the lysins would themselves be degraded by food matrix enzymes, or by re-heating or re-cooking treated food. Consequently, little free lysin is expected in food protection scenarios.

In addition, the widespread use of broad-spectrum antibiotics (e.g., penicillin and tetracycline) has accelerated the distribution of resistance genes within the bacterial community by placing selective pressure not only on the target pathogen, but also on commensal organisms (Nelson 2012). In this regard, the genus- or species-specificity of most endolysins presents an important advantage over classical broad-range antibiotics (Schmelcher 2012).

As Fischetti (2010) pointed out, the co-evolution of bacteriophages and their hosts is believed to have led to lysins binding to and cleaving highly conserved and highly immutable targets in the host bacterium cell wall, presumably making the formation of resistance a rare event and thereby ensuring survival of the phage. The extracellular nature of the target peptidoglycan also limits the number of possible mechanisms of resistance to lysins when applied from outside the target cell. This is in sharp contrast to the resistance mechanisms that arise from using compounds that typically target bacteria by acting inside the cell (Schmelcher 2012).

We conclude that the use and consumption of lysins through ingestion, regardless of level, is unlikely to allow for selection of lysin-resistant *C. perfringens* or of lysin-resistant commensal bacteria in the intestinal tract of humans, for three main reasons:

- Ingested lysins will be inactivated and/or denatured by the low pH of the stomach;
- Endolysin-class proteins will be rapidly digested by proteases in the upper and mid gastrointestinal tract before they reach the colon where commensal bacteria might be affected; and
- The lysins used in Notifier's ENDOLYSIN product are specific for *C. perfringens.*

Therefore, we anticipate that exposure of the resident bacterial microflora in the colon to intact lysins derived from ingestion of foods treated with our product will be highly unlikely. And even if the lysins were to survive to reach the colon, they would only attack *C. perfringens*, which is a minority resident of the colonic microbiome (Todar 2012).

6.5 Low Potential for Allergenicity or Immunogenicity by Endolysins

Although recombinant endolysins have the same amino acid sequence as natively produced bacteriophage endolysins, to which humans and other animals have been exposed throughout evolution, allergenic or immunogenic reactions cannot be ruled out *a priori* because lysins are, after all, foreign proteins. As summarized in Section 2.4.4, it is highly unlikely that ingestion of significant amounts of lysins 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 lysins for known immunogenic/allergenic domains against databases of known allergenic epitopes (e.g. AllergenOnline (2015)). Proteins with greater than 50% identity to known allergens are considered potentially allergenic (Aalberse 2000). CODEX Alimertarius (CODEX Alimentarius 2003), lists 35% as the minimum homology required for categorizing a protein as potentially allergenic.

More detailed sequence searches, at the 80-mer and even at the 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 six lysins that could comprise the ENDOLYSIN product formulation were analyzed using the AllergenOnline database v17 (January 2017; accessed November 18 and 19, 2017; http://allergenonline.org/databasefasta.shtml) to assess whether Notifier's lysins shared aa sequences with known allergens, including newly identified allergens.

PlyCP26F (212 aa)

MIIGSRYGHSENCRGAKGLRDEVDAMKPLHFEFKKIMEQYGHTIIDCCSNANTQNGELSEGARKANAQILDLFISWHGNKGGGQGCEA
WIANNSRAKPYAERMCKNFSSLGFKNRGVKYSDKYYEMRNINAPNIIFETLFLDSEKDISIWSPIPYEVMARYLANAIDPNIPLEKEQDYYR
VCVQRFTNKEDAEKAQQRISNELGYYCFAEKI

PlyCP39O (213 aa)

MKIALRGGHSPNCKGANVLRDEQSCMWALADEVEKVLTSHGHTVVRCETTLSNEREDVRQGAKKGYNCDMFISLHMNASDGRGNGT
EAWVARSARSSIKEIASRLCKNYATLGLQNRGVKEKNYWEMTDTNCPNIIFETMFCDDKHDIDIWASTSWDKLARLIANAIDPNIPLEKE
QDYYRVCVQRFTNKEDAEKAQQRISNELGYYCFAEKI

Psm (342 aa)

MQSRNNNNLKGIDVSNWKGNINFESVKNDGVEVVYIKATEGNYFKDKYAKQNYEGAKEQGLSVGFYHFFRANKGAKDQANFFIDYLN
EIGAVNYDCKLALDIETTEGVGVRDLTSMCIEFLEEVKRLTGKEVVVYTYTSFANNNLDSRLGNYPVWIAHYGVNTPGANNIWSSWVGF
QYSENGSVAGVNGGCDMNEFTEEIFIDSSNFNLDNATTKNVSTKLNIRAKGTTNSKIIGSIPAGETFKIKWVDEDYLGWYYVEYNGVVGY
VNADYVEKLQMATTYNVSTFLNVREEGSLNSRIVDKINSGDIFRIDWVDSDFIGWYRITTKNGKVGFVNAEFVKKL

CP25L (377 aa)

MYINQSNIKFNGLRYGNDPNKIIIHNADATSCSVYDIDRWHKGNGWSGIGYDYFIRKEGSVWTGRPENAIGAHTIGQNSSSIGICLEGAF
MREKPTRAQLNSLYELIADIRARRGNLPVYGHKDFNNTDCPGINFPLEQFKNNSYRPTGGEIVSDNGFYRSDEERTNATIVGEGNIEVLDK
NCKVIENRYISSLDRVFVLGIYPASKYIEIIYPAGNEKYHAYISIENYSRISFDYHMQYKNDNGVTYVWWDSEDVNVKEHNEELQANQKAS
PMYRVGKWLRVTFYRTDGTPSDGFVRYEGEQAVKFYEEEKIKEGIVKVNTYLNVRDSINGNIIGKVFNGEEVSIIWTKDGWYYIDYNTNH
GKKRGYVSSKYVEEV

ZP173 (335 aa)

MLKGIDVSEHQGRIDWERVKGNIDFAILRAGYGRNNIDKQFIRNIEECNRLCIPVGIYWFSYAWNEEMAKNEARYVLEAIKGYRVDYPISY
DLEYDTLNYASKNGVTIGKRLATDMVKAFCDEINRNGYRAMNYTNQDFLLNKFYMNELTNYPLWYAWYNSKLNRDCAIWQYSENGQ
VPGIPGSSVDMNYCYEDFLKKDFTLENATTCNVDTELNIRAKGTTGATIVGSIPAGDRFRIKWVDSDYLGWYYIEYQGITGYVSQDYVEKL
QMATTCNVDSVLNVRAEGNTSSNIVATINPGEVFRIDWVDSDFIGWYRITTANGANGFVKSDFVKKL

ZP278 (351 aa)

MQSRSDSNFKGIDISNWQKGINLNQLKERGYDVCYIKITEGKGYVDPCFEENYNKAIAAGMKVGVYHYWRGTSSAIEQANNIVRTLGNK
HIDCKIAIDVEQTDGLSYGELNNSVLQLAEELERLIGAEVCIYCNTNYARNVLDSRLGKYSLWVAHYGVNKPGDNPIWDKWAGFQYSEN
GTSNVNGSLDLDEFTEEIFINKESSKVTENKLFSTNARALVALDPRDNPSDNYNDLGEIYEGERIQVLAEVCDKEDYLPVKYWKDSEGRES
GKVWIRSKQDYMMIDTYHRVFNVITQLDARYEPSSDSATMGYVKNGERLYVHRTEGNYSLCTYFAGNGYKTAWFTAKYLERI

The results of informatic searches are summarized.

Results

Endolysins 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 lysin was scanned for potentially allergenic or hypersensitivity inducing sequences. The results are summarized in Table 6-2 and described in the accompanying text for each lysin entry. Three of the six plant-produced lysins examined had similarities to known allergens of greater than 50% (Aalberse 2000) and therefore could be considered potentially allergenic.

However, Table 6-2 also shows similarity at the more stringent threshold of >**35% identity** (CODEX Alimentarius 2003). None of the lysins crossed the >35% identity threshold for allergenicity.

Table 6-2. Bioinformatic amino acid scan for potentially allergenic sequences in plant-made lysins

Lysin	≥35% allergen similarity at indicated search granularity? If yes, highest % similarity			Allergenicity Potential
	Full seq	80-mer	8-mer	
PlyCP26F	0	0	0	low
PlyCP39O	0	0	0	very low
psm	0	0	0	low
CP25L	0	0	0	low
ZP173	0	0	0	very low
ZP278	0	0	0	very low

PlyCP26F. A bioinformatic FASTA search of the full 212 amino acids of plant-produced PlyCP26F revealed only a distant relationship to 4 AllergenOnline database entries for bahiagrass (*Paspalum notatum*) pollen allergen sequences, all registering 59.5% similarity but only 25.0% identity.

A more specific bioinformatic comparison at the 80-mer level (sliding window of 133 80-mers in 212 aa) revealed no (zero) hits. An even more precise search at the 8-aa level revealed no (zero) exact matches. In sum, no amino acid sequences in lysin PlyCP26F are found to match known proteinaceous food allergens at >35% identity level and hence the allergenic potential of PlyCP26F should be considered low.

PlyCP390. A bioinformatic FASTA search of the full 213 amino acids of PlyCP390 revealed no sequence matches to known allergens. More precise sliding window 80-mer and 8-mer exact-match searches revealed no sequences with >35% identity to any known allergens. Hence, the allergenic potential of lysin PlyCP390 should be considered very low.

Psm. A bioinformatic FASTA search of the full 342 amino acids of lysin psm revealed 57.4% similarity but only 24.5% identity with a 229-aa protein from the nematode *Strongyloides stercoralis*. Sliding window 80-mer (263 80-mers in 342 aa) and exact match 8-mer searches revealed no (zero) similarities to any known allergen in the database. The allergenic potential of lysin psm should be considered low.

CP25L. A bioinformatic FASTA search of the full 377 amino acids of CP25L revealed only a distant relationship to a known allergenic protein from the saliva of the tsetse fly *Glossina morsitans* morsitans**;** specifically, there was 51.5% similarity but only 23.7% identity to antigen precursor 5 from *Glossina morsitans* saliva protein.

However, more specific sequence searches at the sliding 80-mer (298 80-mers in 377 aa) and exact match 8-mer level revealed no (zero) matches of >35% identity to any known allergens. Hence, the similarity of CP25L to tsetse fly saliva protein can be considered coincidental, and the allergenic potential of lysin CP25L should be considered low.

ZP173. A bioinformatic FASTA search of the full 335 amino acids of ZP173 revealed no matches to any entry in the database; likewise, 80-mer and 8-mer searches revealed no matches to any known allergens at the >35% identity level. The allergenic potential of lysin ZP173 can be considered very low.

ZP278. A bioinformatic FASTA search of the full 351 amino acids of ZP278 revealed no matches to any entry in the database; likewise, 80-mer and 8-mer searches revealed no matches to any known allergens at the >35% identity level. The allergenic potential of lysin ZP278 can be considered very low.

Conclusion

In the six endolysin proteins analyzed for sequence identity, comprising a total of 1,830 amino acids, there were no (zero) peptide sequences matching the sequences in known proteinaceous allergens.

Hence, the potential for allergenicity/hypersensitivity of plant-made lysins is considered low to very low. This low risk is corroborated by the lack of reports in the literature linking bacteriophages or endolysins with development of allergenicity or hypersensitivity.

Furthermore, intact endolysin proteins applied to food post cooking are not expected to survive 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, further lowering the concern over allergenicity.

In addition to the lysin active ingredients, the formulated product ENDOLYSIN may contain small amounts of proteinaceous residues derived from the host plant and/or the process of genetic induction (i.e. vector). When lysins are purified by chromatography in the downstream component of the manufacturing process, proteinaceous impurities would be present at low amounts (<30% of total protein) in the final formulation.

Considering that lysins would be applied to food at the 1-10 ppm level, any residual protein impurity that might pose an allergenic concern would be co-applied with lysins at <0.3-3 mg/kg food, or <0.3-3 ppm, and also face denaturation or degradation through food preparation and/or on-matrix digestion. Hence, the allergenic potential of any proteinaceous impurity in ENDOLYSIN is expected to be very low.

6.6 Safety in Relation to Dietary Intake of ENDOLYSIN

In Section 3 we summarized Notifier's estimated ENDOLYSIN application rate and projected potential intake based on consumption of cooked red meats, poultry and gravy treated with the product. The projected intake was 2.6 mg lysins/person-day from ENDOLYSIN treatment of food, assuming 100% market utilization of Notifier's product. We rounded that figure up to 3 mg/person-day to account for additional lysins that consumers may ingest from foods and the environment and/or from prior treatment of foods with bacteriophage-based antibacterial products. We estimated such additional exposure to be very low at 15% of the 2.6 mg from ENDOLYSIN, for a total of 3 mg/person-day total lysin exposure.

Each Gram+ lysin binds to specific sites on the peptidoglycan layer of the bacterial cell wall and each typically has a very low off-rate (Loessner 2002; Schmelcher 2010). This feature of Gram+ targeting endolysins provides a further safety advantage when used in food, because chelation to the cell wall would act to restrict distribution of the enzyme and shorten the duration of technical effect after binding.

If viewed as food (safety) enzymes, lysins 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 bio-chemical 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 endolysins can be considered one of their most desirable traits (Schmelcher 2012). The fact that these enzymes specifically destroy a target pathogen such as *C. perfringens* 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 0.05-10 mg lysin/kg food, even if all of the lysins were to survive in cooked foods and be ingested at the levels applied, daily per capita ingestion would be 2.6 to 3 mg lysin/person-day. Such a minute amount of lysin would represent <0.004% of the total adult daily protein intake, and is nutritionally insignificant.

No reports have appeared in the literature linking endolysin consumption (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 lysin protein exposure, including immune tolerance.

Importantly, the physicochemical properties of lysins, notably their instability to high heat and their susceptibility to enzymatic digestion (e.g. degradation in the gastric and upper intestinal environments; Nelson (2012)), contribute significantly to their safety profile and support the use of lysins as antimicrobials for food safety interventions.

Risk from consumption of other components of the ENDOLYSIN product, including residual host alkaloids and other host-derived or process impurities (e.g. residual buffers, salts), is deemed to be low. The impurities of highest concern would be nicotine and anabasine, and even at a maximum application rate of 10 mg/kg food at 100% product market penetration, the total additional per capita ingestion of alkaloids would be about 1/5 the amount currently consumed in the average diet (Section 3.3).

7 Supporting Data and Information

Multiple sources of information were used to support the conclusion that the ENDOLYSIN product is GRAS. Table 7-1 lists the various data and other information discussed in this Notice and used in reaching this conclusion, and the source and availability of the information (i.e. Notifier-generated or public domain).

Table 7-1. Information supporting ENDOLYSIN GRAS determination

Topic	Document	Location	Source	Availability
C. perfringens biology; conditions for contamination of food	This Notice	Introduction, pg 1 Section 2.4, pp 19, 28, 31	(Albrecht 2017; Todar 2012; Xiao 2012)	Public
Source, traits and selection of target *C. perfringens* strains	This Notice	Table 2-3, pp 19-21	(Boyd 1948; Eastoe 1959; Epps 1945; Hobbs 1953; Nakamura 1976)	Public
Endolysins' mode of action; specificity; spectrum of activity	This Notice	Section 2.1, pp 12-13 Table 2-1, pg 12	(Briers 2007; Cheng 1994; Fischetti 2010; Gervasi 2014; Loessner 1995; 2002; 2005; Lopez 2004; Nariya 2011; Schmelcher 2010; 2012; Schmitz 2011; Simmons 2010)	Public
History of human exposure to phages and/or endolysins	This Notice	Section 1, pg 9	(Barr 2017; Drulis-Kawa 2015; Fischetti 2010; Hermoso 2007; Jun 2013; Loeffler 2003; Nakonieczna 2015; Nelson 2001; 2012; Rios 2016; Roach 2015; Schmelcher 2012; Sillankorva 2012; Todar 2012; Yang 2014)	Public
Human exposure to phage lysins from food	This Notice	Section 6.1, pp 54-55	(Barr 2017; Drulis-Kawa 2015; Groman 1963; Protass 1966; Schmelcher 2012; Sillankorva 2012)	Public
Human exposure to phage lysins from phage therapy and phage antimicrobials	This Notice	Section 2.3, pp 16-17	(Barr 2017; Fischetti 2005, 2010; Hermoso 2007; Jado 2003; Loeffler 2003)	Public
Safety of enzymes added to food; lack of glycosylation; low risk from minor protein modifications	This Notice	Section 2.3, pg 16 Section 6.6, pp 62-63	(Kamionka 2011; Sewalt 2016) (Federal Register 2010)	Public
Safety of endolysin 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 GRN 775	Section A.2.2, pp 26-28 Section 6.1, pp 28-39	Multiple references	Public

Topic	Document	Location	Source	Availability
Safety of manuf. process	GRN 593 GRN 775	APPENDIX B, pp 51-57 Section 6.1, pp 32-38	Notifier	Public
Safety of host and process impurities	GRN 593 GRN 775 This Notice	Section A.2.2, pp 27-28 Section 6.1, pp 32-39 Section 3.3, pp 51-53 Section 6.2, pp 56-57 Section 6.3, pg 57	(Andersson 2003; Davis 1991; Domino 1993; Leffingwell 1999; Liu 2013; Moldoveanu 2016; Nielsen 2013; Siegmund 1999; Sisson 1990; Stephan 2017)	Public Public To be made public via this GRN
Lysin digestibility / degradability	This Notice	Section 2.4.3, pp 42-46	Notifier	To be made public via this GRN
Low potential for development of bacterial resistance	This Notice	Section 6.4, pp 57-58	(Fischetti 2010; Loeffler 2003; Nelson 2012; Pohane 2015; Resch 2011; Schmelcher 2012; Young 1992)	Public
Low allergenicity or immunogenicity	This Notice	Section 6.5, pp 59-62	(Aalberse 2000; AllergenOnline 2015) Calculated by Notifier	Public To be made public via this GRN
Safe ingestion estimates of lysins applied to meat and other foods from ENDOLYSIN product	This Notice	Section 3, pp 48-53 Table 3-2, pg 52 Table 3-3, pg 53	Intake calculated from application rates and meat consumption data from: (USDA ERS 2014a, 2015); CDC NHANES (Daniel 2011; DeBruicker 2011; Wang 2009); (USDA ERS 2014b); (USDA WASDE 2016); (USDA 2015 Dietary Guidelines); (USDA ERS 2017)	Public
Safety from additive consumption of lysins from applied ENDOLYSIN product and from other sources of lysins	This Notice	Section 3.4, pg 53 Table 3-3, pg 53	Notifier	To be made public via this GRN

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APPENDIX A. ENDOLYSIN Safety Data Sheet

NOMAD

ENDOLYSIN SAFETY DATA SHEET
Version 1.0
DRAFT – Date 01 March 2018
Print Date 03 March 2018

1. PRODUCT AND COMPANY IDENTIFICATION

1.1 Product identifiers

Product name : ENDOLYSIN, mixture of bacteriophage endolysins produced in plants
: Contains one or more of the following proteins (Registry Number = GeneBank Entry No): PlyCP26F (YP_007004008), PlyCP39O (YP_002265435), psm (ZP_02640173) CP25L (YP_008058948), ZP173 (WP_003469359; ZP_02640173), and ZP278 (WP_003469445; ZP_02640278)

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

Identified uses : Antibacterial for *Clostridium perfringens* to be used in foods post-cooking or baking

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

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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 solid product or solutions 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. TOXICOLOGICALINFORMATION

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

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. ECOLOGICALINFORMATION

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

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
Endolysins from plants. See Section 1.1

New Jersey Right to Know Components
Endolysins 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. ENDOLYSIN Manufacturing Process Summary

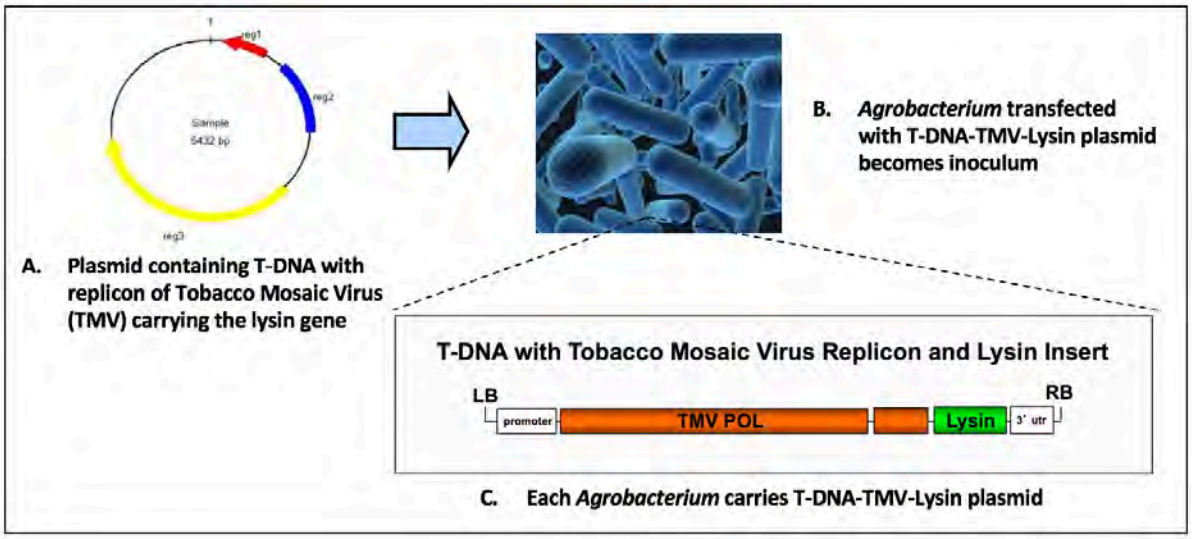
B.1 Introduction and Rationale

Notifier manufactures endolysin proteins recombinantly using a plant-based process very similar to the process described in GRN 593, modified for the current proteins. This approach minimizes concerns over toxicity of lysins to the producing host and offers a more scalable and cost-effective manufacturing option relative to fermentation. In Notifier's process, leaf tissue of the food plants *Beta vulgaris* (beet), *Spinacia oleracea* (spinach), lettuce (*Lactuca sativa*) or the non-food species *Nicotiana benthamiana*, can be transduced to express lysins 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 a vector suspension admixed with a surfactant. The components of the expression system and host plants are prepared independently and subsequently combined. Alternatively, lysins can be produced in the same host plants carrying transgenically the lysin gene and an ethanol-inducible promoter, with induction by dilute ethanol. After induction with either method, lysin 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 biomass. Each lysin is manufactured independently to meet its own active ingredient specification. Notifier's ENDOLYSIN product may be formulated to contain a single lysin protein or blended as a mixture of two or more lysins 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 replicon with inserted lysin gene is depicted in Figure B-1. 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 TMV-Lysin replicon is transfected into *A. tumefaciens* to prepare the inoculum (Figure B-1-**B**). Each bacterium in the inoculum contains the T-DNA-TMV-Lysin plasmid (Figure B-1-**C**).

Figure B-1. Schematic of vector for endolysin expression in plants (source: Nomad Bioscience)



ENDOLYSIN 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 lysins and other antimicrobials.

B.3 Procedure

A flow diagram summarizing the key steps in producing endolysin 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. When manufacturing lysins using native cultivars of *N. benthamiana* as the host plant, chromatographic purification of the process stream is necessary to reduce host alkaloids. Specific steps in downstream purification that are employed to reduce alkaloids in *N. benthamiana*-produced endolysins (process modification) are described in more detail.

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 lysin 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 in Werner et al. (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 endolysin protein.

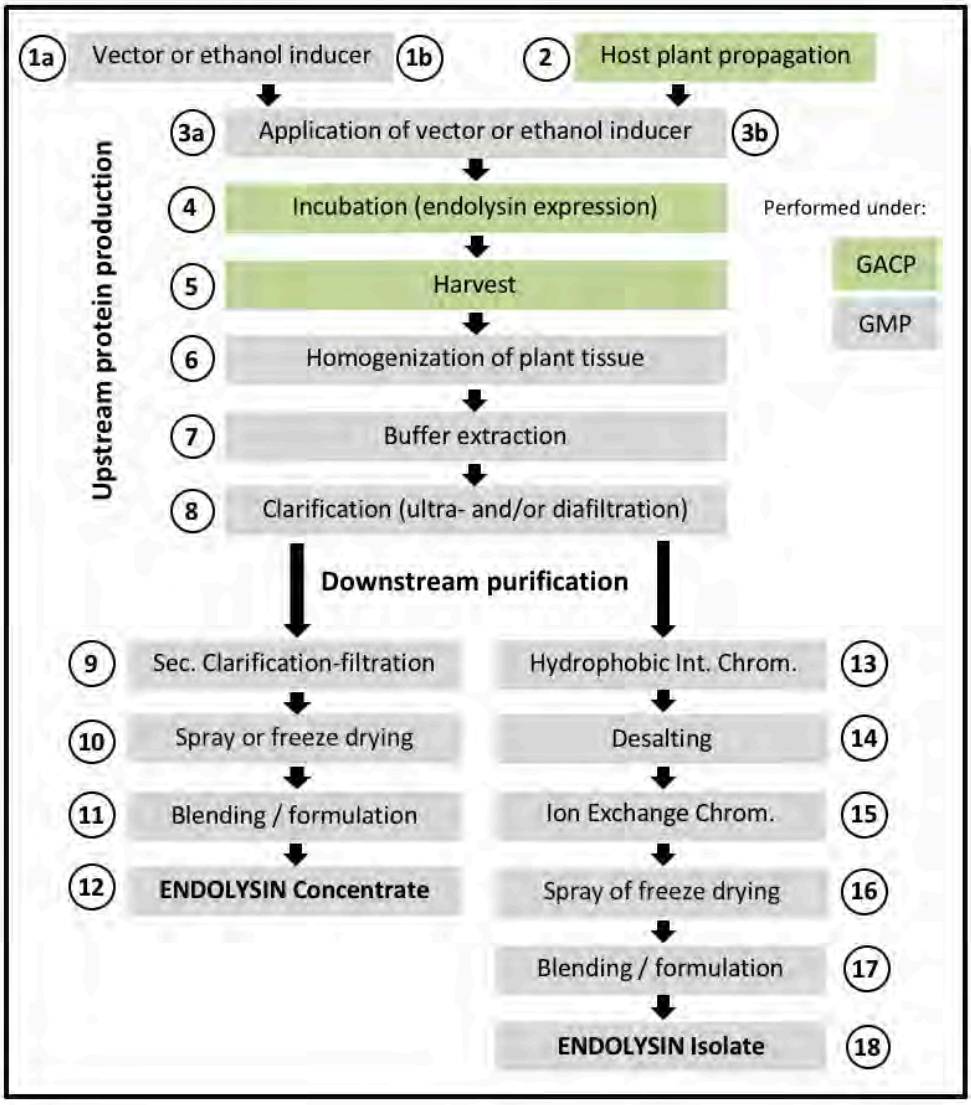
Step 2. Host plant propagation and preparation

For agroinduction, normal seeds of *Spinacia oleracea* (spinach), *Beta vulgaris* (beet), *Lactuca sativa* (lettuce), or *Nicotiana benthamiana* are obtained from qualified seed producers. For ethanol induction, transgenic seeds of these host plants developed by Notifier are used, which contain the gene insert for the desired lysin 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 ENDOLYSIN production in plants



Step 3a. Inoculation of host plants with agrobacterial vector

The *A. tumefaciens* inoculum carrying the selected lysin 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 lysin-encoding RNA transcripts. Amplification of the transcript and translation of the lysin RNA message into lysin protein occurs in the cytoplasm of each plant cell. Neither the vector nor lysin 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 endolysin gene with dilute ethanol (2.5% v/v) releases the replicon leading to RNA amplification and high-level lysin production. To achieve tight control of replicon activation and spread in the non-induced state, the viral vector has been 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, lysin protein accumulates in the tissues of the host plant. The inducer (ethyl alcohol) is evaporated or metabolized 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 lysin protein are harvested typically 8-9 days post inoculation/induction; the time-to-harvest can vary among expressed proteins. Samples of plant biomass are taken for analyses of lysin 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 lysin 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. Drying

The solution from Step 9 can be uses as a concentrated lysin solution, singly or blended with other concentrated lysin solutions to form the final bulk product. However, for storage stability, ease of transport and application, the concentrated bulk solution can be spray dried or lyophilized.

Step 11. Blending, fill and finish

For lysin mixes or blends, the individual lysin bulks are blended to a predetermined ratio, packaged and stored. Samples are taken at each process step and during storage to assess lysin quality per specification as well as product stability.

Step 12. ENDOLYSIN Concentrate

The final semi-purified product from Step 11 constitutes **ENDOLYSIN Concentrate**. This **"restricted"** process to yield a semi-purified lysin-enriched protein mix is only used when producing endolysins from **edible plant species** hosts (i.e. vegetables), because residual host impurities are deemed safe to consume in unrestricted quantities. When using a non-food plant species such as *Nicotiana benthamiana* to express recombinant lysins, chromatographic purification of the process stream is applied to lower the content of residual host-derived alkaloids (see description of alternative downstream purification, Steps 13-15).

Step 13 – 15. Multi-step Chromatographic Purification

An alternative downstream processing (DSP) procedure can be used when a final product with higher purity is desired. This alternative DSP is especially important when manufacturing lysins (or other proteins) using *Nicotiana benthamiana* as the host plant. Briefly, the product-enriched solution is subjected to three additional purification steps. These include hydrophobic interaction chromatography (HIC), followed by desalting, followed by ion-exchange chromatography (either AEXC or CEXC depending on each lysins' isoelectric point and other molecular properties).

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. Hence, this more rigorous DSP procedure can be considered a **"universal" lysin purification method** because it can be applied to the isolation of endolysins expressed in any plant species host.

Steps 16. Drying

After the precursor solution from Step 15 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 17. Formulation, fill and finish

Lysin mixes are blended to produce a final bulk product and packaged. Prior to release, the bulk products are tested to ensure compliance with the respective final product specification for ENDOLYSIN Isolate.

Step 18. ENDOLYSIN Isolate

The final product of higher purity from Step 17 constitutes **ENDOLYSIN Isolate**.

In-Process controls and quality assurance

Notifier applies rigorous in-process controls to 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 is in place to ensure conformance with industry standards and federal and local regulatory guidelines.

B.4 Specification

The target Specification for ENDOLYSIN dry powder produced by the "universal" process adaptation described above is shown in Table B-1.

Table B-1. Specification for ENDOLYSIN product

Target Specification of ENDOLYSIN Product			
Parameter	**Method**	**Specification limit**	**Results of analyses***
Appearance	Visual	Powder, white to beige	Conforms
Specific Activity (endolysin protein basis)	Viability inhibition of *C. perfringens* strain ATCC13124 (NCTC8237) with lysins Psm, CP25L, ZP178, ZP278; strain NCTC11144 for lysins Ply26F, and Ply39O	≥2 $\Delta\log_{10}$ (lysins psm, CP25L, ZP173 and ZP278) ≥1 $\Delta\log_{10}$ (lysins PlyCP26F and PlyCP39O)	≥3 $\Delta\log_{10}$ (lysins psm, CP25L, ZP173 and ZP278) ≥1 $\Delta\log_{10}$ (lysins PlyCP26F, and PlyCP39O)
pH of a 1% solution	Potentiometric	6.5-8.5	7.5 ± 0.5
Heavy metals, total (sum of Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, Sn)	USP38<233> ICP-OES	≤ 30 ppm	< 1 ppm
Heavy metals: Lead	USP38<233> ICP-OES	≤ 5 ppm	< 1 ppm
Heavy metals: Cadmium	USP38<233> ICP-OES	≤ 5 ppm	< 1 ppm
Nicotine (per total lysin blend)**	HPLC/MS	≤ 90 ng/mg endolysin	Ave. 76 ng/mg
Anabasine (per total lysin blend)**	HPLC/MS	≤ 15 ng/mg endolysin	Ave. 2.1 ng/mg
Bioburden	USP32<61>	≤ 10 CFU/25 g sample	0 (absent)
Agrobacterium (vector) (CFU/10 g sample)	Selective plate-based assay	0 (absent)	0 (absent)
Undesirable microorganisms: *Escherichia coli, P. 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)	Specific activity at T_n vs. T_0; plate- based assay	> 6 months	In progress. Accelerated testing showed >9 month stability in solution

*Results of analyses for a dry ENDOLYSIN (mixed-endolysin) product are based on average results obtained from analyses of individual endolysin proteins blended at equal ratios (dwb). **Specifications for host alkaloids nicotine and anabasine are included only for endolysins produced in *Nicotiana benthamiana* and are omitted for endolysins produced in food plant species hosts that do not harbor these alkaloids.

B.5 Manufacturing Facilities

Notifier can manufacture ENDOLYSIN 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 endolysin 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 at pilot scale; 68 mt/shift at commercial scale
- 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 ENDOLYSIN 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.

B.7 Representative Batch–to-Batch Endolysin Manufacturing Consistency

Representative results of batch-to-batch manufacturing consistency for lysins are discussed in APPENDIX C (specifically APPENDIX Section C.9).

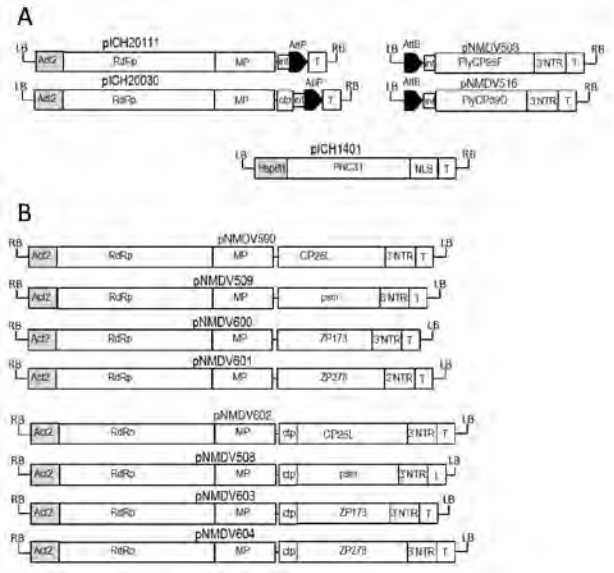
APPENDIX C. Methodology

The methods employed to assess the quality attributes of endolysin proteins are summarized here and include (listed by Section):

C.1 Expression Vector Construction

The vectors used to transiently express the various endolysin genes are introduced into the host plant via viral replicons contained in *Agrobacterium tumefaciens*. The T-DNA regions of obtained 3' provectors pNMDV503 (PlyCP26F) and pNMDV516 (PlyCP39O) and of assembled vectors pNMDV509 (cytosolic psm), pNMDV600 (cytosolic ZP173), pNMDV601 (cytosolic ZP278), pNMDV599 (cytosolic CP25L), pNMDV508 (chloroplast psm), pNMDV603 (chloroplast ZP_02640173), pNMDV604 (chloroplast ZP278), pNMDV602 (chloroplast CP25L) are presented in Figure C-1.

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



Schematic representation of T-DNA regions of lysin expression vectors. TMV-based provector modules **(A)** and assembled vectors **(B)**. LB – left T-DNA border, RB – right T-DNA border, Act2 and Hsp81.1 –promoters, RdRp –RNA-dependent RNA polymerase, MP –TMV movement protein, int – intron, T – nos terminator, ctp - synthetic chloroplast targeting sequence; AttP and AttB - integrase recombination sites, PhiC31 - *Streptomyces* phage C31 integrase, NLS - nuclear localization signal.

Briefly, plant-optimized gene coding sequences were synthetized by Eurofins, Austria for lysins psm, PlyCP26F and PlyCP39O, and by GenScript, USA, for lysins CP25L, ZP173, ZP278. The sequences were inserted as BsaI-BsaI fragments into the pICH31070 Δ lacZ plasmid (magnICON® deconstructed tobacco mosaic virus (TMV) system 3'-provector (Marillonnet 2004), into plasmid pICH29912 for cytosolic expression, and into pICH26201 for chloroplast expression. The assembled TMV-based magnICON® vectors were as described in Marillonnet (2005). The plasmids were used to transform *A. tumefaciens* strain GV3101. *A. tumefaciens* strain containing pICH1401 with expression cassette of *Streptomyces* phage C31 integrase was used for co-infiltration with 5' and 3' provectors.

C.2 Bacterial Vectors and Growth Conditions

The *Agrobacterium tumefaciens* strains containing viral amplicons for expressing and intracellularly targeting various endolysins are listed in Table C-1. *E. coli* strain DH5α was used as a recipient for all cloning procedures. Both *E. coli* and *A. tumefaciens* cells were grown in LB medium at 37 °C or 30 °C, respectively. All media were supplemented, when necessary, with 100 µg/ml ampicillin, 50 µg/ml spectinomycin, 50 µg/ml kanamycin and 25 µg/ml rifampicin. *Clostridium perfringens* were grown in TSB medium at 37 °C in anaerobic conditions using AnaeroGen gas generating system (Oxoid).

Table C-1. *Agrobacterium tumefaciens* vector strains for lysin expression and targeting

Strains	Description	References
A. tumefaciens GV3101	pMP90RK), nopaline, Rif^r	(Koncz 1986)
GV3101 (pICH20111)	Contains TMV virus-based 5' provector for cytosolic expression	(Marillonnet 2004), (2005)
GV3101 (pICH20030)	Contains TMV virus-based 5' provector for chloroplast targeting	(Marillonnet 2004), (2005)
GV3101 (pICH1401)	Contains integrase expression cassette	(Marillonnet 2004), (2005)
GV3101 (pNMDV503)	Contains TMV virus-based 3' provector with PlyCP26F	This GRN
GV3101 (pNMDV516)	Contains TMV virus-based 3' provector with PlyCP39O	This GRN
GV3101 (pNMDV509)	Contains TMV virus-based vector for cytosol -expressed psm	This GRN
GV3101 (pNMDV600)	Contains TMV virus-based vector for cytosol -expressed ZP173	This GRN
GV3101 (pNMDV599)	Contains TMV virus-based vector for cytosol -expressed CP25L	This GRN
GV3101 (pNMDV600)	Contains TMV virus-based vector for cytosol -expressed ZP278	This GRN
GV3101 (pNMDV600)	Contains TMV virus-based vector with chloroplast-targeted psm	This GRN
GV3101 (pNMDV600)	Contains TMV virus-based vector with chloroplast-targeted ZP173	This GRN
GV3101 (pNMDV600)	Contains TMV virus-based vector with chloroplast-targeted ZP278	This GRN
GV3101 (pNMDV602)	Contains TMV virus-based vector with chloroplast-targeted CP25L	This GRN

C.3 Expression of Endolysins in Plants

Spinacea oleracea (spinach) plants as a representative food species host, and *Nicotiana benthamiana* plants as a representative non-food species host, were grown in a controlled-environment chamber at 25 °C with a 16-h light and 8-h dark photoperiod. Four- to six-week-old plants of each species were used for infiltration and for spraying with recombinant *A. tumefaciens*.

A. tumefaciens cultures were grown overnight at 30 °C in Luria-Bertani media containing 50 mg/l rifampicin and other appropriate antibiotics depending on the type of plasmids (50 mg/l kanamycin for selection of integrase, TMV 3‘ provector and TMV assembled vector; 50 mg/l carbenicilin for selection of 5‘ TMV provectors). *Agrobacterium* cultures grown overnight were adjusted to an OD_{600} of 1.5, sedimented at 3220 × *g* for 5 min, and resuspended in equal volume of water.

Agroinfiltration of *N. benthamiana* leaves. Agroinfiltration of *N. benthamiana* hosts was performed as previously described (Starkevic 2015). A mix of three *A. tumefaciens* strains containing the 5‘ provector, 3‘ provector and integrase plasmid constructs was used. Strains containing 5’ and 3‘ provector modules were used at dilution 1:100, and the integrase cassette-containing strain at dilution 1:40. Plant biomass is observed and collected at five-six days post infiltration (DPI).

Agrospraying of *S. oleracea* and *N. benthamiana* plant leaves. For expression of lysins in *N. benthamiana* and in *S. oleracea* using assembled vectors, plant leaves were sprayed with a 1:1000 dilution of *A. tumefaciens* strain containing assembled vectors as described in Hahn (2015). Spraying was performed with bacteria diluted in water containing 0.1% (v/v) Silwet L77 (surfactant). Plant biomass is observed and collected at six to ten days post spraying (DPS).

Preparation of crude protein extracts for bacteriolytic activity tests. For small-scale evaluations, frozen plant material was homogenized with chilled mortar and pestle and mixed with extraction buffer (50 mM NaH_2PO_4, 5 mM DTT, 150 mM NaCl, (pH 7.5) at a ratio of 1 g of tissue to 5 ml of buffer. Cell debris were removed by centrifugation at 3220 g, at 4 °C for 60 min. The supernatant was filtered and taken as total soluble protein. These crude lysin solutions were evaluated for bactericidal activity (Section C.5).

C.4 Purification of Endolysins

Purification of lysins followed the general scheme with the sequence: Homogenization of sample, clarification, HIC chromatography, desalting, and CEXC or AEXC column (Figure C-2). Extraction conditions were optimized for each lysin based on each molecule's pI and other characteristics, as summarized.

ZP173. Homogenized plant material extracted with buffer containing 50 mM NaH_2PO_4, 2 mM DTT, 0.1% Tween 80, (pH 7.0), was clarified and loaded on butyl sepharose FF column. Colum was washed by 50 mM NaH_2PO_4, 2 mM DTT, 1.1 M $(NH_4)_2SO_4$, pH 7.0, 135-145 mS/cm. Lysin was eluted by step gradient at 30% of elution buffer containing 50 mM NaH_2PO_4, 2 mM DTT, pH 7.0. After buffer exchange by concentration/dilution procedure (conductivity <7 ms/cm), the preparation was loaded on SP sepharose FF column. Column was washed by 50 mM NaH_2PO_4, 2 mM DTT, pH 5.0 and eluted by linear gradient (0-100%) of 50 mM NaH_2PO_4, 2 mM DTT, 0.5 M NaCl, pH 5.0.

ZP278. Homogenized plant material extracted with buffer, containing 50 mM NaH_2PO_4, 5 mM DTT, 150 mM NaCl, (pH 7.5), was clarified and loaded on butyl sepharose FF column. Colum was washed by 50 mM NaH_2PO_4, 5 mM DTT, 1,2 M $(NH_4)_2SO_4$, pH 6.5, 145-155 mS/cm. Lysin was eluted by step gradient at 35% of elution buffer containing 50 mM NaH_2PO_4, 5 mM DTT, pH 6.5. After buffer exchange by concentration/dilution procedure (conductivity <7 ms/cm), the preparation was loaded on DEAE sepharose

FF column. Column was washed by 50 mM NaH_2PO_4, 5 mM DTT, pH 7.0 and eluted by linear gradient (0-100%) of elution buffer (50 mM NaH_2PO_4, 5 mM DTT, 250 mM NaCl, pH 7.0 (5.5 – 6.5 mS/cm)).

CP25L. Homogenized plant material extracted with buffer, containing 50 mM NaH_2PO_4, 2 mM DTT, 100 mM NaCl, (pH 7.5), was clarified and loaded on butyl sepharose FF column. Colum was washed by 50 mM NaH_2PO_4, 2 mM DTT, 0.85 M $(NH_4)_2SO_4$, pH 6.5, 108-112 mS/cm. Lysin was eluted by step gradient at 20% of elution buffer containing 50 mM NaH_2PO_4, 2 mM DTT, pH 6.5. After buffer exchange by concentration/dilution procedure (conductivity <5 ms/cm), the preparation was loaded on Q sepharose FF column. Column was washed by 20 mM NaH_2PO_4, 2 mM DTT, pH 8.0 and eluted by linear gradient (0-25 %) of elution buffer (20 mM NaH_2PO_4, 2 mM DTT, 0.5 M NaCl, pH 8.0.

(Purification detail continues after Figure C-2)

Figure C-2. Purification of plant-produced endolysins

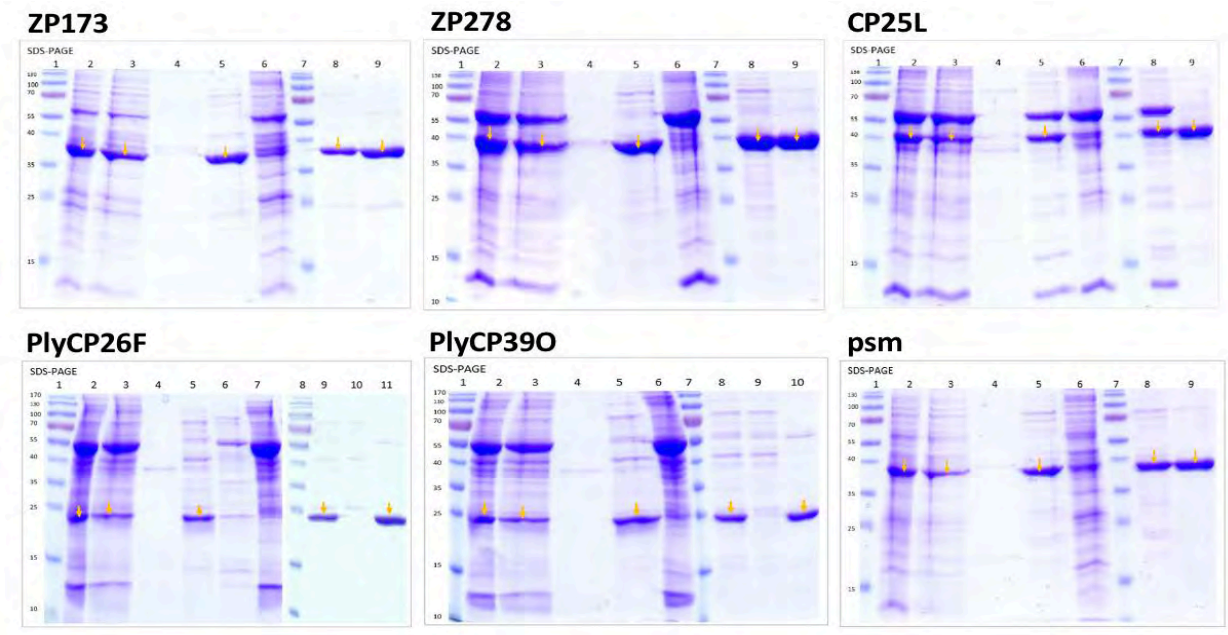


Figure C-2. Purification of plant-produced lysins. ZP173 – lane 1 and 7 - PageRuler™ Prestained Protein Ladder, lane 2 – extracted proteins, lane 3 – loaded proteins on Butyl sepharose, lane 4 – flow-through Butyl sepharose, lane 5 – collected proteins after Butyl sepharose I, lane 6 – collected proteins after Butyl sepharose II, lane 8 - loaded proteins on SP sepharose, lane 9 – collected proteins after SP sepharose. **ZP278** - lane 1 and 7 - PageRuler™ Prestained Protein Ladder, lanes 2 – extracted proteins, lane 3 - loaded proteins on Butyl sepharose, lane 4 – flow through Butyl sepharose, lane 5 – collected proteins after Butyl sepharose I, lane 6 – collected proteins after Butyl sepharose II, lane 8 – loaded proteins on DEAE sepharose, lane 9 – collected proteins after DEAE sepharose. **CP25L** – lane 1 and 7 - PageRuler™ Prestained protein ladder, lane 2 – extracted proteins, lane 3 – loaded proteins on Butyl sepharose, lane 4 – flow through Butyl sepharose, lane 5 – collected proteins after Butyl sepharose I, lane 6 – collected proteins after Butyl sepharose II, lane 8 – loaded proteins on Q sepharose, lane 9 – collected proteins after Q sepharose. **PlyCP26F** – lane 1 and 8 - PageRuler™ Prestained protein ladder, lane 2 – extracted proteins, lane 3 – loaded proteins on Butyl sepharose, lane 4 – flow through Butyl sepharose, lane 5 – collected proteins after Butyl sepharose I, lane 6 – collected proteins after Butyl sepharose II, lane 7 – collected proteins after Butyl sepharose III, lane 9 – loaded proteins on SP sepharose, lane 10 – flow through SP sepharose, lane 11 – collected proteins after SP sepharose. **PlyCP39O** – lane 1 and 7- PageRuler™ Prestained protein ladder**,** lane 2 – extracted proteins**,** lane 3 – loaded proteins on Butyl sepharose**,** lane 4 – flow through Butyl sepharose**,** lane 5 – collected proteins after Butyl sepharose I, lane 6 – collected proteins after Butyl sepharose II, lane 8 - loaded proteins on SP sepharose, lane 9 – flow through SP sepharose, lane 10 – collected proteins after SP sepharose. **psm** – lane 1 and 7- protein ladder**,** lane 2 – extracted proteins**,** lane 3 – loaded proteins on Butyl sepharose**,** lane 4 – flow through Butyl sepharose**,** lane 5 – collected proteins after Butyl sepharose I, lane 6 – collected proteins after Butyl sepharose II, lane 8 - loaded proteins on Q sepharose, lane 9 – collected proteins after Q sepharose.

PlyCP26F. Homogenized plant material extracted with buffer, containing 50 mM NaH_2PO_4, 5 mM DTT, 150 mM NaCl, (pH 7.5), was clarified and loaded of butyl sepharose FF column. Colum was washed by 50 mM NaH_2PO_4, 5 mM DTT, 1.2 M $(NH_4)_2SO_4$, (pH 7.0), 145-150 mS/cm. Lysin was eluted by step gradient at 30% of elution buffer containing 50 mM NaH_2PO_4, 5 mM DTT, (pH 7.0). After buffer exchange by concentration/dilution procedure (conductivity < 8 mS/cm), the preparation was loaded on SP sepharose FF column. Column was washed by 50 mM NaH_2PO_4, 5 mM DTT, pH 6.0 (8-9 mS/cm) and eluted by step gradient at 20% of elution buffer (50 mM NaH_2PO_4, 5 mM DTT, 1.0 M NaCl, pH 6.0).

PlyCP39O. Homogenized plant material extracted with buffer, containing 50 mM NaH_2PO_4, 5 mM DTT, 200 mM NaCl, (pH 7.5), was clarified and loaded on butyl sepharose FF column. Colum was washed by 50 mM NaH_2PO_4, 5 mM DTT, 1.2 M $(NH_4)_2SO_4$, pH 7.0, at 145-150 mS/cm. Lysin was eluted by step gradient at 35% of elution buffer containing 50 mM NaH_2PO_4, 5 mM DTT, pH 7.0. After buffer exchange by concentration/dilution procedure (conductivity < 16 mS/cm), the preparation was loaded on SP sepharose FF column. Column was washed by 50 mM NaH_2PO_4, 5 mM DTT, 120 mM NaCl, pH 6.0 (15-16 mS/cm) and eluted by linear gradient (0-100%) of elution buffer (50 mM NaH_2PO_4, 5 mM DTT, 0.5 M NaCl, pH 6.0).

Psm. Homogenized plant material extracted with buffer, containing 50 mM NaH_2PO_4, 150 mM NaCl, (pH 5.0), was clarified and loaded of butyl sepharose FF column. Colum was washed by 50 mM NaH_2PO_4, 2 mM DTT, 1.2 M $(NH_4)_2SO_4$, pH 6.0, at 140-150 mS/cm. Lysin was eluted by step gradient at 35% of elution buffer containing 50 mM NaH_2PO_4, 2 mM DTT, pH 6.0. After buffer exchange by concentration/dilution procedure (conductivity < 6-7 mS/cm), the preparation was loaded on Q sepharose FF column. Column was washed by 50 mM NaH_2PO_4, 2 mM DTT pH 8.0 (6-7 mS/cm) and eluted by linear gradient (0-100%) of elution buffer (50 mM NaH_2PO_4, 2 mM DTT, 250 mM NaCl, pH 8.0).

SDS-PAGE images comparing the semi-purified plant extracts of several *N. benthamiana*-expressed lysins and the purified products after two-stage chromatographic purification are shown in Figure C-3.

Figure C-3. Comparative purity of semi-purified and purified plant-produced endolysins

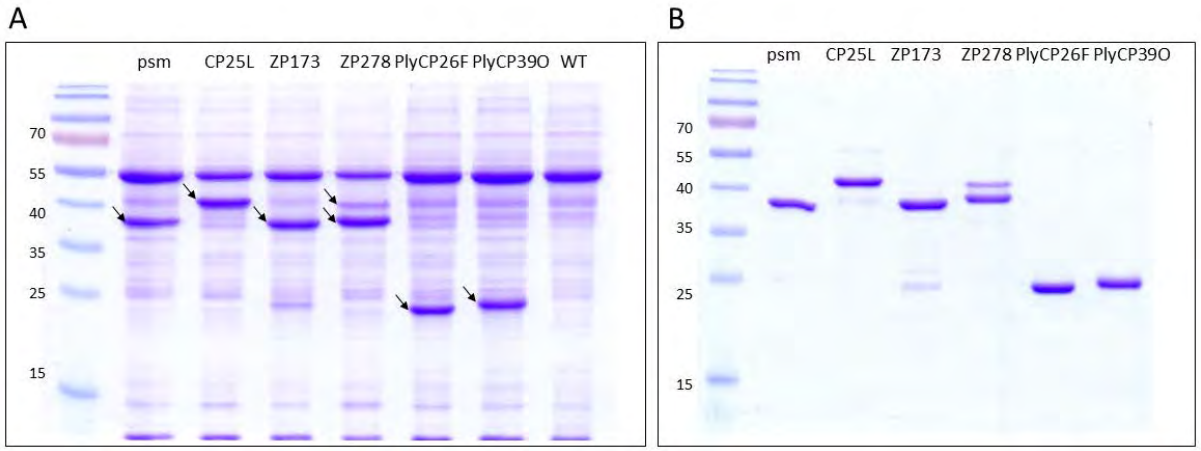


Figure C-3. Coomassie stained SDS-PAGE of crude plant extracts (**A**) and purified lysins (**B**). **A.** Plant material (50 mg) was harvested at 5 or 7 days post spraying (psm, CP25L, ZP173, ZP278) or post infiltration (PlyCP26F, PlyCP39O), ground in liquid nitrogen and extracted with 50 mM sodium phosphate, 5 mM DTT, 150 mM NaCl, (pH 7.5). Four (4) µl of plant extract was resolved in 12.5% polyacrylamide gel for Coomassie staining. MW – PageRuler Prestained protein ladder (Thermo Fisher Scientific Baltics), psm, CP25L, ZP173, ZP278, PlyCP26F, PlyCP39O – extracts of *N. benthamiana* leaves, transfected with lysins expression constructs, Wt – crude extract of non - sprayed *N. benthamiana* leaves (control). Bands corresponding to recombinant lysins are marked by arrows. **B**. Lysins were purified by two-step chromatography as described in APPENDIX B and resolved in 12.5% polyacrylamide gel for Coomassie staining.

C.5 Bactericidal Activity of Endolysins

The methods used to determine the bactericidal effects and potency of endolysins during developmental studies described in this Notice are summarized here. For product Quality documentation, the procedures described in APPENDIX D – Standard Operating Procedure for ENDOLYSIN Efficacy Determination (**Nomad SOP 704-01**) are followed.

Turbidity reduction assay and CFU enumeration. Typical methods are summarized here; detailed conditions for each antibacterial activity experiments are described in Section 2.4. Generally, crude protein extracts or purified lysins were used in bacteriolytic activity determination assays. The indicated *C. perfringens* target strains were grown in TSB under anaerobic conditions to OD_{600}≈0.7, sedimented by centrifugation and resuspended in buffer of choice for each lysin.

In developmental assays, a volume of 950 - 999 µL of bacterial suspension was mixed with 1 - 50 µL of purified lysin or crude protein extract and incubated at ambient temperature. Turbidity (OD_{600}) measurements were determined spectrophotometrically (Genesys 20, Thermo Scientific) every 5-10 minutes. All experiments were performed in triplicate.

Serial dilutions for CFU determination were done in 1xPBS pH 7.3 after 60 min. co-incubation of bacteria with plant extracts. Volumes of 30 µL of each sample were plated on TSA plates: Undiluted, and serially diluted 10, 10^2, 10^3, 10^4 and 10^5 times. CFU were enumerated following overnight incubation under anaerobic conditions at 37°C.

Bactericidal activity of endolysins in cooked meat samples. Typical methods are summarized here; detailed conditions for each antibacterial activity experiments are described in Section 2.4. Formal assessment of efficacy and duration of technical effect on meat matrices follow procedures described in APPENDIX D. Generally, crude protein extracts were prepared from agro-infiltrated *N. benthamiana* plants or a food species and harvested at 6 dpi with buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, (pH 5.0). Protein extracts were diluted to conc. ~0.44-0.66 µg/µL.

Clostridium perfringens type (tester) strain NCTC8237 was grown anaerobically in TSB to OD_{600}=0.24-0.26. Other strains or mixtures of strains are prepared and applied as per methods in APPENDIX D. Turkey breast meat and beef round meat were purchased from a local market to simulate realistic consumer sourcing. Chopped turkey meat was cooked in a pressure cooker for 30 min and chopped beef meat for 60 min.

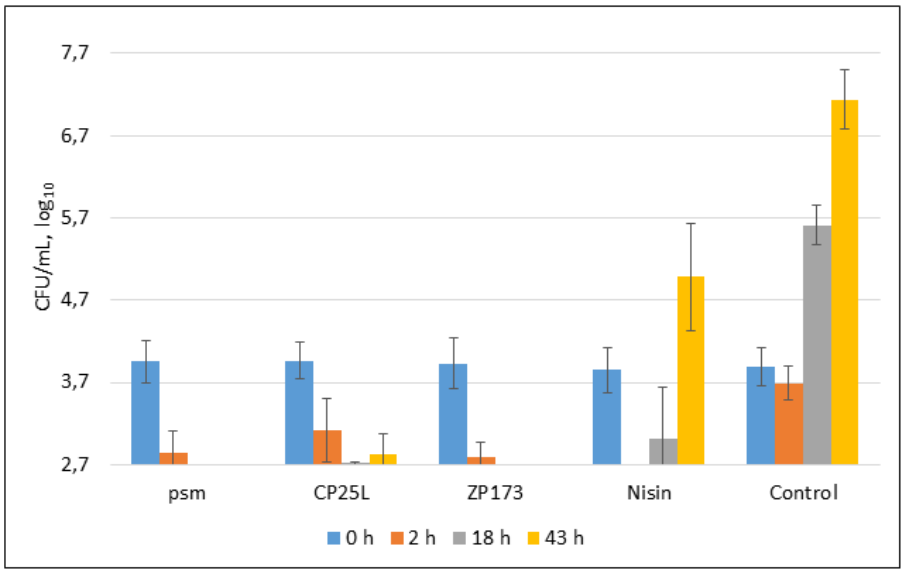
Volumes of 100 µL of bacteria and 50 - 700 µL of protein extract or purified lysins were added to 10 g of cooked meat and vortexed well. To equalize the volume of all samples, 1xPBS, pH7.3 up to 1 ml was added to each meat/bacteria sample. All the samples were placed into 6-well multiwell plates and incubated under anaerobic conditions at room temperature with samples taken typically at 2 h, 18 h and 43 h.

Serial dilutions of analyzed samples were done in 1xPBS pH 7.3 and 30 µL of each sample was plated on TSA plates: Undiluted, and serially diluted 10, 10^2, 10^3, 10^4, and 10^5 times. Plate images for CFU/mL enumeration were taken following overnight incubation under anaerobic conditions at 37°C.

Figure C-4 shows results of a typical suitability evaluation using contaminated turkey meat. Results include the mean ± SD of four (4) independent experiments.

The **results of bacteriolytic efficacy** (activity range against multiple *C. perfringens* strains) at various lysin application rates (potency), and the **suitability of crude and purified endolysins** for controlling *C. perfringens* on beef and turkey matrices are presented in Section 2.4 of this Notice.

Figure C-4. Bactericidal activity of purified endolysins on *C. perfringens*-contaminated turkey meat



C.6 Endolysin Digestibility Under Simulated Gastrointestinal Conditions

Analysis of protein digestion using simulated gastroduodenal fluids *in vitro* can be used to evaluate the post-ingestion persistence of a protein and to assess risk. In these studies, the method of Mandalari et al. (2009) was followed, with indicated adaptations. As in previous notices (e.g. GRN 593), we assessed digestibility using a two-stage approach to simulate (1) gastric followed by (2) duodenal digestibility, as summarized below. The results of endolysin digestibility studies are shown in Section 2.4.4.

Phase I: Gastric digestion

In vitro simulated gastric digestive stability assay of *Clostridium perfringens* phage lysins

Purified and lyophilized plant-produced *Clostridium perfringens* phage lysins and control proteins were dissolved in simulated gastric buffer (0.15 M NaCl, pH 2,5, 1 mg/ml). Pepsin (gastric buffer pH 2.5, 5 mg/ml) was added to give 165 U of pepsin per mg of protein in the final digestion mix containing 1 mg of protein and 0.05 mg of pepsin (pepsin:protein ratio of 1:20, w:w). Digestion was followed by Tricine-SDS-PAGE.

Results of gastric digestion

Controls

The digestibility of two allergenic milk proteins, beta-Lactoglobulin (β-Lg) and alpha–Casein (α-CS) was assessed for gastric digestion comparison of *Clostridium perfringens* phage lysins. Results showed that β-Lg was resistant to gastric digestion *in vitro* at different timepoints (T_0 min – T_{60} min). In contrast α-CS was rapidly degraded by pepsin after 2 min incubation.

Clostridium perfringens phage lysins

ZP173. SDS-PAGE analysis showed that the amount of lysin ZP173 was reduced in gastric buffer after 60 min (P_0 – P_{60} min). During gastric digestion this protein was degraded after 60 min as indicated by loss of the

38.6 kDa polypeptide. Digestion of ZP173 was accompanied by the appearance of a series of fragments of 5-25 kDa (0-30 min). The bands were also digested after 60 min.

ZP278. Lysin ZP278 was digested rapidly by pepsin after 30 min incubation. Low molecular weight material running (5-40 kDa) together with the 40.2 kDa lysin band was further degraded after 10 min digestion.

CP25L. Lysin CP25L was found stable to digestion in gastric buffer (pH 2.5, P_0-P_{60} min) as the 43.4 kDa protein was not degraded after 60 min incubation. However, a degradation pattern for the CP25L protein was observed after 60 min in SDS PAGE gel (T_{60} min); the resultant 10 kDa and 5 kDa fragments weren't further digested by pepsin (partial digestion).

Psm. Lysin psm was stable in gastric buffer (pH 2.5, P_0 – P_{60} min). SDS-PAGE analysis showed that gastric digestion of psm was slower than digestion of CP25L. The 38.6 kDa polypeptide corresponding to the intact protein could still be observed after 30 min. SDS-PAGE showed the presence of a single protein band of 5 kDa after 60 min (T_{60} min) incubation (partial digestion).

Phase II: Duodenal digestion.

Simulated duodenal digestion

Quenched test protein solutions from Phase I of gastric digestion (60 min) were incubated in 4 mM sodium taurocholate, 4 mM glycodeoxycholic acid, 10 mM $CaCl_2$, 25 mM Tris buffer pH 6.5 (25 mM Trizma-HCl 2.5 mM Na-acetate) with and without trypsin and chymotrypsin (1:4:400 (w:w:w)) of trypsin:chymotrypsin:lysin protein (34.5 U trypsin:0.4 U chymotrypsin/1.0 mg lysin protein) at 37 ^{0}C with agitation. Aliquots of reactions were removed at different timepoints and quenched with 5 mM Pefabloc SC. Protein digestion was followed by Tris-tricine SDS-PAGE.

Results of gastro-duodenal digestion

Control

Digestion of beta-Lactoglobulin (β-Lg) from bovine milk was assessed for duodenal digestion and compared to digestion of *Clostridium perfringens* phage lysins. Results showed that β-Lg was digested by trypsin and chymotrypsin in duodenal buffer after 120 min. No turbidity of the β-Lg solution was observed during duodenal digestion.

Clostridium perfringens phage lysins

ZP173. Gastric digestion of ZP173 showed the presence of a single protein band of 3.4 kDa after 60 min (T_{60} min). Duodenal digestion analysis of T_{60} min ZP173 showed that this 3.4 kDa protein was degraded by exposure to trypsin and chymotrypsin (T0). Duodenal digestion of ZP173 without pepsin (P_{60}) showed that ZP173 was digested after 180 min (T_{180}) by trypsin and chymotrypsin. Turbidity of solution with ZP173 and duodenal enzymes was observed during duodenal digestion.

ZP278. Gastric digestion of ZP278 showed degradation by pepsin but a degradation fragment of ~4 kDa was still visible in SDS-PAGE after 60 min (T_{60}). Duodenal digestion of the T60 sample showed that trypsin and chymotrypsin degraded the fragment completely.

In addition, ZP278 was subjected to Phase I gastric digestion without pepsin but exposed to Phase II trypsin and chymotrypsin treatment. This procedure showed degradation of ZP278 after 120 min (T_{120}). Turbidity was observed in the ZP278 solution during duodenal digestion.

CP25L. Gastric digestion of CP25L showed that this lysin was stable in gastric buffer (pH 2.5, P_0-P_{60} min) and was not digested by pepsin at up to 60 min incubation; however, a degradation pattern was observed after 60 min yielding visible 10 kDa and 5 kDa fragments that were resistant to pepsin. The CP25L (T_{60}) sample and its 5K and 10K partial degradation products were fully digested by trypsin and chymotrypsin under simulated duodenal conditions.

Psm. Gastric digestion of Psm showed the presence of a single protein band of 5 kDa after 60 min (T_{60} min). Duodenal digestion demonstrated that this 5 kDa degradation product (after pepsin digestion) was degraded by trypsin and chymotrypsin in intestinal buffer.

Conclusion

The protocol used to assess digestibility of plant-made endolysins in simulated gastric fluid and simulated intestinal fluid (SGF and SIF, respectively) with appropriate controls allowed determination of digestibility of all lysins either in the simulated stomach (by acid and pepsin) and/or the simulated upper intestinal environments (by trypsin and chymotrypsin). The results of these series of studies are shown in Section 2.4.4 of this Notice.

C.7 Amino Acid Sequence Analysis of Endolysins by Mass Spectrometry

Notifier commissioned the Fraunhofer Institute for Cell Therapy and Immunology (IZI; Halle, Germany) to independently analyze the amino acid sequences of its plant-made endolysin proteins, namely, ZP173 (IZI sample No. 95), PSM (No. 97), ZP278 (No. 99), and CP25L (No. 103). For all proteins, the molecular mass was determined and the N- and C-terminal sequences were verified.

Sequencing methodology. Sequence verification of the protein termini was achieved by applying a specialized mass spectrometry technique known as **in-source decay (ISD)**. This technique makes use of N-terminal (a- and c-type) and C-terminal (y- and z-type) fragment ions, which are generated due to highly elevated laser energy levels during ionization. These fragment ions can be used to derive the terminal amino acid sequences of proteins.

ISD spectra do not directly cover the first amino acids of the N- and C- terminus and hence, they often do not allow the unambiguous identification/confirmation of the respective amino acids as well as the exact localization of possible modifications. Hence, to complement ISD, IZI applied **T3-sequencing**. This sequencing method is based on the analysis of selected ISD fragments by the "LIFT" technique, which specifically selects an ISD fragment ion and acquires a fragment (MS/MS) spectrum of it. This fragment spectrum usually allows the direct identification of the first amino acids and their modifications.

Therefore, by applying both techniques, accurate and meaningful information about lysin protein sequences was obtained in all cases.

Analysis of mass spectrometry data. Molecular mass determination was based on the mass-to-charge-ratios (*m/z*) of single and multiple charged molecular ions observed within the acquired mass spectra. Each charge state allowed the determination of one value for the molecular mass. Hence, several molecular mass values were obtained for each protein. The mean of these values was used for comparison with the theoretical molecular mass, which was calculated using the amino acid sequences provided by Nomad Biosciences GmbH (Notifier).

Annotation of ISD and LIFT spectra was carried out with the help of BioTools (Version 3.2, Bruker Daltonics, Bremen, Germany) by *in silico* generation of *m/z* values for fragment ions and their comparison with the

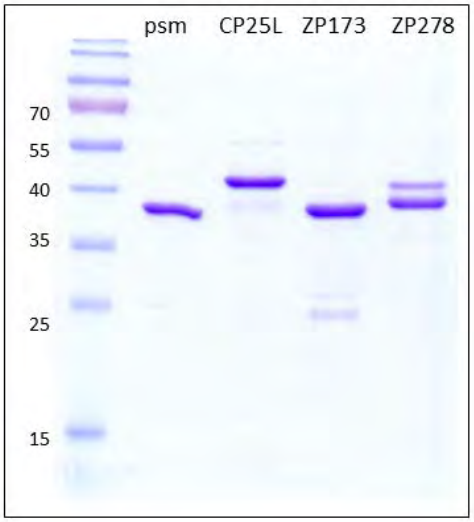
m/z values of the fragment signals observed within the acquired ISD and LIFT spectra. This approach enabled the identification of the terminal amino acid sequences as well as of present modifications.

Molecular mass, ISD and LIFT fragment signals were used to assess the integrity of the protein termini.

C.8 Molecular Analysis of Endolysin Proteins

Purified plant-made endolysins obtained as described in Section C.4 were subjected to molecular analysis to determine molecular mass, confirm each protein's N- and C-termini, and determine whether any truncations or post-translational modifications were present. Lysins that showed the broadest target range and the highest potency were analyzed for molecular properties, and included psm, CP25L, ZP173 and ZP278. Figure C-5 shows the relative purity of each endolysin prior to identity confirmation by mass spectrometry.

Figure C-5. Purified plant-made endolysins prior to identity confirmation by mass spectrometry



Results of protein analyses of plant-produced endolysin **ZP173** performed by the methods described in Section C.6 are shown below to illustrate the data acquired in the analyses and its significance. For brevity, similar data are not shown for the other lysins; instead, a final composite table is shown where all molecular masses and any modifications are shown, together with verification of N- and C-terminal sequences.

Example: Analysis of Endolysin ZP173

Molecular mass analysis. The acquired MALDI-TOF mass spectrum displayed mass signals for the single and the multiple charged molecular ion of ZP173 (sample No. 95). Further mass signals that could belong to truncated and/or modified ZP173 were not detected, indicating that only one proteoform was present. The determined molecular mass displayed a deviation of +34.8 Da compared with the theoretical value, which points towards the presence of an acetylation.

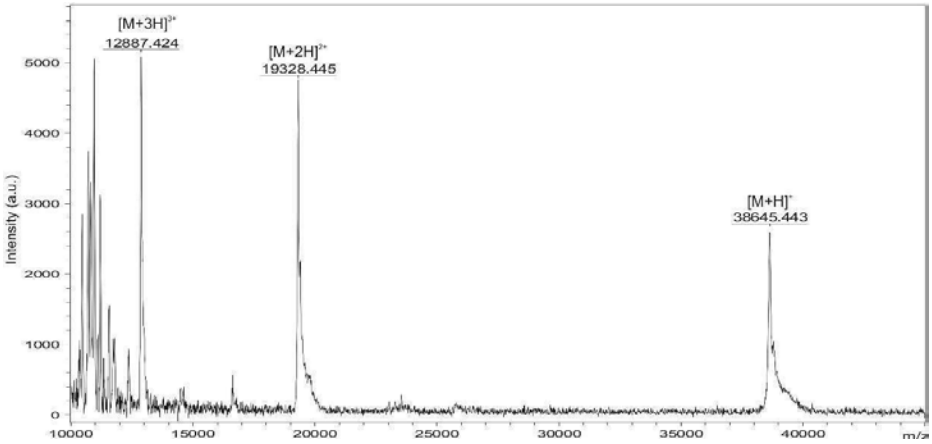
Figure C-6 summarizes the detected m/z values as well as the calculated molecular mass (top table); the center figure shows the acquired MALDI-TOF mass spectrum.

Figure C-6. Molecular analysis of endolysin ZP173

Table 1 – Mass signals for ZP173 (No. 95)

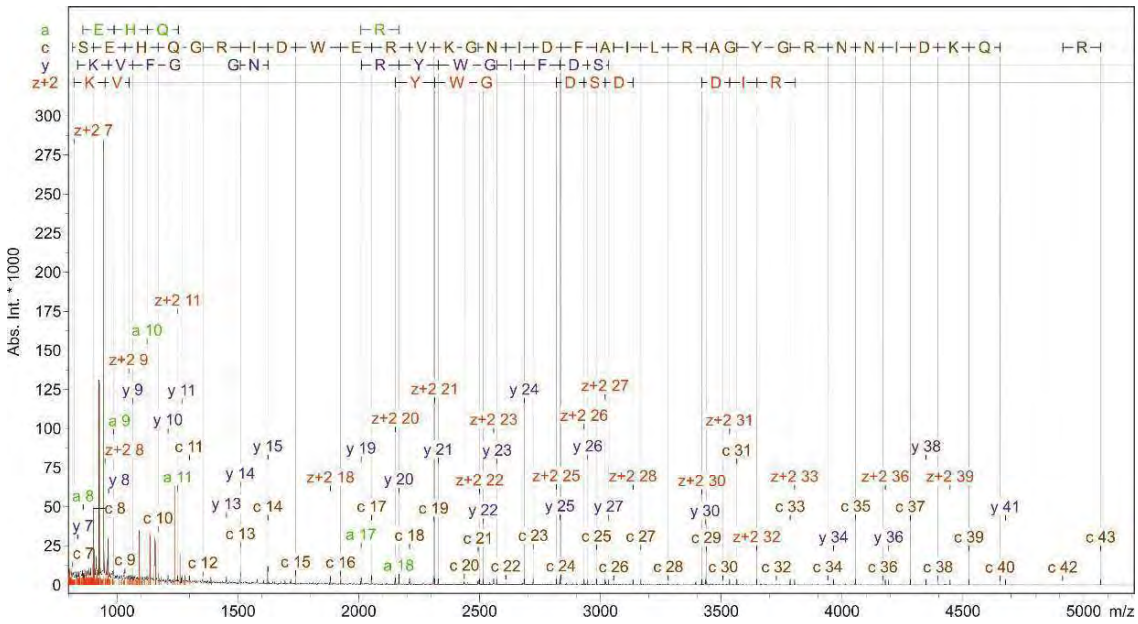
	$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$	Mean
m/z	38645.4	19328.4	12887.4	-
Mass (Da)	38644.4	38654.9	38659.3	38652.9
Dev. (Da)	-15.7*	-5.2*	-0.8*	-7.2*
Modification(s)	• N-terminal acetylation			

*Deviation ≙ $M_{Avg., experimental}$ - $M_{Avg., theoretical}$ (38660.1 Da)



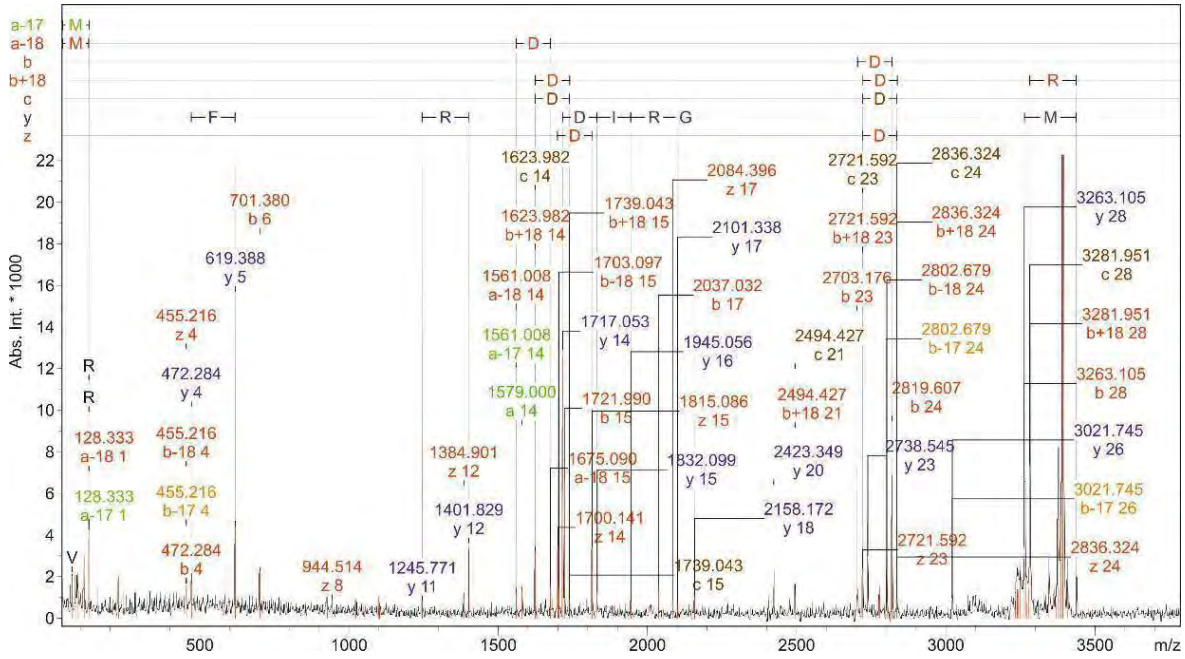
The ISD analysis of ZP173 (sample No. 95) delivered a fragment spectrum with a large number of ISD fragment signals (Figure C-7). Sequence information for the N- and C- terminus of ZP173 was obtained from the a-, c-, y- and z+2-type ion series. These ion series indicate that neither the N- nor the C-terminus were truncated and that an amino acid residue of the N-terminus was acetylated. In principle, this acetylation could be present at the N-terminus as well as at the side chain of the lysine residue at position 3.

Figure C-7. In-Source Decay (ISD) fragment spectrum acquired from lysin ZP173



To elucidate the exact position of the acetylation, a T3-sequencing was carried out for fragment c29 (*m/z* 3437.833), which covers the N-terminal amino acids 1-29. The detected fragment signals indicate that the acetylation was present at the N-terminus (Figure C-8).

Figure C-8. MS/MS spectrum acquired from ISD fragment ion c_{29} of lysin ZP173



These techniques demonstrate that complex yet detailed molecular characterization of protein sequences and alterations can be accomplished.

The same types of molecular analyses were conducted for the other lysins in development, with representative results summarized in Table C-2.

Table C-2 **A** shows that the molecular masses of ZP173 (sample No. 95), PSM (No. 97), ZP278 (No. 99) and CP25L (No. 103) were determined with deviations of less than 20 Da. Table C-2 **B** shows confirmation of N- and C-termini of the plant-made endolysins and references to available bacterial sequences.

Table C-2. Verification of molecular masses of selected plant-made endolysins

A

Sample	*m/z* [M+H]$^+$		Deviation (Da)
	Theoretical	Experimental	
ZP173 (No. 95)	38660.1	38652.9	-7.2
PSM (No. 97)	38635.7	38648.0	12.4
ZP278 (No. 99)	40208.4	40222.4	14.0
CP25L (No. 103)	43400.9	43418.0	17.1

B Confirmation of N- and C-terminal amino acids, molecular masses and PTM of selected endolysins				
Lysin	**Plant-made (determined by Notifier)**			**Bacterial (literature values)**
	N-terminus	**C-terminus**	**Determined molecular mass (vs theoretical)**	**N-terminus (method). Reference**
ZP173	**Confirmed.** N-terminal met is present. N-terminus is acetylated.	**Confirmed**	38652.9 (38660.1)	No reference available
psm	**Confirmed.** N-terminal met is present. N-terminus is acetylated.	**Confirmed**	38648.0 (38635.7)	N-terminal met is present in *E. coli* expressed protein (x-ray structure data) Tamai (2014).
ZP278	**Confirmed.** N-terminal met is present. N-terminus is acetylated.	**Confirmed**	40222.4 (40208.4)	No reference available
CP25L	**Confirmed.** N-terminal met is present.	**Confirmed**	43418.0 (43400.9)	No reference available

For the four lysin proteins shown, ISD fragment spectra with numerous fragment signals were acquired. Based on the ISD and T3-sequencing analyses, these studies concluded that all proteins were intact without any truncation.

Other than N-terminal acetylation (except for CP25L), which is common in plants, no other post-translational modifications (PTM) were detected. Only literature data for bacterial lysin psm was available. All N- and C-termini were confirmed.

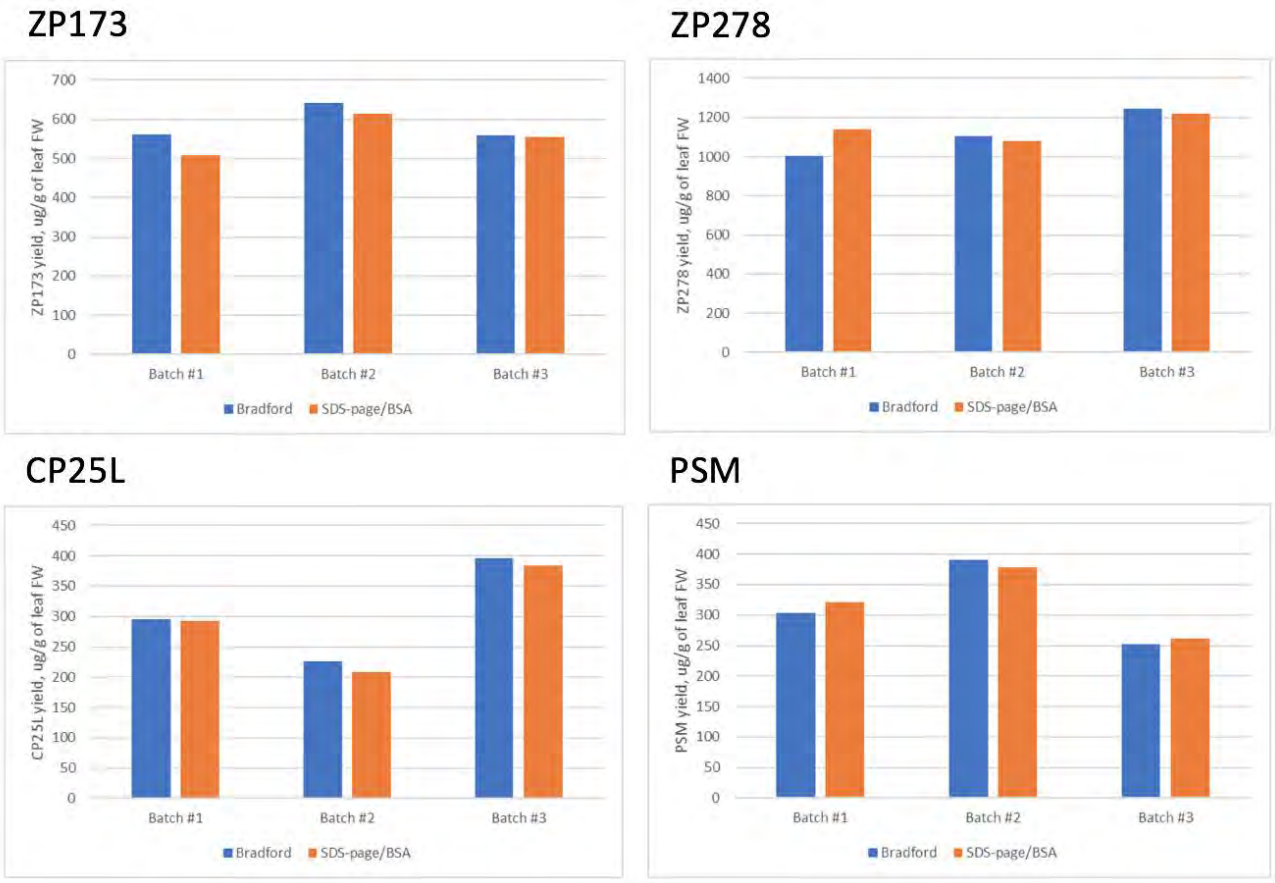
C.9 Batch-to-Batch Endolysin Manufacturing Consistency

For the proteins defined above, multiple manufacturing batches were produced and the results compared, using a minimum of three (3) independent and non-sequential manufacturing runs in each. The nomenclature "Batch 1", "Batch 2" and "Batch 3" in the figures do not denote sequential batches but rather coded identifiers for inclusion in this GRN.

Figure C-9 shows results of such comparisons. The plant-based process utilized by Notifier for lysin manufacturing yields consistent results from batch to batch.

For release, all lysins in every batch, semi-purified or purified, whether used singly or blended into mixtures, must meet their respective specification.

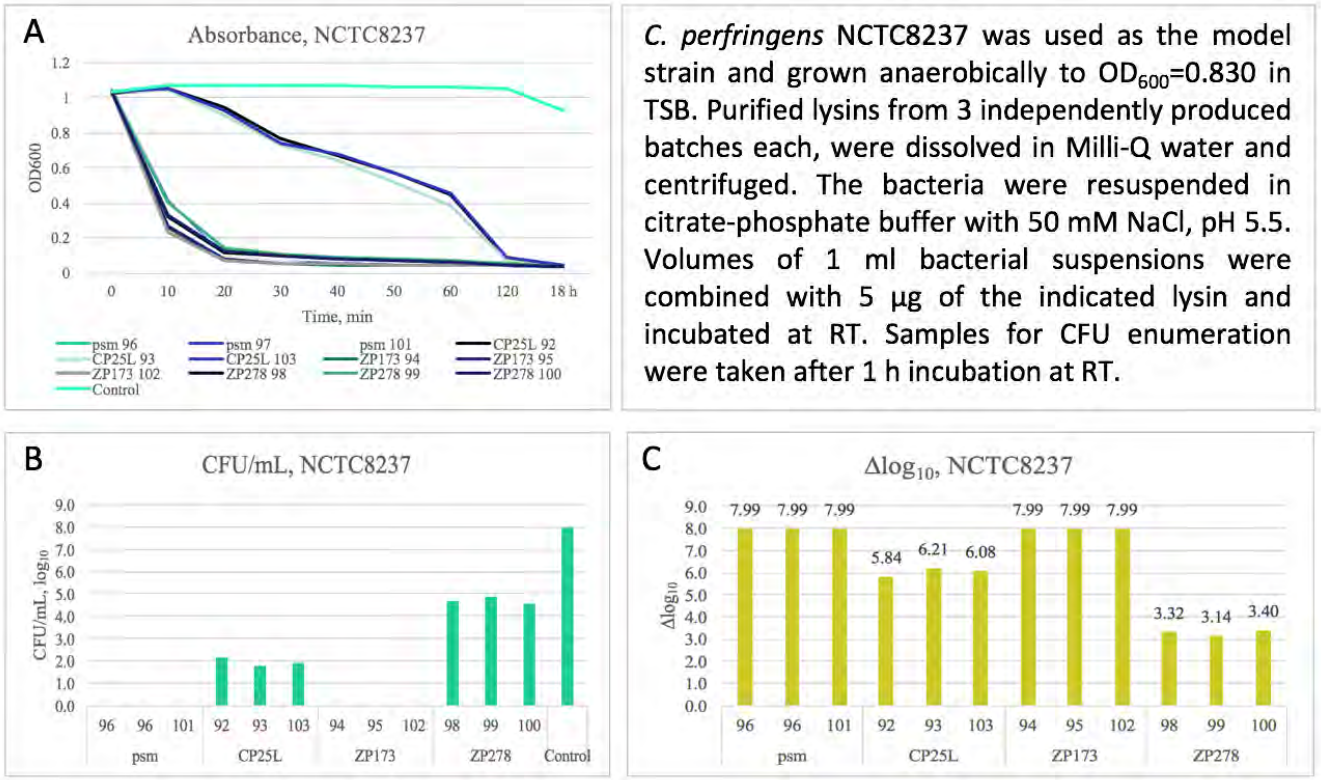
Figure C-9. Batch-to-batch yield comparability of plant-made lysins



In addition to batch-to-batch consistency of endolysin protein yield, the potency of lysins from multiple batches is monitored. Potency against a type or target strain of *C. perfringens* is dependent on several properties of each protein, including correct content and sequence of amino acids for target binding and for catalytic activity, correct protein folding to allow access of targeting and catalytic domains to the cell wall structures, and non-interference of impurities with antibacterial activity of each lysin. Hence potency (activity per unit weight of lysin protein) and its consistency are excellent indicators of reproducible manufacturing of the expected product as well as the stability of each lysin during storage.

The potency of representative lysins from three (3) independent manufacturing runs was compared. As summarized in Figure C-10, lysins produced by the plant-based process described show a high degree of consistency with respect to bactericidal activity per unit weight of protein (i.e. potency) against the reference test strain *C. perfringens* NCTC8237.

Figure C-10. Batch-to-batch consistency of lysin potency against *C. perfringens* NCTC8237



In Figure C-7, Panel A shows high consistency of bactericidal activity (change in OD as a function of incubation time) for the indicated lysins produced in 3 independent manufacturing batches each. Panel B shows the data as log CFU/mL for each batch of lysin, and similarly Panel C shows $\Delta\log_{10}$ CFU/mL, verifying high consistency of potency from batch to batch.

C.10 Analysis of residual host alkaloids in purified *N. benthamiana*-produced endolysins

Determination of nicotine and anabasine via HPLC-MS/MS (Fraunhofer IZI)

Chromatographic purification methods applied downstream can reduce host-derived impurities including alkaloids in *N. benthamiana*-produced food antimicrobials. The multi-modal chromatography step employed for purifying endolysins produced in *N. benthamiana* successfully reduces host-derived alkaloids and other impurities. The process modification is described in APPENDIX B (Section B.3) of this Notice. This purification procedure is an adaptation of the method described in Stephan et al. (2017) and GRN 775 (GRN 775 APPENDIX B, pp 65-72) for other food antimicrobials.

Alkaloid content in crude extracts and chromatographically purified endolysin samples was determined by HPLC/MS analysis and was conducted on behalf of Notifier by the Fraunhofer Institute of Cell Therapy and Immunology (Halle, Germany). For endolysin analysis, the method had a LLOQ of 1 and 5 ng/mL (1 and 5 ppb) for anabasine and nicotine, respectively.

Results of analyses of residual alkaloids in *N. benthamiana*-produced endolysins were used by Notifier to assess the safety of its ENDOLYSIN product.

Table C-3 (identical to Table 6-1) is a summary of representative results from developmental batches of various endolysins, showing nicotine and anabasine values in crude and purified proteins.

Table C-3. Residual alkaloid content in *N. benthamiana*-produced endolysins

Sample	Sample Content (lysin, purity, and concentration)	Nicotine Calculated Concentration (µg/ml)	Anabasine Calculated Concentration (µg/ml)	Nicotine Calculated Concentration (ng/mg protein)	Anabasine Calculated Concentration (ng/mg protein)
1	ZP173 (Plant extract) 400µl, 0.55 mg/ml	73.000	26.60	132727	48363
2	ZP278 (Plant extract) 400µl 1.275 mg/ml	84.500	18.40	66274	14431
3	Psm (Plant extract) 400µl 0.55 mg/ml	183.500	51.30	333636	93273
4	CP25L (Plant extract) 400µl 0.925 mg/ml	174.500	24.90	188649	26919
5	Ply26F (Plant extract) 400µl 1.05 mg/ml	123.500	49.40	117619	47048
6	Ply390 (Plant extract) 400µl 0.825 mg/ml	200.000	40.90	242424	49576
95	ZP173 (purified) 200µl 0.7 mg/ml	0.014	< LOQ	20	< 1.4
99	ZP278 (purified) 200µl 0.9 mg/ml	0.182	< LOQ	202	< 1.1
112	psm (purified) 200µl 0.52 mg/ml	0.028	< LOQ	54	< 1.9
110	CP25L (purified) 200µl 0.31 mg/ml	0.014	< LOQ	45	< 3.2
136	Ply26F (purified) 200µl 0.32 mg/ml	0.029	< LOQ	91	< 3.1
72	Ply390 (purified) 200µl 0.5 mg/ml	0.022	< LOQ	44	< 2
	Average content from all purified lysins			**76**	**< 2.1**

Table C-3 shows the residual content of nicotine and anabasine in crude extracts (top rows) and chromatographically purified endolysins (bottom rows) expressed in *N. benthamiana*. The alkaloids were analyzed by HPLC-MS/MS using an adaptation of the method described in Stephan et al. (2017) for colicins. For endolysins, the method has lower limits of quantitation (LOQs) of 0.005 µg/ml for nicotine (5 parts per billion) and 0.001 µg/ml for anabasine (1 part per billion). Because the various lysins shown in the table can be blended in different ratios, the average content of nicotine and anabasine was quantified for all purified lysins (shaded area) and listed as average values (bottom row).

C.11 Analysis of heavy metal content in purified *N. benthamiana*-produced lysins

Residual heavy metals were analyzed for sample endolysins produced in *N. benthamiana* and purified by the method described in APPENDIX B. Analysis for elemental impurities was conducted by Wolfener Analytik GmbH, Bitterfeld-Wolfen, Germany, on behalf of Notifier. Methods applied were in conformance with DIN EN ISO 11885 (E22), *Determination of Selected Elements by Inductively Coupled Plasma Optical Emission Spectrometry* (ICP-OES), which is similar to USP38<2332>, *Elemental Contaminants in Dietary Supplements*.

Briefly, lyophilized powders containing individual purified lysins were analyzed by ICP-OES for lead and cadmium content first followed by analysis of total heavy metals to include Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb and Sn. Sample preparation included total metal extraction by acid dissolution of sample, followed by determination of metal contaminants by ICP-OES (FSIS CLG-TM3 2016). The method has a LOD of detection of low ppm. Results for lead and cadmium are shown in Table C-4. Analyses for other heavy metals resulted in additive values of <1 ppm; however, confirmatory analyses were in progress at the time of this submission. Because water and nutrients of controlled purity are used in plant cultivation, very low levels of all heavy metals are expected, as supported by results to date.

Table C-4. Summary of heavy metal residues in dried lysins produced in *N. benthamiana*

Endolysin	Lead	Cadmium	Sum of Total
psm	< 1 ppm*	< 1 ppm*	< 1 ppm*
ZP173	< 1 ppm*	< 1 ppm*	< 1 ppm*
ZP278	< 1 ppm*	< 1 ppm*	< 1 ppm*
CP25L	< 1 ppm*	<1 ppm*	< 1 ppm*

Multi-batch (non-sequential) average results of heavy metal content in dried powders of endolysins expressed in *N. benthamiana* and purified by the "universal" method described in APPENDIX B. *Denotes heavy metals values that were below limits of detection by ICP-OES.

As indicated by the results summarized in Table C-4, purified lysins have very low residues of heavy metals. A daily intake of 0.560 kg food treated with 10 mg/kg colicin containing 1 ppm levels of Lb, Cd or other heavy metals would add **~5.6 ng** of these elements to the average US daily diet. The limit allowed from food supplements is 5 μg/day; hence, endolysin-derived heavy metal intake is insignificant compared to other sources of these elements in the diet.

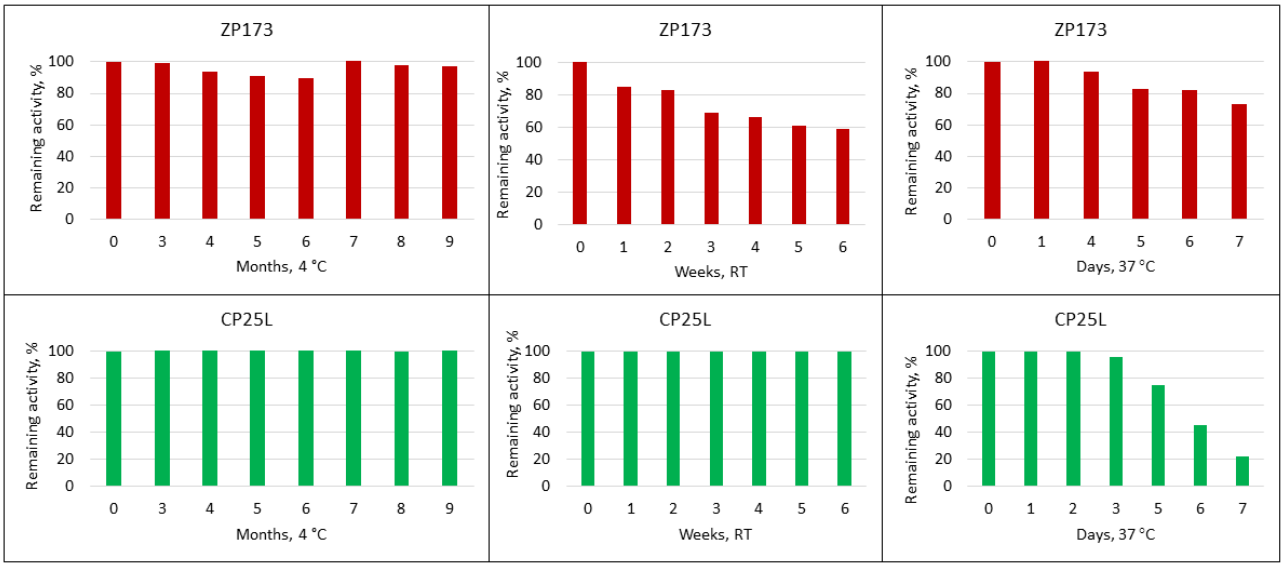
C.12 Stability of plant-made endolysins

The stability of plant-made endolysins was assessed upon storage at different temperatures. In this series of studies, dry powders of purified endolysins were dissolved in purified (milli-Q) water and stored as sterile solutions at three different temperatures, 4°C, RT (22-25°C) and 37°C and sampled over time to determine remaining bactericidal activity against *C. perfringens* reference strain NCTC8237.

Remaining bactericidal activity is expressed as percent of original activity of freshly solubilized lysins at T_0 = 100% (from purified lyophilized stock); the differential CFU is expressed as $\Delta\log_{10}$ CFU/mL of T_n relative to T_0.

Figure C-11 summarizes the stability results obtained to date for lysins ZP173 and CP25L as representative examples. A stability program has been initiated for remaining lysins and lysin mixtures.

Figure C-11. Stability and retention of bactericidal activity of endolysin solutions at 4°C, RT and 37°C



The stability of dry powders of purified lysins at 4°C and RT is being determined as part of our Stability Program (data not shown). To simulate accelerated aging, aqueous solutions of candidate lysins ZP173 and CP25L were stored at various temperatures and evaluated for potency to assess the stability of these proteins.

Results showed that stock solutions of lysins ZP173 and CP25L are equally stable at 4°C for a minimum of 9 months. At room temperature (RT; 22-25°C), solutions of ZP173 and CP25L have a shelf-life of at least 6 weeks, while denaturation of the proteins occurs faster at 37°C (activity half-life of >7 days for ZP173 and approximately 5.5 days for CP25L). These results show that endolysins have a minimum shelf-life of 6 months (the target release criterion) if stored at low temperature, even in solution, and that the proteins retain bactericidal activity at temperatures at which they can control *C. perfringens* in contaminated food products.

APPENDIX D. Standard Operating Procedure for ENDOLYSIN Efficacy Determination

NOMADS	**Standard Operating Procedure**	**NMD 704-01**
	Determination of Efficacy and Duration of Bactericidal Effect of ENDOLYSIN (Lysin Mixtures) on Strains of *Clostridium perfringens* Applied to Meat Matrices	

Author Date of draft	Aušra Ražanskienė 2018-06-26	
Last modified-by date	2018-07-1	
Valid from	2018-07-4	

D.1 Purpose

This protocol (Standard Operating Procedure) describes the methods for evaluating the efficacy and suitability of ENDOLYSIN (lysin mixtures) in reducing contamination by *C. perfringens* strains in meat (e.g. ground turkey and beef) and for evaluating residual technical effect (duration of activity) on these foods. Turkey and beef matrices are used to illustrate procedures. However, this SOP could be used as a guide for determining the bactericidal effects of ENDOLYSIN on other meats, such as chicken, pork, lamb, mutton and veal. In this SOP, the term lysins applies to individual lysins, whereas ENDOLYSIN refers to a formulated product containing a specified mix of lysins.

D.2 Scope

Assays for ENDOLYSIN's efficacy and continued technical effect

Evaluation of efficacy encompasses the analysis of *C. perfringens* populations on contaminated meat samples subsequently treated with mixtures of various plant-made recombinant lysins, or a control carrier solution consisting of plant extract from the same production host but without lysins, and stored for various time periods at different temperatures.

Evaluation of continued technical effect encompasses the analysis of time-dependent re-growth of *C. perfringens* strains on contaminated meat after ENDOLYSIN or carrier control application during prolonged storage of meat at different temperatures.

The effect of ENDOLYSIN on the bacterial populations upon storage periods of 2-43 h are analyzed at different temperatures (22 °C, 37 °C, 45 °C, 50 °C). These temperatures are selected because they bracket a realistic spore germination and vegetative growth range for *Clostridium* spp. in contaminated food.

D.3 Replicates

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

D.4 Definitions

TSB Tryptone Soya Broth medium
OD_{600} Optical density of bacterial solution at 600 nm
RT Room temperature
TSP Total soluble protein
CFU Colony forming unit

D.5 Consumables

Nunclon Delta Surface flasks (Thermofisher Scientific) for cultivation of *C. perfringens* in liquid culture
Nunclon Delta Surface 6 well culture plates (Thermofisher Scientific) for incubation of *C. perfringens* with meat
Anaerogen atmosphere generation system bags and sachets (Oxoid) for cultivation of *C. perfringens* in anaerobic atmosphere
Disposable plastic cuvettes for spectrophotometric measurement of OD_{600}
Sterile Petri dish, 94x16 mm
Sterile disposable 50 ml centrifuge (Falcon) tubes
Sterile 1.5 and 2.0 ml disposable reaction tubes
Sterile disposable plastic spatulas
Sterile disposable pipette filter tips

D.6 Equipment

Incubator (22 °C, 37 °C, 45 °C, 50 °C) for cultivation of *C. perfringens*.
Spectrophotometer for measurement of OD_{600} of bacterial culture
Anaerogen atmosphere generation system jar (Oxoid) for cultivation of *C. perfringens* in anaerobic atmosphere
Table-top centrifuge
Variable volume pipettes
Laminar flow cabinet
Microwave oven
Autoclave
Refrigerators (10 °C and 15 °C)
Freezer -80 °C
Freezer -20 °C
Personal protective equipment

D.7 Chemicals/Media/Solutions

TSB medium (sterile, liquid): For cultivation of *C. perfringens* strains
Tryptone Soya Broth (Oxoid**,** cat. #CM0129)
TSA medium (sterile, solid): For cultivation of *C. perfringens* strains
Tryptone Soya Broth (Oxoid**,** cat. #CM0129)
1.5% (w/v) Agar, bacteriological (Oxoid, cat. #LP0011)

D.8 Biologicals

Bacterial tester strains used in efficacy and technical effect experiments

The *C. perfringens* strains used in the experiments conducted within this SOP are shown in Table D-1.

Table D-1. *Clostridium perfringens* strains used in product efficacy evaluations

Strain	Isolated from	Characteristics
NCTC8237 (ATCC 13124)	-	Type A. a-toxigenic, cpa and pfoA genes present. Type strain.
NCTC11144	Beef, food poisoning outbreak	Type A.
NCTC8235 (ATCC1922)	Stew	Type A, cpa and cpe genes present.
NCTC8239 (ATCC 12917)	Boiled salted beef	NCTC: Type A: a-toxin gene positive. Contains a fragment of the enterotoxin gene. ATCC: Type D: Heat resistance of spores. Agglutinating type 3. The presence of cpa, etx, and cpe genes was confirmed by PCR.
NCTC9851 (ATCC 12925)	Braised heart	Type A. Agglutinating type 11. The presence of cpa and cpe genes was confirmed by PCR.
NCTC8449 (ATCC 12921)	Steamed lamb	Type A. a-toxin gene positive. This isolate also contains a fragment of the enterotoxin gene
NCTC8797	Salted beef	Type A. a-toxin gene positive. This isolate also contains a fragment of the enterotoxin gene

D.9 Precautions

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

D.10 Procedure for Determining Efficacy and Duration of Technical Effect

D.10.1 Endolysins

Lysins are produced in plants as described in the present Notice. The intended product contains individual plant-made lysins or a blend of several lysin proteins selected from the list that includes lysins ZP173 (WP_003469359), ZP278 (WP_003469445), psm (YP_699978.1), CP25L (YP_008058948), PlyCP26F (YP_007004008) and PlyCP39O (YP_002265435).

Plant-made lysins blended into the ENDOLYSIN product can be supplied in various forms, including: 1) lysin-containing plant total soluble protein (TSP) extracts; 2) dry (e.g. lyophilized or spray dried) lysin-containing plant TSP; or 3) dry (e.g. lyophilized or spray dried) purified lysins. ENDOLYSIN formulations may be delivered to the customer as dry powder, ready-to-use solution, or concentrated liquid with defined concentrations of lysins.

Before use, the supplied lysin formulations should be diluted/dissolved in the appropriate volume of deionized water and stored at low temperature (e.g. 2-8 °C).

D.10.2 Verification of basic functionality of lysin (blend) solution

The antimicrobial activity of the separate lysins or of prepared ENDOLYSIN solution is analyzed by a growth inhibition assay (CFU counts).

C. perfringens strains are grown in TSB under anaerobic conditions to OD_{600} appr. 0.8 and suspended in citrate-phosphate buffer, 50 mM NaCl, pH 5.5 to OD_{600} of approximately 1. Lysins are added to bacterial suspension at final concentration of 10 µg/ml. Serial dilutions for cfu counts are done in PBS, pH 7.3 after 60 min. of co-incubation with lysins.

All activities are expressed as $\Delta\log_{10}$ CFU/mL. Strain NCTC8237 (ATCC 13124) is used as the indicator for lysins ZP173, ZP278, psm and CP25L (at least 3 $\log_{10}$ CFU reduction should be achieved) and strain NCTC11144 is used as the indicator for lysins PlyCP39O and PlyCP26F (at least 1 $\log_{10}$ CFU reduction should be achieved).

D.10.3 Preparation, contamination and treatment of sample meat matrices

10.3.1 Purchase of meat in local retail outlets

No special sourcing of meat samples is used to ensure that ENDOLYSIN activity is evaluated in representative consumer products. Raw turkey or raw beef fillets are purchased at retail outlets one day before study start. The meat is stored at 4 °C, the packaging is disinfected with 70% ethanol before opening, and the meat is not washed or pre-treated before experimental exposures. A matrix summarizing how meat samples are prepared for these experiments is shown in Table D-2.

Table D-2. Matrix for preparation of turkey and beef cuts

Test matrix	**Ground meat**
	Raw turkey and raw beef prior to grinding
Initial meat block	Turkey breast, beef fillet
Preparation	The meat block is chopped into small cubic pieces of 5 mm using a clean knife. 10 g of chopped meat is cooked in a 50 ml Falcon tube for 30 min and stored at -70 °C. The meat is thawed before use and thoroughly mixed using a sterile steel weighting spatula.

10.3.2 Preparation of bacterial cultures used to experimentally contaminate meat

The meat test matrices are experimentally contaminated with a 1:1:1:1:1 mixture of 5 *C. perfringens* Type A enterotoxigenic strains shown in Table D-1.

Before inoculation the strains are thawed and individually inoculated to TSB broth anaerobically. Individual TSB broth overnight cultures (37 °C) are diluted to OD_{600}=0.13 with fresh TSB broth and grown to OD_{600}~0.23 which corresponds to ~1.5x 10^7 CFU/ml (~7.2 log CFU/ml). Individual cultures are diluted with TSB broth to OD_{600}=0.0002 and mixed 1:1:1:1:1.

10.3.3 Contamination of meat

Ground cooked meat (10 g) is mixed in 50 ml Falcon tube with 3 ml of citrate-phosphate buffer with 856 mM NaCl, pH 5.5 and 100 µl of diluted mixed bacterial culture. A summary matrix of the process is shown in Table D-3.

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

Test matrix	Ground cooked turkey breast meat or ground cooked beef fillet ~10 g weight
Containment	Falcon 50 ml tube
Density of pathogenic *C. perfringens* suspension for lysin efficacy evaluation	~ 10^5 CFU/ml (correspond to 1 ml of bacterial culture OD_{600}=0.001.) For test with mix of strains, five *C. perfringens* strains are grown to OD_{600} appr. 0.23 in TSB anaerobically. Each strain is diluted to OD_{600} =0.0002 and mixed in equal amounts to get 1 ml of bacterial culture of OD_{600}=0.001.
Application of *C. perfringens* suspension	Pipetting and vortexing
Dose of *C. perfringens* suspension	10 µl/g
Expected bacterial load of contaminated meat for lysin efficacy evaluation	10^3 CFU/g

10.3.4 Application of lysin (blend) solution

Contaminated meat is treated with carrier or lysin blend solution by pipetting solubilized lysins on the ground meat mix and thorough mixing by vortexing. The procedure is summarized in Table D-4.

Table D-4. Matrix for application of ENDOLYSIN to meat samples

Test matrix - specific	Ground cooked turkey breast meat or ground cooked beef ham meat ~10 g weight
Containment	Falcon 50 ml tube
Application of ENDOLYSIN/carrier solution	Pipetting, vortexing
Application rate	~2.5-5 mg lysin/kg (individual lysins) 10 – 20 mg lysin mix/kg 1:1:1:1 of psm, ZP173, ZP278 and CP25L

10.3.5 Incubation of meat samples

The contaminated and lysin- or control-treated cooked meat samples are aliquoted into 6-well culture plates (Nunc) and sealed in Anaerogen atmosphere generation system bags with added sachets (Oxoid). The samples are stored at one of several controlled temperatures, such as 22 °C, 37 °C, 45 °C, or 50 °C, to simulate a range of food contamination and vegetative growth conditions. Samples are taken at 2 h, 18 h and 43 h.

D.10.4 Quantification of ENDOLYSIN efficacy and duration of effect on-matrix

The efficacy and duration of ENDOLYSIN treatment are determined by dilution-plating followed by CFU enumeration as functions of sampling time. Specifically, 50 µl microbial suspension aliquots from each well of the plates described above are transferred into 1.5 ml microcentrifuge tube using a pipet with a cut tip. A 1:10 dilution series of concentrated microbial suspension (50 µl microbial suspension + 450 µl citrate-phosphate buffer with 256 mM NaCl, pH 5.5) is prepared. Subsequently, 50 µl aliquots of undiluted and diluted microbial suspensions are plated on TSB agar plates. The plates are incubated for 18-24 h at 37 °C and the CFU are enumerated.

The CFU per g sample value is calculated as follows:

$$\frac{\text{Total CFU}}{\text{g Meat}} = \frac{\text{Actual CFU x Dilution Factor}}{\text{0.05 ml Plating Volume}}$$

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

D.10.5 Statistical analysis

The efficacy of the ENDOLYSIN treatment in reducing the number of viable pathogenic *C. perfringens* in the experimentally contaminated meat samples, and the duration of (residual) technical effect of ENDOLYSIN treatment, are evaluated by comparing the data obtained with the carrier-treated control samples and ENDOLYSIN-treated samples by one-way ANOVA (Tukey´s multiple comparisons test) and unpaired parametric t-test using GraphPad Prism v. 6.01.

END NOTICE.

consultants to the regulated industry

Center for Regulatory Services, Inc.

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Woodbridge, VA 22192-5755
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smedley@cfr-services.com

October 23, 2018

Denis Wafula, Ph.D.
Division of Biotechnology and GRAS Notice Review (HFS-255)
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Dear Dr. Wafula:

SUBJECT: RESPONSE to EMAIL October 17, 2018 –Questions
GRAS Notice 802: ENDOLYSIN use as antimicrobial agent
NOMAD BIOSCIENCE GmbH

Enclosed you will find additional information as requested for the support of GRAS notice for ENDOLYSIN to be used as an antimicrobial for controlling *Clostridium perfringens* as submitted by NOMAD BIOSCIENCE GmbH. The questions were received by email dated October 17, 2018.

We have provided a narrative that includes every point raised and the response.

We are providing a CD that includes the narrative as well as new (publically available) reference material.

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

Sincerely,

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

Attachments

Question Response Narrative (email, Hard Copy and CD-Copy)
Cited References (CD-copy)

cc: Yuri Gleba, Nomad

04/12/2019

GRN 802

While we understand that the intended use of your product is as an antimicrobial to control *Clostridium perfringens* in certain temperature-abused foods, please confirm that it is not intended to be used in lieu or in conjunction with standard methods for preventing temperature abuse in cooked foods. Also confirm that the product is not meant to be used as a substitute to standard methods for the safe handling of food.

Response from NOMAD: **04/12/2019**

ENDOLYSIN is an antimicrobial for the control of Clostridium perfringens in certain temperature-abused foods. This product is not intended to be a substitute to standard culinary hygiene and safe preparation and handling of food. This product is intended for use in addition to standard methods and practices for preventing temperature abuse in cooked food that can lead to the growth of C. perfringens.

Notifier's Responses to CFSAN Reviewers' Question for GRN 802

On October 16, 2018, CFSAN provided a list of questions concerning Notifier's GRAS notice for its ENDOLYSIN food antimicrobial to control *Clostridium perfringens* (GRN 802). We thank the Agency's reviewers for their thorough review of the GRN and for their questions and clarifications. Notifier has developed responses to the Reviewers' questions, provided below, and arranged by repeating the Agency's numbered questions in ***bold italic*** font, followed by Notifier's corresponding answers.

1. ***In assessing potential toxicity of proteins, notifiers typically run bioinformatic analyses to make sure that their substance does not have significant homologies to known toxins. It appears that the bioinformatics analyses in the notice were done using databases that contain only known or suspected allergens. Please indicate whether bioinformatics analyses for homologies to known toxins were done for the endolysins and what the assessment was.***

A. Notifier conducted food allergen database searches to assess the allergenic potential of endolysin proteins. The method used and the results obtained were documented in GRN 802, Section 6.5, pp 59-62. Food allergen searches were prioritized because of the nature of the product and its intended use in food.

Although not mentioned in the GRN, Notifier also conducted searches for endolysin homologies to polypeptide toxins. Endolysins can be considered toxins to bacteria, but not toxins to mammalian cells. The reason is that endolysins are highly specific bacteriophage-derived antibacterial proteins with characterized modes of action and no mammalian target structures. Their high specificity for pathogenic bacteria explains why numerous lytic phages that encode endolysins are GRAS food processing aids (e.g. GRNs 218, 528, 435, 468, 672, 752, 757) and why both phages and lysins have been used and considered for use, respectively, in medical therapy (e.g., Hermoso 2007; Jado 2003; Fischetti 2005; Kaur 2012, Nakonieczna 2015, Seal 2012).

Notifier consulted the database of bacterial exotoxins for human (DBETH; Chakraborty 2012) to search for human pathogenic bacterial toxins. None of the endolysin amino acid sequences listed on page 13 of GRN 802 generated any toxin domain hits ("0") in FASTA searches. The DBETH server was interrogated because it simultaneously searches several databases containing entries for toxins, including protein families databases (PFAM-A and PFAM-B), clusters of orthologous groups (COG), simple molecular architecture research tool (SMART), conserved domains database (CDD), and entries in the institute of genomics research (TIGR) domain collection. None of these satellite searches retrieved homologies between endolysins and known toxins.

Searches in the antiSMASH database for microbial secondary metabolites were less useful because the searches must be done at the genome level. Endolysins are expressed in plants using codon optimization, which complicates genomic cluster analysis across species; hence, searching for homology at the protein level is preferred.

Searches of the toxin and toxin targets database (T3DB; Wishart 2015) revealed no ("0") homologies with any protein toxin sequences in the collection.

Negative results when searching for homologies between endolysins and mammalian-relevant toxins were not unexpected. On page 15 of GRN 802 we listed the modes of action of the endolysins under development by Notifier. These include:

1) N-acetylmuramidases (lysozymes) cleave the β-1-4-glycosidic bond between N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) in bacterial cell walls. Lysozyme is an antibacterial enzyme present in tears, egg white, etc.;
2) N-acetyl-β-D-glucosaminidases (glycosidases) cleave the β-1-4-glycosidic bond between NAM and NAG, which are structures present only in bacterial cell walls;
3) N-acetylmuramoyl-L-alanine amidases cleave the amide bond between sugar and peptide in NAM and L-alanine in the peptide side chain of different bacterial peptidoglycan products; and
4) L-alanoyl-D-glutamate endopeptidases are interpeptide bridge-specific endopeptidases that cleave the peptide moiety of the bacterial cell wall peptidoglycan.

Hence, endolysins could be considered "toxins" only for target bacterial species. In the searches identified above we found no evidence that they are toxins for mammalian cells due to their specialized mode of action and narrow substrate requirements. In sum, the most relevant information from the standpoint of food safety is the allergenic potential of endolysin proteins, which we included in the GRN.

2. On page 51, you reference GRN 775 as citation to support the following statement:

"...nornicotine and anatabine … have similar activities but are present at very low levels in the plant and pose no significant risk"

Whereas GRN 775 will be publicly available through the FDA website, it is not considered peer-reviewed material. Furthermore, each GRN is considered a separate entity, and if specific citations are mentioned in previous GRNs, the notifier must give specific information (i.e. page numbers/sections) from that specific GRN. Please provide specific citations that support your statement. The notifier should also note that at the time of submission of this notice, GRN 775 is still under evaluation and FDA had not issued its opinion.

A. Notifier relied on peer-reviewed published sources for the levels of host alkaloids in *Nicotiana benthamiana* as well as internal data generated by Notifier and by a contracted independent analytical laboratory. The information cited and the assessment of safety from consumption of trace levels of residual host alkaloids were included in Notifier's GRN 775 submission (Section 3.3, pp 25-27; Section 6.1.4, pp 32-37), which Notifier recognizes is still under review by CFSAN. For clarity, the information requested by FDA for GRN 802 is provided below.

The relative levels of alkaloids in *N. benthamiana* (Sisson 1990) in proportionately decreasing concentrations in the biomass are (with nicotine set at "100"):

Nicotine (100) > anabasine (10) > anatabine (1) > nornicotine (0.2)

Anatabine plus nornicotine represent only ~1-2% of the total alkaloid content of this host.

The mammalian toxicities of these alkaloids are available from the literature. The (LD_{50}) of anatabine (CAS 2743-90-0) is 1.62 mg/kg mouse, intravenous; 22.0 mg/kg guinea pig, subcutaneous (BPDB 2018); and 100 mg/kg human, oral (acute toxicity estimate ATE SDS; Carbosynth 2017).

The mammalian toxicity (LD_{50}) of nornicotine (CAS 494-97-3) is 3.4 mg/kg mouse, intravenous; 14.7 mg/kg mouse, intraperitoneal, and 3 mg/kg rabbit, intravenous (PubChem 2018).

The toxicities of anatabine and nornicotine by species and routes of administration are similar to that of nicotine (CAS 54–11–5), the LD_{50} of which is 3.3 mg/kg mouse oral; 50 mg/kg rat, oral (De Landoni 1991), 9.2 mg/kg dog, oral (CDC 2018; and >6.25 mg/kg (est) human, oral (Mayer 2014).

Because toxicity is directly proportional to dose, and anatabine and nornicotine would be present at ~1-2% the level of residual nicotine in the ENDOLYSIN product, the contribution of these two minor alkaloids to the total alkaloids consumed from all ENDOLYSIN-treated food products (<206 ng/person-day; page 52, GRN 802), would be inconsequential and within the error of estimated exposure.

3. Please provide the details (i.e. dates, database, and search terms) of your literature search to capture all material relevant to your safety conclusion.

A. Notifier provided the results of literature searches used to support its safety conclusion about endolysins, as well as whether the sources were internal or from the public domain, in Table 7-1, pp 64-65 of GRN 802: "Supporting Data and Information." When conducted, the results of on-line database searches were also referenced. When search results yielded relevant information Notifier made a PDF copy of the database entries for inclusion in the References Folder of the GRN. Table 7-1 also listed specific sections and pages of prior (published) GRNs when these documents were cited as sources, as well as section/pages cited within GRN 802.

Notifier used common search engines and search strategy to acquire historical information with which to document endolysin safety. Typically, Google Scholar (https://scholar.google.com/) and/or PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) were used as search tools. Files positively identified were downloaded, cited in the GRN, and included in the References listing and as PDFs in the References Folder provided to FDA with the GRN. Searches that did not produce the desired information on the subject were not included in the GRN; in such instances, a statement was included in the text indicating that the search did not identify a target topic.

For example, in Section 6.1, pg 55 of GRN 802, we stated: "*Unfortunately, no literature reports were found that estimated the natural level of endolysin exposure.*" To arrive at that statement, search words on this topic included: Natural, environmental, commensal, exposure, food, disease, endolysin, and lysin. Searches on this subject were conducted on 1 Dec 2017, 10 Apr 2018, 22 Jun 2018 and 7 Jul 2018. Multiple searches were conducted during GRN document development to assess whether any new literature on the topic had been published since the previous search.

In the search for the topic described above, the top 10 articles appearing by priority of "relevance" are included in References: "Results of 7 Jul 2018 search – endolysin exposure" in this response letter. None of the top 10 publications listed included information about natural or environmental exposure of humans to bacteriophage endolysins. Therefore, results of these searches were used to support our statement in the GRN (i.e. "*…no literature reports were found that estimated the natural level of endolysin exposure*").

Similarly, on pg 55 of the GRN we stated: "*No reports have appeared in the literature linking bacteriophage or phage-produced endolysin consumption (ingestion) with onset of disease, progression of disease, morbidity or mortality*." Using the same search strategy defined above, these searches were conducted on 1 Dec 2017, 5 Mar 5 2018 and 22 Jun 2018. Search terms in

combination included: *Clostridium, perfringens*, endolysin, lysin, human, consumption, ingestion, disease, pathology, morbidity, mortality and etiology. The results of the 22 Jun 2018 search are included in References: "Results of 22 Jun 2018 search – endolysin safety" in this response letter. None of the top 10 articles identified and ranked were relevant to the subject matter searched. We used this search result to support our statement in the GRN (i.e. "*No reports have appeared in the literature linking bacteriophage or phage-produced endolysin consumption (ingestion) with onset of disease, progression of disease, morbidity or mortality*").

In addition to its safety assessment of endolysins in Section 6.1, pp 54-55 of GRN 802, Notifier documented its safety assessment for host impurities (Section 6.2, pp 56-57) and process impurities (Section 6.3, p 57). Sections 6.4 and 6.5 discuss safety risks from the development of bacterial resistance and from allergenicity, respectively. Section 6.6 summarizes our assessment of safety in relation to the amounts expected to be ingested from ENDOLYSIN-treated food. Numerous citations were provided in all these sections. As mentioned, summaries of documents referenced by category, section or page number, were included in Table 7-1. PFDs of cited references were included in the References Folder of GRN 802.

We hope this addresses the Agency's request for clarification. If not, it would be helpful if Reviewers provided a more specific request for additional information so that Notifier can better address their concerns.

4. In section 6.5 (pg. 60), you state:

> ***"Three of the six plant-produced lysins examined had similarities to known allergens of greater than 50% (Aalberse 2000) and therefore could be considered potentially allergenic."***

You have also concluded that these three proteins with greater than 50% similarity had "low" allergenicity potential compared to "very low" for the other three proteins. Yet, according to Aalberse (2000), "cross-reactivity is rare below 50% identity." Combined with your statement that there is a "lack of reports in the literature linking bacteriophages or endolysins with development of allergenicity or hypersensitivity", therefore, it is unlikely that any of the 3 lysins with >50% similarity would have any more allergenicity potential than the other 3 that do not. Please discuss or consider removing the artificial distinction regarding the allergenicity potential of the proteins.

A. Notifier asserts that all endolysin proteins comprising the ENDOLYSIN product have a low potential for allergenicity. As such, Notifier agrees with FDA that the subjective distinctions in the allergenicity ratings can be removed from the GRN.

5. The notifier provides only specifications for the final product from the "universal" lysin purification method. The notifier is requested to provide the specifications for the lysin product produced using the "restricted" production process.

In APPENDIX B of GRN 802 we described our plant-based biomanufacturing process for producing endolysins. The process is applicable to food species plants such as spinach, red beet and lettuce, as well as to non-food species such as *Nicotiana benthamiana*. Because food species plants can be consumed in unlimited quantities, a simpler purification process can be applied to isolate endolysins since any remaining host impurities are not a safety concern. When using *N.*

benthamiana as the host, a more stringent purification process is used to reduce host alkaloids in the final product and therefore reduce their content to less than the daily amount of alkaloids typically consumed in a normal US diet. The purification process applied to food species hosts is termed "restricted" because it is only used for food species. The process modification made to reduce alkaloids from *N. benthamiana* can also be applied to food species, albeit without concern for residual alkaloids. This latter process can be broadly applied and hence it is referred to as a "universal" purification method. Since submission of GRN 802, Notifier published much of the information contained in the notice in Kazanaviciute et al. 2018. This reference is provided in our response to FDA.

In GRN 802 Notifier provided a target Specification for the more stringent process (universal) applied to *N. benthamiana*-produced endolysins in Table 2-2 (pp 17-18). The specification release criteria and tolerances are the same for the two variations of the process. That is, regardless of production host, the blended endolysins comprising the ENDOLYSIN product need to meet minimum criteria set in the specification; the difference being limits for alkaloid content.

As requested by FDA, Notifier is providing the two specification tables for endolysins produced by the "universal" process (Table 2-2 from GRN 802) and its equivalent for the "restricted" process ("Table 2-3"). Both specifications appear immediately after our response narratives.

6. ***In the cover letter and on page 10 (under conditions of use), it is stated that the product is also intended for use in 'baked goods and other cooked foods' along with meat and poultry-based foods. However, in estimating dietary exposure (pages 48-53) only exposure to meat and poultry-based foods are discussed. Please provide a rationale for not discussing 'baked foods and other foods' and provide an example of such foods. If the intended uses are inclusive of foods other than meat and poultry, please recalculate estimated dietary exposure with all the proposed uses of the ingredient***

A. Notifier clarifies the Conditions of Use for its ENDOLYSIN product to include cooked meats, including red meats and poultry, and meat- and poultry-derived products such as gravies or meat sauces. Because food preparation terms can have broad meanings, Notifier clarifies that the Conditions of Use of its product do not imply application to bakery items (e.g. bread, cookies, cakes, etc.), which are also "baked" foods. Low moisture foods including bakery items are expected to be less susceptible to the germination of *C. perfringens* spores.

To clarify, our Conditions of Use statement in GRN 802 emphasized that the ENDOLYSIN food antimicrobial is intended to be applied to "cooked" foods as a category and not to raw or uncooked foods. The terms "cooked" or "cooking" were used as general statements to indicate thermal food preparation. Examples of the types of cooked foods (and foods meant for cooking prior to treatment with product and prior to consumption) in which the product could be used were listed in the statement. Notifier did not evaluate the performance of its product on every single food type in which the product could be used. Hence, the statement "…and other foods" suggested that the examples of foods listed were illustrative and not necessarily comprehensive.

Nevertheless, Notifier does not anticipate a significant excursion beyond the food definitions or categories that it has provided in the GRN. The term "baked" was used to also indicate a general method of thermal food preparation. For example, one can roast, barbeque or bake a turkey. Pan

drippings used to make gravy or sauce after the turkey is baked are also "baked" food. After cooking, including baking, and concomitant reduction in temperature, foods can become contaminated with *C. perfringens de novo* or through germination of heat-resistant spores, especially if they are stored under inappropriate conditions including temperature abuse.

Therefore, the major categories of food products in which ENDOLYSIN could be applied to control *C. perfringens* are cooked red meats, poultry, sauces and gravies and categories of cooked products that contain meat. Consequently, Notifier finds that no additional intake or exposure calculations are necessary as they are already accounted for in Section 3, pp 48-53, of GRN 802.

Appended Specification Tables

Table 2-2. Target Specification of ENDOLYSIN Product (All plants including *N. benthamiana*)

Target Specification of ENDOLYSIN Product (All plants including *N. benthamiana*)			
Parameter	**Method**	**Specification limit**	**Results of analyses***
Appearance	Visual	Powder, white to beige	Conforms
Specific Activity (endolysin protein basis)	Viability inhibition of *C. perfringens* strain ATCC13124 (NCTC8237) with lysins Psm, CP25L, ZP178, ZP278; strain NCTC11144 for lysins Ply26F, and Ply39O	≥2 $\Delta\log_{10}$ (lysins psm, CP25L, ZP173 and ZP278) ≥1 $\Delta\log_{10}$ (lysins PlyCP26F and PlyCP39O)	≥3 $\Delta\log_{10}$ (lysins psm, CP25L, ZP173 and ZP278) ≥1 $\Delta\log_{10}$ (lysins PlyCP26F, and PlyCP39O)
pH of a 1% solution	Potentiometric	6.5-8.5	7.5 ± 0.5
Heavy metals, total (sum of Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, Sn)	USP38<233> ICP-OES	≤ 30 ppm	< 1 ppm
Heavy metals: Lead	USP38<233> ICP-OES	≤ 5 ppm	< 1 ppm
Heavy metals: Cadmium	USP38<233> ICP-OES	≤ 5 ppm	< 1 ppm
Nicotine (per total lysin blend)	HPLC/MS	≤ 90 ng/mg endolysin	Ave. 76 ng/mg
Anabasine (per total lysin blend)	HPLC/MS	≤ 15 ng/mg endolysin	Ave. 2.1 ng/mg
Bioburden	USP32<61>	≤ 10 CFU/25 g sample	0 (absent)
Agrobacterium (vector) (CFU/10 g sample)	Selective plate-based assay	0 (absent)	0 (absent)
Undesirable microorganisms: *Escherichia coli, P. 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)	Specific activity at T_n vs. T_0; plate- based assay	> 6 months	In progress. Accelerated testing showed >9 month stability in solution

*Results of analyses for a dry ENDOLYSIN (mixed-endolysin) product are based on average results obtained from analyses of individual endolysin proteins blended at equal ratios (dwb).

Table 2-3. Target Specification of ENDOLYSIN Product (Food Plant Species)

Target Specification of ENDOLYSIN Product (Food Plant Species)			
Parameter	**Method**	**Specification limit**	**Results of analyses***
Appearance	Visual	Powder, white to beige	Conforms
Specific Activity (endolysin protein basis)	Viability inhibition of *C. perfringens* strain ATCC13124 (NCTC8237) with lysins Psm, CP25L, ZP178, ZP278; strain NCTC11144 for lysins Ply26F, and Ply39O	≥2 $\Delta\log_{10}$ (lysins psm, CP25L, ZP173 and ZP278) ≥1 $\Delta\log_{10}$ (lysins PlyCP26F and PlyCP39O)	≥3 $\Delta\log_{10}$ (lysins psm, CP25L, ZP173 and ZP278) ≥1 $\Delta\log_{10}$ (lysins PlyCP26F, and PlyCP39O)
pH of a 1% solution	Potentiometric	6.5-8.5	7.5 ± 0.5
Heavy metals, total (sum of Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, Sn)	USP38<233> ICP-OES	≤ 30 ppm	< 1 ppm
Heavy metals: Lead	USP38<233> ICP-OES	≤ 5 ppm	< 1 ppm
Heavy metals: Cadmium	USP38<233> ICP-OES	≤ 5 ppm	< 1 ppm
Bioburden	USP32<61>	≤ 10 CFU/25 g sample	0 (absent)
Agrobacterium (CFU/10 g sample)	Selective plate-based assay	0 (absent)	0 (absent)
Undesirable microorganisms: *Escherichia coli, P. 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)	Specific activity at T_n vs. T_0; plate- based assay	> 6 months	In progress. Accelerated testing showed >9 month stability in solution

*Results of analyses for a dry ENDOLYSIN (mixed-endolysin) product produced in a food species host (spinach; *Spinacia oleracea*) based on average results obtained from analyses of individual endolysin proteins blended at equal ratios (dwb).

References and Databases Cited in Notifier's Response

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Wishart D, D Arndt, A Pon, et al. T3DB: The toxic exposome database. *Nucleic Acid Res* 43 (database issue) DOI:10.1093/nar/gku1004.

Results of 7 July 2018 on-line search (Google Scholar; summarized results for top 10 hits)

Search criteria: Relevance; key word combination "and": natural, environmental, exposure, commensal, food, endolysin, lysin, disease

Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. D Nelson et al. 2001.

From endolysins to Artilysin®: novel enzyme-based approaches to kill drug-resistant bacteria. H Gerstmans et al 2016.

Bacteriophages and their endolysins for control of pathogenic bacteria. R Keary et al. 2013.

The cell lysis activity of the Streptococcus agalactiae bacteriophage B30 endolysin relies on the cysteine, histidine-dependent amidohydrolase/peptidase domain. DM Donovan et al. 2006

Bacteriophage endolysins as novel antimicrobials. M Schmelcher et al. 2012

Bacteriophages and bacteriophage-derived endolysins as potential therapeutics to combat Gram-positive spore forming bacteria. A Nakonieczna 2014

Endolysins of *Bacillus anthracis* Bacteriophages Recognize Unique Carbohydrate Epitopes of Vegetative Cell Wall Polysaccharides with High Affinity and Selectivity KF Mo et al. 2012

Bacteriophage endolysins: a novel anti-infective to control Gram-positive pathogens. VA Fischetti 2010

The 500 Dalton rule for the skin penetration of chemical compounds and drugs. JD Bos 2000

Immunocompatibility of bacteriophages as nanomedicines. T Kaur 2012

Results of 22 Jun 2018 on-line search (Google Scholar; summarized results for top 10 hits)

Search criteria: Relevance; key word combination "and": *Clostridium, perfringens*, endolysin, lysin, human, consumption, ingestion, disease, pathology, morbidity, mortality, etiology

The murein hydrolase of the bacteriophage ϕ3626 dual lysis system is active against all tested Clostridium perfringens strains. M Zimmer 2002

Characterization of bacteriophages virulent for *Clostridium perfringens* and identification of phage lytic enzymes as alternatives to antibiotics for potential control of the bacterium. BS Seal 2013

Identification and characterization of a putative endolysin encoded by episomal phage phiSM101 of *Clostridium perfringens*. H Nariya 2011

The Identification and Characterization of *Clostridium perfringens* by Real-Time PCR, Location of Enterotoxin Gene, and Heat Resistance. KA Grant 2008

Genomic analysis of Clostridium perfringens bacteriophage ϕ3626, which integrates into guaA and possibly affects sporulation. M Zimmer 2002

A thermophilic phage endolysin fusion to a Clostridium perfringens-specific cell wall binding domain creates an anti-Clostridium antimicrobial with improved. SM Swift 2015

Genome sequencing and analysis of a type A Clostridium perfringens isolate from a case of bovine clostridial abomasitis. VJ Nowell 2012

Molecular characterization of podoviral bacteriophages virulent for Clostridium perfringens and their comparison with members of the Picovirinae. NV Volozhantsev 2012

Expression and delivery of an endolysin to combat *Clostridium perfringens*. T Gervasi 2014

Application of *Lactobacillus johnsonii* expressing phage endolysin for control of *Clostridium perfringens*. T Gervasi 2014

End file

04/12/2019

GRN 802

While we understand that the intended use of your product is as an antimicrobial to control *Clostridium perfringens* in certain temperature-abused foods, please confirm that it is not intended to be used in lieu or in conjunction with standard methods for preventing temperature abuse in cooked foods. Also confirm that the product is not meant to be used as a substitute to standard methods for the safe handling of food.

Response from NOMAD: **04/12/2019**

ENDOLYSIN is an antimicrobial for the control of Clostridium perfringens in certain temperature-abused foods. This product is not intended to be a substitute to standard culinary hygiene and safe preparation and handling of food. This product is intended for use in addition to standard methods and practices for preventing temperature abuse in cooked food that can lead to the growth of C. perfringens.

From:	Kristi Smedley
To:	Wafula, Denis
Subject:	RE: Response Letter for GRAS Notice No. GRN 000802
Date:	Friday, May 10, 2019 3:07:44 PM
Attachments:	image011.png image017.png image029.png

Dr. Wafula:

Thank you for this letter, we appreciate your work on this notice.

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: Wafula, Denis [mailto:Denis.Wafula@fda.hhs.gov]
Sent: Friday, May 10, 2019 2:30 PM
To: Kristi Smedley
Subject: Response Letter for GRAS Notice No. GRN 000802

Hello Dr. Smedley,
Find attached our response letter to GRN 000802. If you have any questions, please do not hesitate to contact us.
Best regards,
Denis

Denis Wafula, Ph.D.
Staff Fellow
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Office: 2404021314
denis.wafula@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Friday, April 12, 2019 10:59 AM
To: Wafula, Denis <Denis.Wafula@fda.hhs.gov>
Cc: 'DANIEL TUSE' <daniel@dt-cg.com>
Subject: RE: Comment from Reviewers GRN 802

Denis:

Is this the format you need?

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: Wafula, Denis [mailto:Denis.Wafula@fda.hhs.gov]
Sent: Friday, April 12, 2019 10:38 AM
To: Kristi Smedley
Subject: RE: Comment from Reviewers GRN 802

Hello Dr. Smedley,
Thanks for the answer, for ease of records and because the reply is considered an amendment to the notice, I will be glad if you provide the answer after the comment on the attachment.
Regards,
Denis

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Friday, April 12, 2019 10:29 AM
To: Wafula, Denis <Denis.Wafula@fda.hhs.gov>
Cc: 'DANIEL TUSE' <daniel@dt-cg.com>; Gleba@nomadbioscience.com; Anatoli Giritch <gyrych@nomadbioscience.com>
Subject: RE: Comment from Reviewers GRN 802

Dr. Wafula:

Thank you for your email of this morning. We are providing the affirmation you discussed, below:

ENDOLYSIN is an antimicrobial for the control of *Clostridium perfringens* in certain temperature-abused foods. This product is not intended to be a substitute to standard culinary hygiene and safe preparation and handling of food. This product is intended for use in addition to standard methods and practices for preventing temperature abuse in cooked food that can lead to the growth of *C. perfringens*.

Should you need this in letter format, please let me know and I will immediately comply with this request.

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: Wafula, Denis [mailto:Denis.Wafula@fda.hhs.gov]
Sent: Friday, April 12, 2019 9:41 AM
To: Kristi Smedley
Cc: 'DANIEL TUSE'
Subject: Comment from Reviewers

Hello Dr. Smedley,
Please find attached a comment from our reviewers. Briefly, due to the intended uses of your product there is concern that some users might view it as a substitute for proper handling of food. The reviewers would like an affirmation that the product is not meant to replace such methods. If you have any questions, do not hesitate to contact me.
Best Regards,
Denis

From: Wafula, Denis
Sent: Tuesday, March 26, 2019 9:27 AM
To: 'Kristi Smedley' <smedley@cfr-services.com>
Cc: 'DANIEL TUSE' <daniel@dt-cg.com>
Subject: Extension of Review timeframe for GRN 000802

Hello Dr. Smedley,
This email is to inform you that, in accordance with 21 CFR 170.265 (b)(2), FDA is extending the normal 180-day review timeframe by 90 days for GRN 000802 (Endolysin).
We are finalizing the response and I hope you will get it soon (I estimate in a week or two). Do not hesitate to call or email me if you have any questions.

Regards,
Denis

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Tuesday, October 23, 2018 11:04 AM
To: Wafula, Denis <Denis.Wafula@fda.hhs.gov>
Cc: 'DANIEL TUSE' <daniel@dt-cg.com>
Subject: RE: Questions from Reviewers for GRN 000802

Dr. Wafula:

We have considered the questions raised by the GRN 802 reviewers and provided a response to each of the issues raised.

For your convenience we have provided the cover letter and the narrative of the response as an attachment to this email.

You will also receive by FEDEX (tomorrow) a hard copy of the signed letter and narrative, as well as a CD copy of the cover letter, narrative, and ALL cited references.

We would be happy to provide any clarification of our response. Please contact Daniel or I for any needed additional information.

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: Wafula, Denis [mailto:Denis.Wafula@fda.hhs.gov]
Sent: Tuesday, October 16, 2018 3:37 PM
To: Kristi Smedley
Subject: Questions from Reviewers for GRN 000802

Hello Dr. Smedley,
Find attached questions and comments to the notifier from the reviewers of GRN 000802. If you or the notifier have any questions, please contact me and I will be glad to help. Also, remind the notifier not send any confidential information or a new GRAS notice when answering the questions because that will complicate and slow down the review.
Best regards,

Denis

Denis Wafula, Ph.D.
Staff Fellow
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Office: 2404021314
denis.wafula@fda.hhs.gov

From: Wafula, Denis
Sent: Monday, August 20, 2018 4:22 PM
To: 'Kristi Smedley' <smedley@cfr-services.com>
Subject: Filing Letter for GRN 000802

Hello Dr. Smedley,
Find attached the filing letter for GRN 000802. Please review the letter for accuracy and let me know if you have any questions.
Best Regards,
Denis

Denis Wafula, Ph.D.
Staff Fellow
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Office: 2404021314
denis.wafula@fda.hhs.gov

247 pages have been removed in accordance with copyright laws. The removed references are:

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AllergenOnline. 2015. University of Nebraska, Lincoln, food allergen bioinformatic database. http://www.allergenonline.org/. Accessed May 2015.

Andersson, C., Wennström, P., & Gry, J. (2013, December 1). Nicotine alkaloids in Solanaceous food plants. Zenodo. http://doi.org/10.5281/zenodo.818223

Barr JJ., "A bacteriophages journey through the human body", Immunol Rev. 2017 Sep;279(1):106-122. doi: 10.1111/imr.12565.