consultants to the regulated industry

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

March 30, 2018

775

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 COLICIN from Nicotiana benthamiana
For use as antimicrobial agent

Enclosed you will find the GRAS notice for COLICIN as manufactured using a new host plant, Nicotiana benthamiana as submitted by NOMAD BIOSCIENCE GmbH. COLICIN is an antimicrobial agent and is the subject of previously filed GRNs 593 and 676. This GRAS notice is specific to the change in host plant.

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)
COLICIN GRN NARRATIVE of Notice (CD-Copy)
Appendices (CD-copy)
Full Complement of References (CD-copy)

cc: Yuri Gleba, Nomad

RECEIVED
APR - 2 2018
OFFICE OF
FOOD ADDITIVE SAFETY

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

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

2. Submission Format: *(Check appropriate box(es))*
☐ Electronic Submission Gateway
☒ Electronic files on physical media with paper signature page
☐ Paper
If applicable give number and type of physical media
Submission consists of three (3) CDs 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 593
☐ b) GRAS Affirmation Petition No. GRP _____
☐ c) Food Additive Petition No. FAP _____
☐ d) Food Master File No. FMF _____
☒ e) Other or Additional *(describe or enter information as above)* GRN 676

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).*

COLICIN is a food safety processing aid consisting of antimicrobial proteins produced in plants. The product is designed for application to vegetables, fruits or meat products in food processing facilities, and is not meant for application to raw agricultural commodities. COLICIN comprises a single colicin protein or a mixture of colicin proteins blended to achieve maximum potency against enteropathogenic strains of *Escherichia coli*. Specifically, COLICIN is intended to prevent or minimize contamination of food products by pathogenic *E. coli*, including ETEC, EHEC and STEC. COLICIN is applied as a spray, solution, dip or package additive to control *E. coli* on fresh or processed vegetables and fruits, including ready to eat produce, and on whole meat cuts and meat prior to grinding, at an application rate of 1-10 mg COLICIN (total colicin protein) per kg of treated food product (approximately 0.5-5 mg/lb).

This notice covers a change in host plant for the previously notified COLICIN substance, Nicotiana benthamiana.

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 V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	Substance Category *(FOR FDA USE ONLY)*
1	Colicin M Colicin K Colicin Ib		AAA23589.1 AAB41288.1 AAA23188.1	Plant, recombinant Plant, recombinant Plant, recombinant	
2	Colicin U		CAA72509.1	Plant, recombinant	
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s))*, and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

Detailed information regarding the identity, safety and suitability of the notified substances is incorporated in the GRAS Notifice documents provided.

3. Synonyms

Provide as available or relevant:

1	
2	
3	

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 COLICIN *(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. ☒ NOMAD BIOSCIENCE GMBH *(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.

NOMAD BIOSCIENCE GMBH *(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)*

NOMAD BIOSCIENCE GMBH *(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)	Kristi O. Smedley, Ph.D.	03/30/2018

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 COLICIN Antimicrobial produced in Nicotiana benthamiana (PDF of Notification)	
	COLICIN GRN References - For FDA Internal Review Only (PDFs of All Cited References - Not for Republication)	

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

March 30, 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: COLICIN Antimicrobial Produced in *Nicotiana benthamiana*

Dear Dr. Mattia,

Nomad Bioscience GmbH ("Nomad"; "Notifier") is submitting this GRAS notice ("Notice") with the goal of expanding the list of acceptable host plants for manufacturing Nomad's COLICIN antimicrobial processing aid to control *Escherichia coli* (*E. coli*) in food. Notifier has concluded using scientific procedures that COLICIN produced via a modified *Nicotiana-benthamiana*-based manufacturing process is GRAS.

In GRN 593 Nomad described a plant-based manufacturing process for its COLICIN antimicrobial product. Plants listed as production hosts included the food species spinach, red beet and lettuce. Colicin proteins are produced recombinantly in the plants and extracted and formulated into an antimicrobial product for controlling pathogenic strains of *E. coli* bacteria in fruits and vegetables. Notifier received a No Questions letter from FDA for GRN 593 on 18 December 2015. Subsequently, Notifier submitted suitability data for COLICIN produced in the same food plant species for use as an antimicrobial on various meat products. That Notice (GRN 676) was jointly reviewed by FDA and FSIS. Notifier received a No Questions letter from FDA for GRN 676 on 15 May 2017 and USDA listed COLICIN in FSIS Directive 7120.1 rev. 42 (2017).

This current Notice aims to expand the list of plants that can be safely used as colicin production hosts to include the non-food species ***Nicotiana benthamiana***. This host offers advantages in its ease of cultivation and protein yield and is already the preferred host plant for producing biologic products for human use. Because the colicin proteins are extracted and purified prior to formulation, we assert that the safety and utility of the COLICIN product is retained regardless of which plant species are used as production hosts.

The compositional properties of colicin proteins produced in *Nicotiana benthamiana*, including their amino acid sequence, lack of glycosylation, activity profile and antibacterial host range, are identical to those of colicin proteins produced recombinantly in food plant species. Further, because the resulting proteins are the same, the application rate of colicin proteins so produced is the same as the application rate of colicins derived from food species hosts (e.g. 1-10 mg COLICIN/kg treated food).

The active ingredients (i.e. colicin proteins), the main process used to manufacture them, the product specification, and the use rate on foods for *Nicotiana*-produced colicins are highly similar to those described in GRN 593 and GRN 676; therefore, while describing the differences, this Notice references GRN 593 and GRN 676 for brevity and to rely on FDA's original review memoranda for our prior GRAS notices.

Nicotiana benthamiana is a non-food species that does not interbreed with commercial varieties of *Nicotiana*, such as *N. tabacum*. This species does best when cultivated indoors under controlled environmental conditions. It is a preferred host for producing plant-made pharmaceuticals and biologics because of its permissiveness in expressing a wide range of recombinant proteins. The FDA is already well-acquainted with *N. benthamiana* because it has been employed in the GMP-compliant manufacture of several human vaccines and therapeutic products. As described in several INDs, these *Nicotiana*-produced biologic products have been evaluated extensively for safety in human clinical studies with close FDA (CBER and CDER) oversight.

This Notice provides substantiating evidence, on the basis of published studies and scientific procedures, for classifying *N. benthamiana* as a safe host for producing recombinant proteins, such as colicins, for use as food processing aids.

Our submission includes a CD with electronic versions (PDF) of the following documents:

FDA Form 3667 Nomad Bioscience COLICIN Antimicrobial Produced in *Nicotiana benthamiana*
GRAS Notice COLICIN Antimicrobial Produced in *Nicotiana benthamiana*
Copies of references cited

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

Sincerely,

(b) (6)

Yuri Gleba, Ph.D.
Chief Executive Officer

Table of Contents

Table of Tables

Table of Figures

1. General Introduction and Claim of Exemption from Premarket Approval Requirements

Nomad Bioscience GmbH ("Nomad"; Notifier) COLICIN product is a mixture of colicin proteins produced recombinantly using a plant-based manufacturing process to match the amino acid sequence of naturally occurring colicin-family antimicrobial proteins. On August 7, 2015, Notifier submitted a GRAS Notice to FDA/CFSAN asserting that COLICIN had been determined to be generally recognized as safe through scientific procedures, and that the product should therefore be exempt from the requirement of premarket approval under proposed 21 CFR 170.36(a). FDA assigned the notification the identifier GRN 593, and subsequently reviewed the merits of Notifier's submission claims. On 18 December 2015, Notifier received from FDA a "No Questions" letter for GRN 593" for COLICIN antimicrobial as a processing aid for fresh and minimally processed produce (fruits and vegetables) under the conditions of intended use. Subsequently, Notifier submitted suitability data for the same COLICIN product for use as an antimicrobial on various meat products. That Notice (GRN 676) was jointly reviewed by FDA and FSIS. Notifier received a "No Questions letter for GRN 676" from FDA on 15 May 2017 and USDA listed COLICIN in FSIS Directive 7120.1 rev. 42 p. 35 (FSIS 2017).

Both GRN 593 for COLICIN's use on produce and GRN 676 for COLICIN's use on meats cited recombinant production of colicin antimicrobial proteins using one of several host plants selected from a group of food species, such as spinach, red beat or lettuce, which are all GRAS by virtue of their traditional use as food. In this present Notice, Notifier claims that additional plant species, including non-food species, could be used for manufacturing colicin proteins recombinantly without jeopardizing COLICIN's GRAS designation.

Notifier provides evidence supporting expansion of the original list of production host plants cited in GRN 593 and GRN 676 for manufacturing colicin proteins to include ***Nicotiana benthamiana***. The solanaceous species *N. benthamiana* is not currently GRAS. The main reasons for this are that *N. benthamiana*: (1) has not been traditionally used as a food crop, (2) contains low levels of bioactive alkaloids including nicotine and anabasine, and (3) has not been previously used in the production of food additives or processing aids, in direct contrast to the already accepted use of *N. benthamiana* in the safe production of biopharmaceuticals and biologics for human clinical and veterinary indications.

Importantly, it is not the manufacturing host plant that is the product applied to food, but rather the recombinant colicins extracted from the host that will be applied as food antimicrobials. The process used for colicin manufacture includes removal and dilution of host- and process-derived impurities to an extent that renders the final formulation nontoxic. This is analogous to *Nicotiana* species having been used as hosts in the production of biopharmaceutical proteins that have already shown very high safety in preclinical and clinical studies under FDA IND.

Nomad has determined that the safety of colicin proteins produced recombinantly using *N. benthamiana* is comparable to colicin proteins produced from food species, and in fact present no greater risk to consumers than consumption of certain plant alkaloids endogenously present in plants such as potato, pepper, eggplant, tomato and cauliflower, for example, which are all foods and recognized as safe based on historical consumption patterns worldwide.

Notifier contends that under the conditions of product manufacture and use described herein, COLICIN produced from *N. benthamiana* is also generally recognized as safe and therefore should be exempt from premarket approval procedures under proposed 21 CFR 170.36(a)(I).

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

COLICIN

1.4 Conditions of Use

COLICIN is intended to prevent or minimize contamination of vegetables, fruits, or meat products by pathogenic strains of *E. coli*. Specifically, the intended use of COLICIN is as a spray, dip, wash, or marinade to control enteropathogenic strains of *E. coli* on fresh or processed produce or meats such as beef, pork, lamb, mutton, veal, etc. at an application rate of 1-10 mg COLICIN per kg of food product (approximately 0.5-5 mg/lb). COLICIN is used in a food processing environment and not on raw agricultural commodities.

Due to the use of the non-food plant host *N. benthamiana* in manufacturing, only one grade of COLICIN, namely **COLICIN Isolate**, is claimed in this Notice for use as an antimicrobial on the same food products defined in GRN 593 and GRN 676. The colicin proteins comprising the formulated **COLICIN Isolate** are subjected to a more stringent purification procedure prior to formulation than are their food plant species-produced counterparts. This constitutes a necessary downstream adaptation of the process to ensure removal of alkaloid impurities to levels that would not pose a risk to consumers.

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 this Notice, it is Nomad Bioscience's conclusion that COLICIN, formulated from colicin proteins produced in *N. benthamiana*, is generally recognized as safe when used as a food safety antimicrobial at application rates not exceeding 10 mg COLICIN/kg treated food.

1.6 Not Subject to Preclearance

Notifier has concluded that COLICIN, as manufactured via its plant-based process using *N. benthamiana* as the production host species, 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 conclusion 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 COLICIN antimicrobial.

(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, Specification, Technical Effect

2.1 Identity, Structural and Functional Information

Identity

COLICIN is comprised of one or more members of the colicin family of proteins that belong to a group of antimicrobial proteins and peptides known as bacteriocins. All colicins are synthesized naturally in the intestinal tract of humans and other animals and in other natural environments by colicinogenic strains of the commensal (indigenous) bacterium *E. coli*, and act against non-colicin-producing strains of *E. coli*, including human enteropathogenic strains (termed EHEC/STEC/ETEC depending on their toxic profile).

The product COLICIN can be formulated to contain a single colicin or a mixture of two or more individual colicin proteins, depending on the breadth of application needed in various food products. Full details on colicins produced in food species plants such as spinach, red beet and lettuce, including their safety and range of biological activities, were provided in GRN 593 (use on produce) and GRN 676 (use on meat).

Detailed information about the components of COLICIN were presented in GRN 593. In that notice and in GRN 676 we listed 9 colicins as candidate components of the product, including Colicins E1, E7, I, M, N, K U, 5 and B. Studies since those submissions have allowed us to identify the colicins having the most potent and broadest spectrum of activity and we have produced these in *N. benthamiana*. Hence, in this current Notice, we list four (4) *N. benthamiana*-produced colicins as candidate components of the final formulation.

Table 2-1 lists the colicins currently included in this Notice and that may be used singly or in combination to achieve the desired antibacterial suitability on food products. These *N. benthamiana*-produced colicins can also be blended with food plant species-produced GRAS colicins identified in GRN 593 and GRN 676.

Table 2-1. Active components of COLICIN product formulation

Colicin	GenBank No.	Mode of Action	Receptor / Translocator	Targets
M	AAA23589.1	Peptidoglycanase	FhuA / TonB-ExbBD	ETEC; *Salmonella*
K	AAB41288.1	Pore-forming	Tsx / OmpAF, TolABQR	ETEC
U	CAA72509.1	Pore-forming	OmpA / OmpF, TolABQR	ETEC
Ib	AAA23188.1	Pore-forming	Cir / TonB-ExbBD, Cir	ETEC; *Salmonella*

*ETEC designation is used here to include Big Seven enteropathogenic strains of *E. coli*, including enterotoxigenic (ETEC), enterohemorrhagic (EHEC) and Shiga-toxin-producing serotypes (STEC).

All colicins, including those listed in GRN 593 and GRN 676 and the subset of *N. benthamiana*-produced colicins listed in this Notice, are produced naturally in the human gastrointestinal tract by commensal strains of *E. coli* or related species and demonstrate complementary modes of action (See Table A-1 in GRN 593, p 20; from (Yang 2014)).

Colicin M, colicin K, **colicin U**, **colicin Ib** are produced in *N. benthamiana* and are particularly effective for food protection against pathogenic strains of *E. coli*. All colicin proteins studied share a high safety profile.

Structural information on COLICIN components

All plant-made colicin proteins have been expressed in several food and non-food host plants. Regardless of host plant used, all plant-made colicins conform to their predicted compositions and share the amino acid sequences of the bacterially produced native colicin proteins.

Table 2-2 below summarizes the confirmation of identity of each *N. benthamiana*-produced colicin using methods described in GRN 593 (peptide fragment mass fingerprinting/MALDI TOF MS) (Schulz 2015). In the current Notice colicin sequencing by MALDI-ISD was also employed, as we described in Stephan (2017).

Table 2-2. Identity confirmation of *N. benthamiana*-produced colicins by mass spectrometry

Colicin	*N. benthamiana*-produced (determined by Notifier)			Bacterial (literature values)
	N-terminus	C-terminus	Full-length coverage	N-terminus (method). Reference
M	Confirmed. N-terminal met is present; thr3 is acetylated	Confirmed	100%	N-terminal met verified by Edman degradation (Dreher 1985)
K	Confirmed. N-terminal met is present	Confirmed	95%	(Pilsl 1995)
U	Confirmed. N-terminal met is cleaved off; mature protein starts with N-terminal pro	Confirmed	89%	(Šmajs 1997)
Ib	Confirmed. N-terminal met is cleaved off; mature protein starts with N-terminal ser; ser2 is acetylated	Confirmed	MALDI-TOF MS/MS and MALDI-ISD confirm intact protein	N-terminal met is cleaved off; mature protein starts with N-terminal ser (DNSC method; Edman degradation). (Konisky 1972; Mankovich 1986)

In previous notices, nine (9) recombinant colicins were described as candidate components of the COLICIN product, to be used either singly or in combination. Structural information for plant-made **colicin E1**, **colicin E7, colicin Ia, colicin M, colicin N, colicin K, colicin U, colicin 5** and **colicin B** was provided by Notifier in GRN 593 (Section 2.3, pp 8-14 of that Notice), which included structural information on each component of COLICIN, the lack of glycosylation in each protein, physical properties, and confirmation of colicin molecular mass and amino acid sequence by MALDI-MS, including comparisons of the recombinant and native bacterial proteins. The peptide mass and peptide fragment fingerprinting methods used for protein sequence verification were described in GRN 593 APPENDIX C, Section C.2: Methods for confirming colicin amino acid sequences by MALDI-MS (pp 61-64).

The results in Table 2-2 confirm that the amino acid sequences of the 4 *N. benthamiana*-produced colicins studied to date (**colicin M, colicin K, colicin U and colicin Ib**) match the corresponding bacterial sequences, have no truncations, and have minor and typical plant-type modifications as also described in GRN 593.

Quantitative composition

COLICIN is preferentially prepared as a dry powder as described in GRN 593 and in the current Notice (APPENDIX B). The bulk product is dissolved/diluted in water to a concentration of 0.05 mg/mL (50 mg/L) for spraying at a rate ≤20 mL solution/kg (9 mL/lb) of product. Alternatively, food products can be dipped in a solution of COLICIN at a concentration of 0.1 mg/mL (100 mg/L). COLICIN can also be added to packaged food products or infused internally at a rate not to exceed 10 mg/kg (<4.6 mg/lb).

Colicin proteins produced in *N. benthamiana* are subjected to the more stringent purification process previously described for COLICIN Isolate. The use of chromatographic separation step further removes host impurities including bioactive alkaloids.

Colicins can be prepared singly or in combination with other colicins. For mixtures, each colicin protein is manufactured separately then combined in defined ratios. The decision to formulate a single colicin or mixtures of colicins depends on the food application and the pathogen(s) targeted for control. Regardless, the total amount of colicin protein(s) formulated in the COLICIN product to be applied to food is ≤10 mg/kg (≤~5 mg/lb). A COLICIN formulation consisting of a single component comprises only the specified colicin as the active ingredient. A COLICIN formulation consisting of two or more different colicins comprises two or more active ingredients having synergistic or additive potency. A preferential use range on a protein basis is 1 to 10 mg (total) COLICIN per kg of food product.

Modes of action

Colicins' modes of action fall into two major categories, namely, those that form pores in the cell membranes of susceptible target bacteria and those that act by enzymatic degradation of cellular macromolecules. The mode of action of individual colicins used by Notifier in its COLICIN food processing aid have been described in detail in GRN 593, specifically in Section 2.2 Mode of Action (pp 7-8) and APPENDIX A, Section A.3.1: Biological Activity of COLICIN on target pathogenic *E. coli* strains (pp 37-44). Colicin mixtures show additive potency against *E. coli* because they attack different cellular/molecular targets. We reported the potencies of *N. benthamiana*-produced colicins in Stephan et al. (2017).

2.2 Method of Manufacture

Notifier uses a plant-based manufacturing process for producing COLICIN proteins; the method is an adaptation of the process used to manufacture biopharmaceuticals, which have been administered in multiple human clinical trials under FDA INDs. The colicins are produced using recombinant technology to yield concentrated extracts. Preferred host plants originally included the food species **spinach** (*Spinacia oleracea*), **red beet** (*Beta vulgaris*) and **lettuce** (*Lactuca sativa*). In the present submission, Notifier asserts that other plant hosts can be used to produce colicin proteins without impacting the safety of the final product. Such species include members of the genus *Nicotiana*, specifically the species ***N. benthamiana***.

The plant-based, colicin manufacturing process was described in GRN 593 APPENDIX B: COLICIN Manufacturing Process (pp 51-57). The **process modification** described in this Notice (see APPENDIX B should enable expansion of the list of candidate host production plant species, to include ***Nicotiana benthamiana***.

Regardless of the host plant used in manufacturing, the plant-derived biomass remaining after colicin 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

The characteristic molecular properties of all colicin proteins that can comprise the final COLICIN product were defined in GRN 593, Section 2.3 Characteristic Properties (p 16). Colicins, by definition, are unstable to proteolytic digestion (Cascales 2007; Murinda 1996; Zhang 1992). Colicins are either denatured by heat (cooking) or stomach acid and are degraded rapidly upon exposure to gastric and intestinal enzymes.

Using plant-produced colicins, Notifier has confirmed the rapid degradation of colicins in simulated gastric fluid and simulated intestinal fluid. Results of these digestibility and degradation studies were presented in GRN 593, Section A.2.3 (pp 28-32) and APPENDIX C: Methodology (pp 58-61), and in Schulz et al. (2015).

Importantly, Notifier studied at the molecular level the allergenic potential of candidate colicins for use in food (GRN 593, Section A.2.5, pp 34-37) and determined, from published information, that colicins have a low potential for inducing immune or allergic responses.

Formulation

COLICIN is provided as a dry powder. When using *N. benthamiana* as the expression host, the proteins are subjected to chromatographic purification to yield the purer **COLICIN Isolate**. The bulk product is dissolved and/or diluted in water according to instructions and applied as a wash, spray, dip, package fill or marinade depending on the intended use.

Content of potential human toxicants in COLICIN

There are no known human toxicants when the product COLICIN is produced using food species of plants. There are two potential human toxicants that may remain in low levels in the COLICIN product when it is manufactured using the host plant *N. benthamiana*, namely, the alkaloids nicotine and anabasine.

The host plant *N. benthamiana* shares metabolic pathways with other members of the family Solanaceae, which includes tomato, pepper, potato, eggplant and others. All these plants contain low residual yet measurable levels of pyridine alkaloids. The purpose of including a chromatographic purification step during downstream processing is to produce colicins with levels of residual alkaloids that are no higher than the levels consumed in common vegetables in a typical diet.

Specification

The general process used to manufacture COLICIN using food species plant hosts was presented in GRN 593, APPENDIX B: COLICIN Manufacturing Process, specifically in Section B.3: Procedure and Section B.4: Specifications, pp 52-56). The **process modification** implemented to produce colicins from *N. benthamiana* (APPENDIX B) entails changes to intermediate biomass extraction steps plus chromatographic purification to yield active ingredients that are individually ≥70% purity and have a reduced content of host alkaloids.

Individual colicins are blended at prescribed ratios into a mixed COLICIN product. The final product may contain only *N. benthamiana*-produced colicins, only food plant species-produced colicins, or a mixture of colicins produced in *N. benthamiana* and in a food plant species host.

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

> A COLICIN antimicrobial product consisting of one or more *N. benthamiana*-produced colicins to include colM, colK, colIb or colU, mixed or blended in any combination, or mixed or blended in any combination with GRAS colicins produced in food plant hosts as defined in GRN 593 and GRN 676, applied at a rate not exceeding 10 mg total colicins per kg of food product treated, and in which maximum levels of nicotine and anabasine impurities in the blend do not exceed 75 ng/mg and 15 ng/mg, respectively.

The current **target Specification** for a COLICIN product consisting of 100% *N. benthamiana*-produced colicins is summarized in Table 2-3. The upper limits for nicotine and anabasine of 75 ng/mg and 15 ng/mg, respectively, for a blend of colicin proteins were set as defined in Section 6.2 (Overall Safety Summary) based on assessed risk.

Current results achieved and listed in the Specification are derived from analyses of colicin proteins produced in multiple independent developmental batches, ranging from 9-12 manufacturing runs for colM, 4-7 for colK, 8-11 for colU, and 13-16 for colIb. Details on the methods used to quantify these parameters are found in APPENDIX C. Nicotine and anabasine limits are introduced for *N. benthamiana*-produced colicins; limits for these alkaloids are omitted when using food species hosts because they are devoid of such impurities.

Table 2-3. Target specification of COLICIN (mixed colicins) product

COLICIN Produced in *N. benthamiana*			
Parameter	**Method**	**Specification limit**	**Results of analyses***
Appearance	Visual	Powder, white to beige	Conforms
Specific Activity (colicin protein basis)	Viability inhibition; *E. coli* DH10B strain	$\geq 2.5 \times 10^6$ AU/g	$2.15 \pm 0.89 \times 10^9$ AU/g
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	$\leq$ 30 ppm	In progress
Heavy metals: Lead	USP38<233> ICP-OES	$\leq$ 5 ppm	< 1 ppm
Heavy metals: Cadmium	USP38<233> ICP-OES	$\leq$ 5 ppm	< 1 ppm
Nicotine (per total colicin blend)	HPLC/MS	$\leq$ 75 ng/mg colicin	Average 48.76 ng/mg
Anabasine (per total colicin blend)	HPLC/MS	$\leq$ 15 ng/mg colicin	Average 9.54 ng/mg
Bioburden	USP32<61>	$\leq$ 10 CFU/25 g sample	0 (absent)
Agrobacterium (CFU/10 g sample)	Selective plate-based assay	0 (absent)	0 (absent)
Undesirable microorganisms: *Escherichia coli, 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	> 8 months

*Results of analyses for a dry, 100% *N. benthamiana*-derived COLICIN (mixed-colicin) product are based on average results obtained from analyses of individual colicin proteins blended at their optimal ratio (dwb), typically 3x colM, and 1x each of colicins K, U and Ib (w/w).

2.4 Technical Effect and Suitability of Use

In GRN 593, the properties, modes of action and antibacterial potency of plant-produced colicins were first described as well as the efficacy of these proteins when applied to *E. coli*-contaminated produce. In GRN 676, the efficacy and suitability of colicins as antimicrobials on multiple meat products were described. Results of some of those studies were also published by Notifier and collaborators (Schulz 2015); a copy of the published manuscript is included in this package for Agency review.

Regardless of host plant used, all plant-made colicins conform to their predicted compositions and share the amino acid sequences of the bacterially produced native colicin proteins. The activity is not altered by the plant host. Therefore, based on our meeting with the Division of Biotechnology and GRAS Notice Review staff (December 5, 2017) no additional data to support the utility was required. However, to support this decision, Notifier replicated some of the studies demonstrating efficacy and duration of technical effect with *N. benthamiana*-produced colicin mixtures on meat matrices.

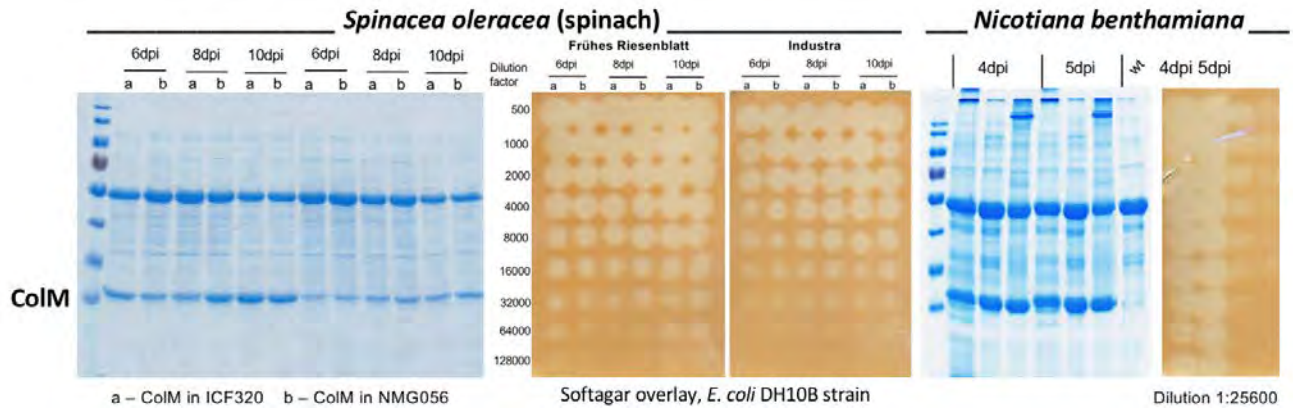
Salient points first described in GRN 593, GRN 676 and in Schulz et al. (2015) about COLICIN's efficacy and suitability in controlling *E. coli* are reproduced herein for convenience to reviewers. The general protocol used in proof-of-principle studies with *N. benthamiana*-produced colicin proteins are summarized below. For on-matrix efficacy evaluations, Notifier's **Standard Operating Procedure (SOP) No. NMD 901-01** is followed, as the SOP is independent of manufacturing host. The SOP was appended to GRN 676 and is appended to this Notice for convenience (see APPENDIX A). See also Schulz (2015) for additional detail.

Verification of technical effect of colicins produced in food species and *N. benthamiana*

Comparability of expression and efficacy *in vitro* of a model colicin (Colicin M) produced in a food species host (spinach) and in *N. benthamiana* was demonstrated (Schulz 2015). To expand briefly, ICF320 and NMG056 strains of *Agrobacterium tumefaciens* were transformed with pNMD10221 for cytosolic expression of ColM. Spinach plants were agroinfiltrated with a suspension of the vector (1:100 dilution). The host consisted of ***Spinacia oleracea*** variety Frühes Riesenblatt (5-weeks old) and variety Industra (7.5-weeks old). The plant phenotype was analyzed and samples were harvested after 6, 8 and 10 days post infiltration (dpi) and frozen in liquid nitrogen.

Total soluble extracts were prepared by adding of 5 vol of 150 mM NaCl after grinding in liquid nitrogen. Extracts were analyzed by loading 15 µl TSP extracts (1:1 diluted with 2x Laemmli buffer) on a 12% SDS-Gel and by activity determination on soft-agar overlay after 1:500 pre-dilution in 1% milk powder and 5 µl sample applied to the plates. A similar procedure was used to induce production of ColM and other colicins in ***N. benthamiana***. Figure 2-1 shows the expression of ColM in two varieties of ***S. oleracea*** and in ***N. benthamiana***. The time-course of accumulation of the colicin protein in green tissue and its antibacterial activity against a tester strain of *E. coli* are both evident regardless of expression host plant.

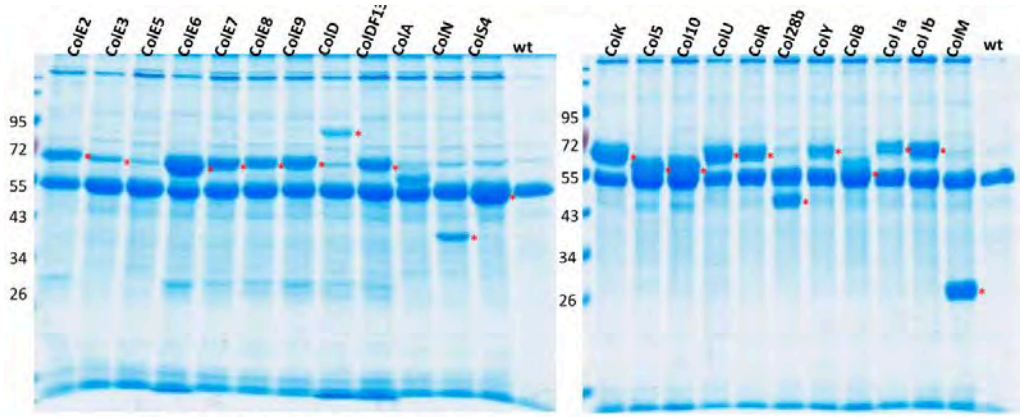
Figure 2-1. Expression and activity of colicin M produced in *S. oleracea* and in *N. benthamiana*



Panels at left show expression of Colicin M (ColM) in spinach between 6 and 10 days post agroinfiltration using two different ColM-expressing vector strains, with corresponding clearing zones in soft agar overlays using *E. coli* tester strain DH10B. Panels at right show results of expression of ColM in *N. benthamiana*, with corresponding clearing zones on soft agar overlay.

Figure 2-2 shows expression of colicins in *N. benthamiana*. With the exception of ColE5 and ColE1, all colicins express well in *N. benthamiana*, reaching levels of 1.5-5.3 g/kg FW biomass, or 13-50% TSP. See also Schulz (2015) for additional detail.

Figure 2-2. Expression of colicins in *N. benthamiana*



12% SDS-PAGE and Coomassie staining, 7.5 µl of TSP extracts correlate to 1.5 mg FW

Colicins expressed in *N. benthamiana* were isolated as described (Schulz 2015) and their antibacterial activity on two sample food matrices (beef and lamb) were determined. Confirmation of amino acid sequence and other structural properties, multi-batch manufacturing results, and the stability of *N. benthamiana*-produced colicins, are shown in APPENDIX C.

Demonstration of efficacy and suitability of *N. benthamiana*-produced colicins

Preparation of colicin and carrier test solutions

The full protocol (Nomad Bioscience **SOP NMD 901-01**) for determining COLICIN's efficacy on meat matrices independent of colicin source plant was submitted for FDA and USDA review in GRN 676 and is appended to this Notice for convenience (APPENDIX A).

Briefly, colicin-containing or colicin-free ("carrier") control extracts of *N. benthamiana* were used in this series of experiments. Colicin treatment consisted of *N. benthamiana*-produced colicin extracts applied at 3+1+1+1+1+1 mg colicin M+E7+Ia+K+5+U, respectively, per kg food equivalent. Carrier control solution consisted of the same volume of vehicle (*N. benthamiana* plant extract) without colicin.

Preparation of *E. coli* inocula

Each *E. coli* pathogenic strain in the USDA's Big Seven list plus STEC strain O104:H4 (responsible for outbreak in Germany in 2011) is grown individually and diluted from exponential overnight cultures to OD_{600} = 0.3. The diluted suspensions are mixed 1:1:1:1:1:1:1:1, diluted further according to desired contamination level, and the CFU/ml is verified by dilution plating and enumeration on Sorbitol-MacConkey (SMAC) medium. Two levels of bacterial contamination are used:

High contamination level

USDA Big 7 strain mix + O104:H4 wild-type strains: 1 x 10^4 to 1 x 10^5 CFU/g (4-5 log/g food)

Low contamination level

USDA Big 7 strain mix + O104:H4 nalidixic acid resistant mutants: 1-5 x 10^1 CFU/g (1-1.5 log/g food)

Preparation and contamination of meat samples

Packages of various fresh meats purchased at local markets were sterilized and the meat was trimmed. Images of samples prior to contamination and treatment are shown below in Figure 2-3.

Figure 2-3. Images of representative meat types used in colicin evaluation

Panel A: Beef raw untrimmed round roast used for preparation of beef steak (85 g) and beef cubes (~100 g, panel B) which were used in high and low contamination level exposures. Panel C: Lamb loins used for trimming to pieces of 85 g used in low contamination level exposures. Panel D: pork steak (85 g) used in high contamination level exposures. Panel E: Some beef samples were ground after contamination and colicin treatment.

As detailed in SOP NMD 901-01 (APPENDIX A), the appropriate (see above) mixed-pathogen bacterial suspension is applied to meat samples by dipping the meat in the suspension (12 ml/85 g) for whole cuts or by tumble mixing cubed samples (10 ml/kg). The bacteria are allowed to adhere to the samples for 30 min at room temperature, after which time very little fluid remains on the sample surfaces (low runoff).

COLICIN and control solution application

The COLICIN solution or the plant background (control) solution were applied to samples with a hand sprayer at a rate of 42 ml/kg meat. The treated samples were allowed to air dry for 30 min at RT. Some cubed meat samples were contaminated, treated, and then ground (ø6 mm and ø3 mm dye) prior to storage (Figure 2-3 **E**). The total time the meat samples were held at RT between colicin treatment and storage in bags was ~1 h for whole/cubed samples and ~2 h for ground meat.

Incubation and storage of treated samples

Quadruplicate samples of meat trimmed to steaks or ground meat were weighed and aliquots of ~40 g each were placed in BagFilter® 400P storage bags. The bags were stored at various temperatures (4°C for pork; 10°C and 15°C for beef and lamb) for storage period up to 144 h post treatment.

Determination of COLICIN's technical effect on food matrices

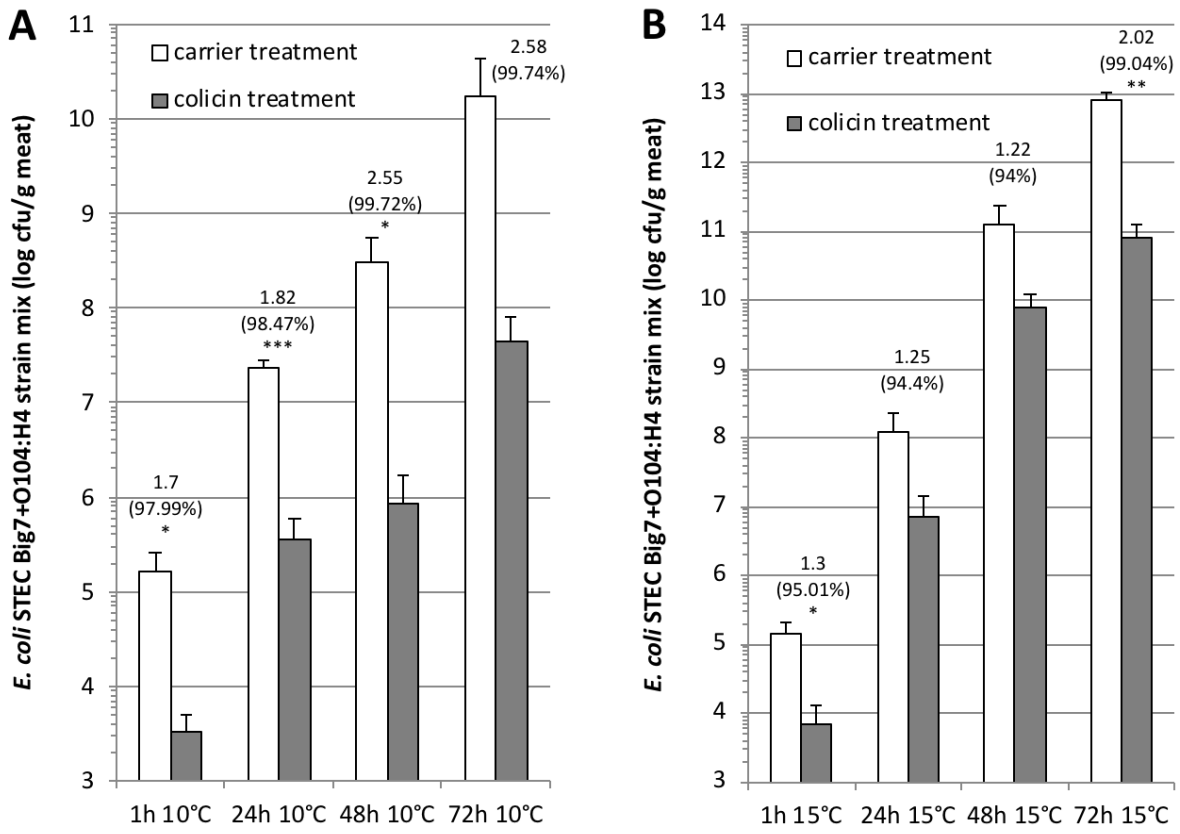
About 160 ml of buffered peptone solution was added to each ~40 g aliquot of sample (4 vol.; 1:5 dilution). The samples were homogenized in Bag Mixer® 400CC Homogenizer (settings: gap 0, time 30 sec, speed 4). Aliquots of the resulting bacteria-containing suspension from the filtered part of the bag were transferred into sterile tubes. The samples were concentrated by centrifugation, serially diluted, and 100 µl of each were plated on Sorbitol-MacConkey (SMAC) agar.

The medium used for dilution plating was supplemented either with 0.05 µg/ml cefixime and 100 µg/ml X-Gluc for the high contamination level treatments, or with 25 µg/ml nalidixic acid and 100 µg/ml X-Gluc for the low contamination level treatments; the latter using nalidixic acid resistant derivatives of all strains. Plates were enumerated for CFU after overnight incubation at 37°C. The technical effect of COLICIN relative to the carrier control was determined by comparing differential log CFU/g meat sample.

Figure 2-4 is an example of the data generated with mixtures of colicins in on-matrix studies against a mixture of *E. coli* STEC strains (results for high contamination level, beef matrix, shown as example). GRN 676 contains representative efficacy results for various colicin mixtures on all meat matrices evaluated. Results of *N. benthamiana*-produced colicins are comparable to those reported in GRN 676.

The results summarized in Figure 2-4 show that even when **beef steak meat** is contaminated at very **high levels** (target 4-5 $\log_{10}$ CFU/g meat) and stored at low yet growth-permissive temperatures for *E. coli* ("worst-case" meat processing scenario), treatment with colicin mixtures can significantly reduce on-matrix contamination by >99% (>1-2.5 $\Delta\log_{10}$ CFU/g meat).

Figure 2-4. Efficacy of *N. benthamiana*-produced colicin mixtures on beef matrix at high *E. coli* contamination levels and stored at 10°C and 15°C

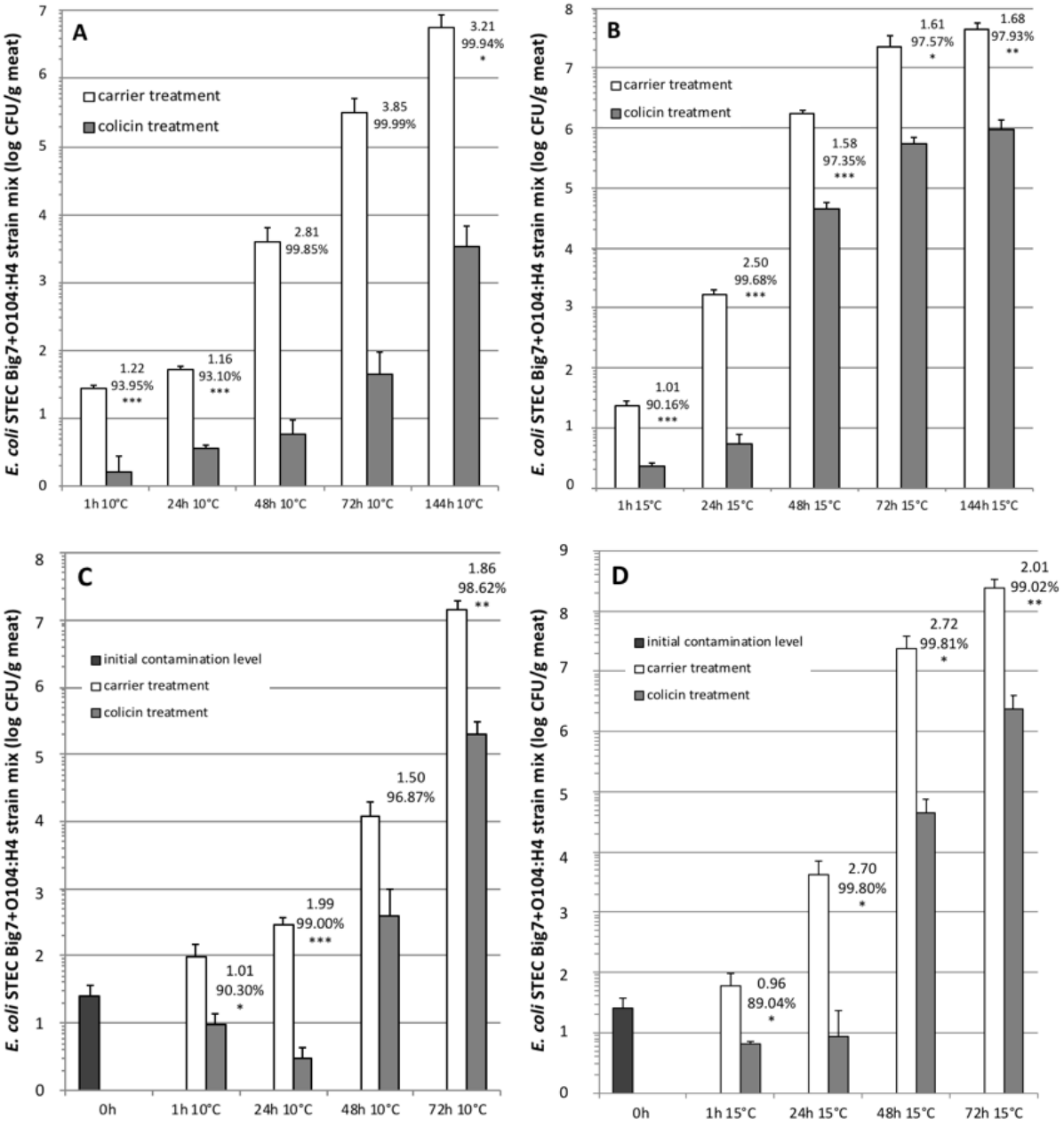


Panels A and B show differential log CFU/g beef samples contaminated with very high levels (target 4-5 $\log_{10}$ CFU/g meat) of a mixed-strain *E. coli* suspension followed by treatment with colicin mix (3+1+1+1+1 mg/kg colicins M+E7+ Ia+K+U) or carrier vehicle (*N. benthamiana* plant-protein extract w/o colicin) and followed by storage at either 10°C and 15°C, respectively. $\Delta\log_{10}$ CFU and (%CFU reduction) shown. Significance level: *= p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001.

Similar experiments to those reported in GRN 676 using **low levels** of bacterial contamination were also performed with *N. benthamiana*-produced colicins, for comparison. Figure 2-5 shows the results of this series of experiments. Mixed colicins from *N. benthamiana* were sprayed per Nomad's SOP NMD 901-01 onto **beef samples** that had been contaminated with lower levels (0.1-1.5 $\log_{10}$ CFU/g meat) of a mixed-strain *E. coli* suspension. Bacterial growth post treatment with a control solution (*N. benthamiana* plant extract with no colicins) and a solution of mixed colicins is shown as $\log_{10}$ CFU/g meat after suboptimal storage at 10°C (Panels A and C) and 15°C (Panels B and D).

Reductions in viable bacteria of >1 to nearly 4 log CFU/g meat were seen compared to controls. These results are comparable to results with whole beef cuts and beef cuts prior to grinding reported in GRN 676.

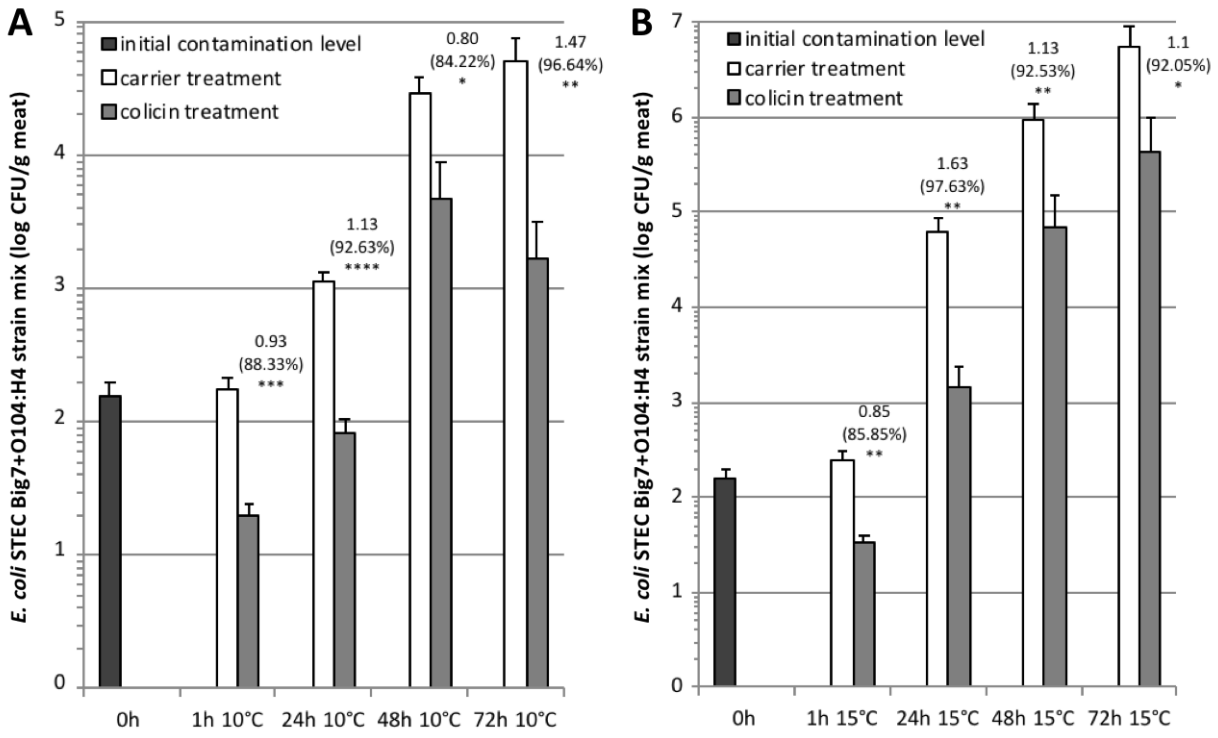
Figure 2-5. Efficacy of *N. benthamiana*-produced colicin mixtures on beef matrix at low *E. coli* contamination levels and stored at 10°C and 15°C



Efficacy of *N. benthamiana*-produced colicin mix (3+1+1+1+1 mg/kg colicin M, E7, Ia, K, U) applied to beef meat matrix (A and B) prior to grinding or to beef whole cuts (steaks) (C and D) and contaminated with low levels (target 1-1.5 $\log_{10}$ CFU/g meat) of a mixed EHEC *E. coli* suspension, after storage at suboptimal temperatures of 10°C (Panels A and C) and 15°C (Panels B and D). $\Delta\log_{10}$ CFU and (% CFU reduction) shown. Significance level: *= p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001.

Similar on-matrix studies with *N. benthamiana*-produced colicins were conducted with **lamb loin meat**. Using similar exposure and storage conditions and **moderate levels** of mixed-strain *E. coli* contamination (2-2.2 $\log_{10}$ CFU/g lamb meat), the mixed colicin solution significantly reduced CFU/g relative to control. Figure 2-6 shows results of on-matrix studies with lamb when post-treatment meat was stored at 10°C and 15°C.

Figure 2-6. Efficacy of *N. benthamiana*-produced colicin mixtures on lamb matrix at moderate *E. coli* contamination levels and stored at 10°C and 15°C



Efficacy of *N. benthamiana*-produced colicin mix (3+1+1+1+1 mg/kg colicin M, E7, Ia, K, U) applied to lamb loin meat matrix and contaminated with moderate levels (target 2-2.2 $\log_{10}$ CFU/g meat) of a mixed EHEC *E. coli* suspension, after storage at suboptimal temperatures of 10°C (left panel) and 15°C (right panel). $\Delta\log_{10}$ CFU and (%CFU reduction) shown. Significance level: *= p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001.

Collectively, these representative on-matrix results reproduce the results reported in GRN 676 and allow us to conclude that, functionally, there are no significant differences in efficacy or on-matrix suitability of colicins regardless of which plant host is used in their production.

Duration of *N. benthamiana*-produced COLICIN's technical effect

Results of Notifier's studies provided in GRN 593 showed that the duration of COLICIN's technical effect on *E. coli*-contaminated produce (vegetables such as arugula; fruits such as apple and cantaloupe) was typically 1 – 72 h post application (in some cases activity was detected up to 144 h) depending on the conditions used. Similarly, a detailed description of the methods used to assess the duration of technical effect and the results obtained on various meat matrices (beef, pork, lamb) were presented in GRN 676 (Section 2.4.3 Duration of COLICIN's technical effect; pp 30-33). Again, significant technical effects of COLICIN relative to control (plant extract/no colicin) applied to meats appears to fall in the range of **duration of from 1 to 72 h post application.** In most studies, beyond 144 h the growth (CFU/g food) becomes comparable between

colicin and control groups (GRN 676), although the residual activity at that time point may be due to differential growth kinetics caused by the initial reduction of cell numbers by colicins.

These prior results have now been reproduced with *N. benthamiana*-produced colicins as reported in this section of the Notice. As can be seen in Figures 2-4 through 2-6, initial bactericidal activity gives way to normal growth of surviving *E. coli* cells; hence, the technical effect of colicin application is temporary.

COLICIN is a food processing aid

The FDA defines processing aids in 21 CFR 101.100(a)(3) as "substances that are added to a food for their technical or functional effect in the processing but are present in the finished food at insignificant levels and do not have any technical or functional effect in that food." COLICIN, including COLICIN produced in *N. benthamiana*, meets this definition based on the following criteria:

a. COLICIN provides temporary antibacterial effect lasting 1-144 h, typically 72 h, post application even when bacterial contamination levels are high (4-5 log CFU/g), as assessed on-matrix on several types of vegetable, fruit and meat products (GRN 593; GRN 676; this Notice);

b. COLICIN residual proteins are present in the finished food product initially at insignificant levels of less than 10 ppm. Those initial levels would be expected to decrease rapidly over time as the colicin proteins would dissolve and degrade through enzymatic activity on the food matrix (especially in fresh meats) and become indistinguishable from the matrix itself; and

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

As such, COLICIN-treated food products should be exempt from the FSIS labeling requirement.

2.5 Effect of COLICIN Application on Organoleptic Properties of Food

The methods applied to determine COLICIN efficacy and duration of technical effect as reported in this Notice and in prior notices (GRN 593 and GRN 676) used food matrices inoculated with a mixture of pathogenic strains (USDA Big 7 + O104:H4) of *E. coli*. As such, no organoleptic evaluations were conducted.

Solutions of *N. benthamiana*-produced mixed colicin proteins formulated as purified COLICIN Isolate are generally visually clear and have no objectionable odor. The solubles in the COLICIN product are applied at a level of 6-10 mg/kg food (6-10 ppm) initial concentration and become diluted by diffusion into the matrix upon storage. There is no masking of the color of meat after COLICIN application. No organoleptic changes are anticipated after application, although an independent evaluation will be conducted later in product development.

2.6 Non-Interference of COLICIN with Pathogen Detection Methods

The component colicin proteins in the COLICIN Isolate product formulation are specific for *E. coli* strains (and some *Salmonella* pathovars) and exert antibacterial effects quickly even at low levels (6-10 ppm). In liquid suspension cultures, multi-log reductions in CFU are seen within minutes of colicin application (GRN 593), and multi-log CFU reductions were reported on-matrix for colicins applied to produce (GRN 593) or various meats (GRN 676).

Uniformly, rapid and significant reductions in CFU/g were seen at the earliest time of sampling (1 h), followed by normal growth of surviving bacteria in COLICIN-treated samples relative to control treatments (GRN 676, Section 2.4.2 Suitability of COLICIN for use in processing meat products; pp 19-29).

Inspection of vegetables or meat products for the presence of pathogens can be done by taking samples from food surfaces prior to and after COLICIN application. As shown in the studies reported in GRN 593, GRN 676 and this Notice, viable bacteria will grow after COLICIN treatment if incubated at a permissive growth temperature. Therefore, COLICIN application should not interfere with pathogen determination methods used in food processing, including protein-protein interactions for ELISAs, or PCR-based amplification reactions.

Importantly, in our studies, neither inhibition of bacterial growth nor interference with growth-based assays were observed in carrier (vehicle control) solutions from *N. benthamiana*, supporting the fact that there are no additional host- or process-derived antimicrobials present in the purified product, or, if present, they are present at levels too low to exert an antimicrobial effect.

2.7 Occupational Safety Related to Use of COLICIN Product

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

The antibacterial proteins comprising COLICIN are GRAS (GRN 593) and are made endogenously in the human intestine and in the gut of many domestic animals to which we are exposed. The colicin proteins used in the COLICIN product have low allergenic potential. In GRN 593 and GRN 676, the host plants listed as candidate hosts to produce colicins were food species (e.g. lettuce, red beet and spinach), which can be consumed at unrestricted levels. Therefore, any residual proteins and plant extractives remaining in the COLICIN formulation are GRAS.

In addition, the excipients in the formulation are food grade and approved for food use. Hence, no significant occupational exposure hazards were identified in either GRN 593 or GRN 676 for either food processing workers or federal inspectors at the food processing facilities.

In contrast, the use of *N. benthamiana* as a production host in this Notice requires more stringent purification of the colicin proteins to reduce to low-risk levels the residual alkaloids that are present in this non-food species. Therefore, for *N. benthamiana*-produced COLICIN released to meet its target Specification, we believe that only minimal personnel protection should be required during product preparation for application, and during application and disposal.

Regardless of the production host plant, protective devices such as a mask, goggles and gloves are suggested as a precaution to prevent inhalation, eye and skin exposure to particulates (if the product is formulated as a solid). Aerosols may be generated when the product is applied as a spray, but spray cabinets should obviate exposure. Specific use procedures, personnel protection practices, and additional safety, use, storage and disposal information will be included in the product label and safety data sheet, as well as included in individual HACCT plans.

2.8 Overall Technical Conclusion

The identities of *N. benthamiana*-produced colicins have been confirmed to match the amino acid sequences of food-species-produced colicins, which are equivalent to natural colicins produced by commensal bacteria in the human GI tract (APPENDIX C Section C.2). The technical effect of *N. benthamiana*-produced colicins, individually and as mixtures, are comparable to those reported in GRN 593 (fresh and minimally processed produce) and in GRN 676 (meat cuts).

The difference between the manufacturing process described in GRN 593 and GRN 676 and that of the current Notice involves the use of a non-food species host plant; consequently, a process modification is needed to include *N. benthamiana* as a safe manufacturing host for colicin production. Further, a target Specification has been revised accordingly to include maximum limits for host impurities plus maximum limits for the host alkaloids nicotine and anabasine.

Our internal analytical results, coupled with food intake and exposure estimates from publicly available sources, support safe application of *N. benthamiana*-produced colicins to various food products at levels that can induce the bactericidal technical effect without undue risks to consumers.

Because the colicin proteins are consistent in composition and activity regardless of expression host, and the *N. benthamiana* source stream is higher in purity than its food species-source counterparts, we do not anticipate interference with pathogen monitoring or detection methods on any foods treated with COLICIN product. Additionally, we have assessed risk of exposure to host impurities and especially to residual host alkaloids and conclude that there should not be undue risk to food processing plant operators or to inspection personnel at facilities where COLICIN is being applied. Simple precautions to avoid inhalation of aerosols and exposure to eyes or skin via standard industry containment practices (e.g. spray cabinets) and/or personal protection devices (e.g. goggles, mask) should further minimize occupational risk.

3 Dietary Exposure

3.1 COLICIN Application Rates and Dietary Intake

Regardless of manufacturing host plant, purity of colicins or their formulation, the application rate of colicin proteins used singly or as mixtures, to any food product including vegetables, fruits or meats, should not exceed 10 mg COLICIN per kg of treated food. Application rates of 1-10 mg COLICIN/kg product have been shown effective in controlling *E. coli* Big Seven and O104:H4 pathogens on all food product classes claimed in GRN 593 and GRN 676.

3.2 Estimated Dietary Exposure to Colicins from All Sources

In GNR 593 and GRN 676, we provided extensive documentation of the potential exposure to colicins from use of Notifier's COLICIN products applied to fruits, vegetables and meats, and from natural sources separate from exposure via ingestion of the COLICIN product. These natural sources included:

- Exposure from biosynthesis by commensal microflora in the human gut
- Exposure from vegetables and soil
- Exposure from colicinogenic bacteria naturally present in food (meats and produce)

A maximum COLICIN application rate of 10 mg total colicins per kg treated food product and upper limits of intake of various foods (10 ppm application rate at highest or "worst case" food intake levels) were used in all our calculations. This COLICIN application rate is the same regardless of which plant host is used to manufacture the colicin proteins, as the proteins are purified prior to formulation. Estimates for colicin intake from raw or uncooked foods and for cooked foods were provided. Table 3-1 summarizes additive exposure to colicins and estimates the intake by food source.

For perspective, natural, endogenous colicins produced through intestinal biosynthesis by colicinogenic strains of enteric bacteria in humans is estimated to be ~3 mg per day, chronically (GRN 593, Section A.2.1; pp 21-25). Because of the localization of endogenous colicin synthesis in the colon and the average human

daily fecal output (0.030 kg), the intestinal concentration of colicins is estimated to be 100 ppm (3 mg/0.030 kg). This figure is an estimate and could well vary from person to person depending on many factors, including diet. Hence, the worst-case level of intake of colicins from COLICIN applied to foods, assuming traditional food consumption and preparation patterns, is of the same order as that produced endogenously. Therefore, the risk posed from consuming colicins applied to foods should be minimal.

Table 3-1. Estimated human exposure to colicins from consumption of various foods treated with a maximum of 10 mg/kg COLICIN and from various natural sources

Source of exposure	Estimated daily per capita colicin exposure	
	If food is not cooked	If food is cooked
COLICIN treatment, total of all red meat products consumed[1]	**1.5 mg**	**nil**
COLICIN treatment, total of all produce consumed[2]	**4.1 mg**	**nil**
Meat (ingestion of naturally present colicins in meats) [1,2]	<0.3 mg	nil
Produce (ingestion of naturally present colicins in fruits, vegetables)[1,2]	<0.1 mg	nil
Total (all food sources)[1]	6 mg	nil

[1]Detailed in Notifier's GRN 676. [2]Detailed in Notifier's GRN 593.

3.3 Dietary Exposure to Host- and Process-Derived Impurities

Although the safety of colicins *per se* has been documented (GRN 593 and GRN 676), the use of non-food plant species as manufacturing hosts introduces the potential for new risks not found in the original list of production plant varieties. A detailed description of risk factors and their mitigation is found in Section 6 of this Notice.

On a protein basis, the COLICIN product consisting of a blend of colicins produced from *N. benthamiana* has a target purity of ≥70%. The residual host-derived constituents of chromatographically purified colicins 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 colicin protein/kg food treated, proteinaceous host-derived impurities introduced to the treated food would be ≤3 mg/kg food (≤3 ppm). With a US food consumption average of 0.56 kg/day, the **exposure to host-derived proteins would be ≤1.7 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 COLICIN 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. 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 produce and meats defined in GRN 593 and GRN 676, we estimated the per capita daily exposure levels.

Colicin blends are not composed of equal amounts of each colicin, and each colicin has a different level of residual alkaloid due to slight differences in extraction buffers used. Typically, colM is applied at 3 mg/kg food, whereas all other colicins are applied at 1 mg/kg. If all colicins are made in *N. benthamiana*, a typical blend would be 3 mg colM plus 2-3 other colicins at 1 mg each for a total of 5-6 mg/kg application rate.

Average values for residual nicotine and anabasine (Table 6-3) were used to estimate intake. Since submission of GRN 593 and GRN 676, Notifier found that effective control of *E. coli* can be achieved with colicins M, Ib, K and optionally U, so the functional blend can contain 5-6 mg total colicin protein. At the colicin ratios mentioned, that blend would contribute ≤290 ng nicotine/kg food treated. Similarly, the anabasine level added per kg food treated would be ≤57 ng.

To estimate a maximum level of exposure at the maximum total colicin rate of 10 mg/kg food, the values for nicotine and anabasine were increased proportionately (from 5-6 mg to 10 mg colicin) to 480 ng/kg and 95 ng/kg, respectively. The **average** (in 5-6 mg/kg colicin application rate) and **maximum** (in 10 mg/kg colicin application rate) consumer exposure to residual alkaloids is calculated from the per capita daily consumption by food type, as shown in Table 3-2.

Table 3-2. Estimated human exposure to host alkaloids from consumption of various foods treated with COLICIN produced in *N. benthamiana*

	Meat	Vegetables	All Foods
Daily food consumption	150 g/day	410 g/day	560 g/day
Nicotine in treated food	Ave 290 ng/kg Max 480 ng/kg	Ave 290 ng/kg Max 480 ng/kg	Ave 290 ng/kg Max 480 ng/kg
Daily nicotine intake	**Ave <45 ng/day** **Max <75 ng/day**	**Ave <120 ng/day** **Max <198 ng/day**	**Ave <165 ng/day** **Max <275 ng/day**
Anabasine in treated food	Ave 57 ng/kg Max 95 ng/kg	Ave 57 ng/kg Max 95 ng/kg	Ave 57 ng/kg Max 95 ng/kg
Daily anabasine intake	**Ave <9 ng/day** **Max <15 ng/day**	**Ave <25 ng/day** **Max <40 ng/day**	**Ave <35 ng/day** **Max <55 ng/day**
Total alkaloid intake	**Ave <55 ng/day** **Max <90 ng/day**	**Ave <145 ng/day** **Max <240 ng/day**	**Ave <200 ng/day** **Max <330 ng/day**

Daily per capita food consumption estimates were derived from multiple public sources, as detailed in GRN 593 and GRN 676. Nicotine and anabasine estimates were derived from average levels measured by Notifier in 4 different purified colicins (see Section 6). **Average** residue levels were calculated for a 4 to 5-colicin 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 food times the daily food consumption yields the estimated intake level of each alkaloid.

It bears mentioning that *N. benthamiana*-produced colicins can be blended with different colicins produced in food plant-species to further increase potency or host range; in such instances the level of *N. benthamiana* alkaloids in the final COLICIN 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 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 foods are treated with a maximum of 10 mg/kg *N. benthamiana*-produced COLICIN and the product achieves 100% market penetration, exposure to solanaceous alkaloids from COLICIN would be only about 1/3 of what is currently, and safety, consumed in the average diet.

The amount of nicotine absorbed in second-hand (passive) smoke is 100 μg/day for those reporting exposure, and 20 μg/day for those not reporting exposure, due to the pervasive nature of environmental nicotine (Karačonji 2005). For additional perspective, a typical cigarette may contain from 2 to >10 mg of nicotine, of which 85-90% is absorbed into the blood stream through inhalation (Digard 2013). Because exposure is very rapid, up to ~9 mg nicotine may be systemically absorbed within 5 minutes of smoking (Digard 2013). That level corresponds to up to 130 μg/kg nicotine exposure for a 70-kg person, which produces pharmacologic but not toxic effects. Nicotine-containing gum has ≥4 mg nicotine per dose (4.2 mg for Nicorette®) of which only 63% is absorbed via the oral route over a 45 min period (Digard 2013). This translates to 2.65 mg nicotine/70 kg person, or an exposure level of ~40 μg/kg. This oral dose produces a mild, non-toxic pharmacologic effect. And these values are from only a single exposure to nicotine-containing products; multiple exposures per day are common. Although the nicotine analog anabasine may be similarly bioactive as nicotine, it is present at only ~1/10th the level of nicotine in *N. benthamiana*.

For comparison, the level of potential nicotine plus anabasine ingestion from COLICIN-treated foods in a "worst-case" scenario (Table 3-2) is <0.330 μg/day, which, for a 70-kg person, translates to 4.7x10^{-3} μg/kg. This is an insignificant amount compared to dietary or environmental exposure to the same alkaloids. In a worst-case scenario, treatment of all food with *N. benthamiana*-produced colicins would raise the current exposure level to pyridine alkaloids from 1 μg/day (dietary) to 1.3 μg/day (dietary + COLICIN).

4 Information on Any Self-Limiting Levels of Use

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

5 Experience Based on Common Use in Food Before 1958

COLICIN (mixture of colicin proteins) from any source has not yet been commercialized and colicins have not previously been used in food.

6 Basis for Conclusion of *N. benthamiana*-Produced COLICIN's GRAS Status

Notifier has used scientific procedures to conclude that its COLICIN antimicrobial is GRAS when produced from edible species of plants (GRN 593 and GRN 676) and when produced from *N. benthamiana* (present Notice) under the conditions of intended use. Notifier has relied on detailed physicochemical analyses of its colicin proteins and on in-house and publicly available information on *N. benthamiana* impurities that may remain in the final product in low amounts. We assert that the low residual levels of host alkaloids in the COLICIN product should not pose a risk to consumers, as those levels are lower than the levels consumers ingest in their present diets. We further assert that the additive content of alkaloids (COLICIN-derived and dietary) should not expose consumers to undue risk because of the small contribution of the product to total alkaloid intake. Section 6 of this Notice summarizes the data and information on *N. benthamiana*-produced colicins that were used by Notifier to support its GRAS conclusion.

In Section 7 (Supporting Data and Information), Table 7-1 presents a tabulated summary of the information cited in this Notice from all sources.

6.1 Safety Assessment of *N. benthamiana*-Produced COLICIN

Because the colicins produced in *N. benthamiana* are compositionally the same proteins produced in food species hosts previously described in GRN 593 and GRN 676 and will be used at the same maximum application rate of 10 mg colicins/kg food, the most significant difference between colicins produced in food species and in *N. benthamiana* are the host-derived impurities that may remain in the final product. For comparison, the composition of each manufacturing host species is described with the goal of estimating risk and providing perspective on the level of risk.

6.1.1 Composition of food plant species used as manufacturing hosts

The plant host species and the process used to manufacture colicin proteins were first described in GRN 593, and the GRAS status of the production food host species has been substantiated. A summary of key properties and a risk assessment were included in GRN 593 and are summarized herein for convenience.

The well-known food species **beet**, **spinach** and **lettuce** can be used for production of recombinant colicins. Notifier's manufacturing process is such that the choice of plant host used does not impact the safety of the colicin product. Notifier initially selected three food species for colicin production on the basis of productivity and historical safety, even though these species are known to contain toxic components.

Beet

Beet (*Beta vulgaris*) can be used as a manufacturing host plant because it meets manufacturing requirements and has a well-established record of safety. Beet has a long history of safe ingestion in human food, with first accounts of its cultivation and use dating back to ancient Greek and Roman times (Nottingham 2004). Beet belongs to the Chenopodiaceae family (now classified in Amaranthaceae) and is a close relative of spinach, chard and quinoa (Purdue University 2012; USDA NRCS BEVU2 2012). While beet tubers and beet sugar may be more common in US diets, beet greens (leaves, stems) are consumed world-wide with no known adverse effects, with the possible exception of enhancement of **renal calculi** formation due to the plant's **oxalic acid** content which, like spinach, can be as high as 0.75-1% in leaves (Duke 1983; Wake Forest Baptist 2014). However, it is thought that calculi formation may afflict a minority of individuals who chronically over-consume beet greens or spinach, or suffer from primary hyperoxaluria (New York Times 2008). Beet per-capita consumption in the US averages about 0.4 lbs/yr (USDA ERS 2014a).

Oxalic acid and other low molecular weight impurities are separated in the purification process and thus oxalates are not expected to present a health risk from use of the product, especially at the low use levels anticipated. There have been reports of allergenicity associated with exposure to beet pollen allergens, or allergens appearing in the beet root (i.e. not leaves), but these events were reported in occupational settings (i.e. commercial beet greenhouse operators; beet sugar refinery workers) and do not appear to afflict the general population (Fowler 2000; Luoto 2008; Thermo Scientific Food Allergen Database 2012). Beet allergens are not a concern in colicin protein manufacture because these impurities are not found in leaf tissue.

Spinach

Spinach (*Spinacia oleracea*) can be used as a manufacturing host plant because it meets manufacturing requirements and has a well-established record of safety (Gaikwad 2010). Spinach is in the same taxonomic group as beet (*Chenopodiaceae/Amaranthaceae*; (USDA NRCS SPOL 2012)). Like beet, spinach has endogenous **oxalates** and also **purines** that can lead to the formation of renal calculi in susceptible individuals who consume the plant frequently or in large amounts.

Like other common foods, spinach contains **salicylates** that can lead to **allergic reactions** in individuals sensitive to salicylate analgesics (e.g. aspirin). Despite the presence of low amounts of these components, spinach is a well-established food with a long history of safe human consumption, with an annual per capita intake of 2.6 lbs (USDA ERS 2014a). Regardless, the low molecular weight and soluble components and any allergenic proteins can be removed during the extraction and purification process used to isolate colicins, and therefore host-derived impurities in the product should not present a significant health risk.

Lettuce

Lettuce (*Lactuca sativa*) can be used as a manufacturing host plant because it meets manufacturing requirements and has a well-established record of safety. Lettuce belongs to the Asteraceae family of plants that includes sunflowers and aster (USDA NRCS LASA3 2015). Unlike beet and spinach, lettuce does not contain significant amounts of potentially toxic organic acids or metabolites, although some reports have suggested that the species accumulates nitrates and/or **lithium** from certain soils (Hullin 1969).

Lettuce has a very long history of human consumption and has been cultivated since Greek and Roman times (Wikipedia contributors 2015). In the USA, lettuce is consumed in higher quantities than are beet or spinach, with 2012 estimated per capita intake of more than 25 lbs of head and romaine lettuce alone (USDA ERS 2014a). There were no reports found of allergenic components present in lettuce.

6.1.2 Host and process impurities when using edible species and their impact on safety

Plant hosts

As summarized above, edible food plants such as **red beet, spinach** or **lettuce** used in manufacturing recombinant colicins are in fact “food" and are therefore generally recognized as safe for ingestion at unrestricted levels. The level of host- or process-derived impurities in the product depends on the extent to which the colicin protein is purified. For example, one or more colicin proteins from food species that are formulated as **COLICIN Isolate** may be 70-90+% purity for in-package fill and hence lower levels of host or process residuals, and even lower levels of any one impurity, in the product are expected. In less pure preparations such as **COLICIN Concentrate** (30-60% purity), which may suffice for vegetable washing applications, there would be additional residual materials from the host plant. Because the host-derived residuals are from an edible plant, no safety risk issues are expected even in the less pure formulations. Therefore, the only remaining potential risks would come from the vector or induction process used to express the colicin genes and/or any residual reagents added in the manufacturing process.

Agrobacterial vector and ethanol induction

Gene induction for colicin synthesis can take place via agrobacteria or via chemical induction (e.g. ethanol) in transgenic hosts, as defined in GRN 593. When agroinfection is used, the bacterial vectors carrying viral amplicons (TMV or PBX) with the colicin genes are recombinantly produced and become part of the manufacturing process. When ethanol induction is used, the colicin genes are a stable but silent trait in the host and are expressed when a dilute solution of ethanol is applied to the plant (Werner 2011). The ethanol is removed during the process of colicin purification (pilot studies; data not shown).

The current agrobacterial induction process yields non-viable vector after the plant biomass is treated and the colicin protein is extracted. The question can be asked about potential safety risks from vector that may remain viable after bioprocessing. This issue can be addressed by noting that the *Agrobacterium* industrial strain used in the production of colicins is very similar to wild-type strains of *Agrobacterium* from which it

was derived. Notifier employs a weakened strain of the vector that contains mutations that severely constrain its environmental viability and competitiveness, so it is environmentally safe.

Agrobacterium tumefaciens and related agrobacteria are natural soil inhabitants, ubiquitous plant colonizers, can effect lateral gene transfer in plants, and are present at levels as high as 30 million CFU/g of soil (Bouzar 1993; Krimi 2002; Mougel 2001; Penyalver 1999; Stockwell 1993; Vicedo 1993). *A. tumefaciens* builds cellulosic bridges to plant structures, sticking to the plant during washing and consumption. Humans, therefore, unavoidably consume agrobacteria in their natural diet. No ill effects have been reported from consumption of agrobacteria present in produce (i.e. it is not an animal or human pathogen), even at levels far exceeding those that might result from the current manufacturing process.

Lastly, the agrobacterial vector carries genes that encode RNA amplicons, which replicate in the plant cells' cytoplasm and are translated by cellular machinery to yield colicin protein. The RNA replicons cannot integrate into the plant genome or be passed on via seed. They cannot integrate into the human genome or replicate in mammalian cells. Humans are routinely exposed to significant amounts of TMV and PVX through consumption of uncooked vegetables (e.g. spinach, beets, collard greens, tomato, cucumber, etc.). A large percentage of smokers have serum antibodies against TMV (Liu 2013) with no ill effects. Neither virus as used in these expression systems (Gleba 2014) has ever been implicated as a health risk to humans.

Therefore, even if viable vector became an impurity in the final product, the levels would be lower than what humans naturally consume from ingesting produce. Further, the difference between the production vector and natural agrobacteria is the presence of the colicin-encoding gene. This is not a safety concern because the colicin-encoding gene is already present and expressed by commensal *E. coli* strains in the human gut. Likewise, the only difference between native and transgenic host plants carrying the inducible colicin gene is the colicin expression machinery, which is present in colicinogenic strains in our gut.

Abiotic process impurities

Heavy metal content in purified colicins has been measured as <1 mg/kg dry powder, and daily exposure is projected to be <5.6 ng/person-day from COLICIN-treated food, or well below the 5 μg/day limit set for such products as food supplements (see APPENDIX C). With respect to abiotic safety risks from process reagents, all processing aids, aqueous buffers/salts and solvents are selected from lists of approved food additives and food processing aids. Any residual levels of processing reagents would introduce no more risk than those derived from the consumption of other food products that also utilize them.

6.1.3 Properties of *Nicotiana benthamiana* as a preferred manufacturing host

Although the original list of production hosts described in GRN 593 included only food (edible) species, Notifier has determined that additional plant species could be included in the manufacturing host list, and that these additional hosts also yield colicin proteins that are safe when produced using Notifier's manufacturing process, even though such species have not been traditionally consumed in human diets.

The concern in using non-food species derives from toxic components of the host that may remain in the final colicin product at levels to cause a safety risk. Therefore, 100% pure colicins produced in food species are expected to have equivalent safety features as 100% pure colicins produced in non-food species. Since colicins expressed in various food and non-food plant hosts have the same amino acid composition and do not undergo any differential postranslational modifications, an assessment of risk needs to be approached not from the standpoint of the safety of the colicin proteins themselves (established in GRN 593 and GRN 676), but rather from potentially toxic levels of host and process impurities that may remain in the final product.

Genetic homogeneity. *Nicotiana benthamiana* is an ideal plant host for producing a wide range of biologics, including food additives. A thorough review of *N. benthamiana* was published by Goodin et al. (Goodin 2008). Native to Western Australia, specimens of the species were reportedly first collected and brought to England aboard the *HMS Beagle* during the ship's third voyage (1843-1846). The specimens were eventually transferred to the Royal Botanic Garden, Kew, and the holotype first described as a new taxon in 1929 (Goodin 2008). Despite *N. benthamiana* having become a popular tool to study plant-pathogen interactions, relatively few accessions may have been the source of cultivars in use today.

The United States Department of Agriculture–Agricultural Research Service (USDA-ARS) National Plant Germplasm System has two accessions (plant introduction [PI] numbers 555478 and 555684), the Botanical Garden of Nijmegen (Netherlands) lists two accessions, the Institut fur Pflanzengenetik und Kulturpflanzen-forschung Gatersleben (IPK, Germany) has one, and the Australian Plant Genetic Resource Information Service (AusPGRIS) lists five accessions but only two are presently available for distribution. The Kentucky Tobacco Research and Development Center (KTRDC) at the University of Kentucky presently maintains six accessions of *N. benthamiana*, of which two are the USDA-ARS accessions and another two are almost certainly redundant (Goodin 2008).

Goodin et al. (2008) also used amplified fragment length polymorphism (AFLP)-based cluster analyses in an attempt to determine whether any genetic variation existed among 11 research accessions. They used DNA obtained from plants grown from seed submitted by researchers in five countries. When grown to maturity, no obvious differences in growth habit, foliage, or flowers were noted, with the exception of a single novel accession of *N. benthamiana* in the KTRDC collection, which was a much larger, coarser plant with larger flowers than the research accessions. Comparisons among the other 10 accessions averaged 0.924, indicating that they are very closely related and could possibly be derived from one source.

These published comparisons suggest that *N. benthamiana* accessions used in research and in the manufacture of plant-made pharmaceuticals are very similar in genotype and phenotype, including metabolic features. Therefore, importantly, with respect to the use of *N. benthamiana* for manufacturing food additives, it can be concluded that the exact cultivar used should not be impactful on the plant's compositional makeup or its inherent safety.

Virus and viral vector permissiveness. *Nicotiana benthamiana* is highly susceptible to infection by many plant viruses, at least in part due to a natural mutation in an RNA-dependent RNA polymerase gene (NbRdRP1m; (Yang 2004)), making it a preferred species for studying plant-viral interactions. *N. benthamiana* EST (expressed sequence tags) are generally highly homologous to those of agriculturally relevant solanaceous crops, such as tomato, potato, pepper, and petunia, which collectively had a 2016-17 farm-gate commercial value of nearly US $7 billion in the United States alone (USDA ERS Cash Receipts by Commodity database, 2015-2018; https://data.ers.usda.gov/reports.aspx?ID=17845). Thus, functional genomics projects concerned with host–pathogen interactions conducted in *N. benthamiana* will most likely reveal genes that play similar roles in agronomically important crops.

More recently, *N. benthamiana* has been the preferred host for heterologous protein expression in plants using plant virus-derived transient expression systems based on tobacco mosaic virus (TMV), potato virus X (PVX), and other vector backbones. A recent review (Gleba 2014) catalogs efficient expression of heterologous proteins using *Agrobacterium*-mediated import of viral replicons, typically using *N. benthamiana* as the production host.

***Nicotiana benthamiana* as a biomanufacturing platform.** The homogeneity of the host, the ease of viral transduction for heterologous protein expression, and the high levels of accumulated protein product

achievable after 7-10 days post infection, have made *N. benthamiana* a widely adopted "platform" species for plant-made pharmaceutical (PMP) R&D. PMP is a rapidly developing field and its progress has been the subject of a number of reviews, many of which cover *N. benthamiana* as the preferred host (Cañizares 2005a; Cañizares 2005b; Chen 2011; Daniell 2009; Gleba 2004; Gleba 2005, 2007; Karg 2009; Klimyuk 2014; Komarova 2010; Lico 2008; McCormick 2008a; Mett 2008; Mortimer 2012; Pelosi 2012; Pogue 2010; Rybicki 2009; Sainsbury 2009; Saunders 2013; Smith 2009; Tusé 2011; Whaley 2011; Yusibov 2008) and others.

It is poignant that most of the currently announced or planned clinical trials of PMP are based on biopharmaceuticals produced using transient expression systems with *N. benthamiana* as the host. Those include recombinant biosimilars, antigens, and immunoglobulins produced using agroinfection (Icon Genetics/Nomad Bioscience, Germany; Mapp Biopharmaceutical, USA; Fraunhofer Institute for Molecular Biotechnology (IMB), USA; iBio, USA; PlantForm, Canada, etc.) or a non-viral transient system (Medicago, Canada).

Ease of scale-up and regulatory compliance. The popularity of transient systems reflects their speed and yields, as well as their scalability, and importantly, acceptance by the regulatory agencies. There are several plant-based cGMP-compliant or GMP-certified manufacturing facilities currently in operation, including those of Kentucky Bioprocessing (KBP), USA; iBio CMO, USA; Medicago, Canada; Icon Genetics, Germany; and Fraunhofer IMB, Germany. Most of the PMP products that have or will enter clinical studies are manufactured in *N. benthamiana*; notably those manufactured by MAPP, KBP, Fraunhofer IMB, iBio CMO, Icon Genetics and Nomad Bioscience.

Manufacturing processes for biopharmaceuticals using *N. benthamiana* as the host are in compliance with quality and safety criteria specified in various FDA guidance documents and the Code of Federal Regulations (CFR), specifically CFRs treating GACP, cGMP and GLP. Universally, the products produced in *Nicotiana* have shown a high level of safety in clinical studies, even products whose release specifications for purity were set at only ≥90% (Tusé 2011). This means that *Nicotiana* host-derived impurities allowed in clinical products at up to 10% levels have failed to produce adverse events in clinical studies, even when the products were injectable vaccines co-administered with an adjuvant (McCormick 2008b; Tusé 2015).

6.1.4 Host impurities from *Nicotiana* species and their impact on safety

Humans and other animals consume a wide range of plants. Historical (evolutionary) trial and error, safety, nutritional value, ease of cultivation, and organoleptic qualities no doubt contributed to the selection of preferred species for dietary consumption. Plants of the genus *Nicotiana* 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.

For example, in the proceedings of an Office of Technology Assessment conference on alternative uses of tobacco in agriculture, Wildman (1983) demonstrated from analytical studies that tobacco-derived protein fractions could be used as animal feed, with calculated nutritional value comparable or exceeding that of alfalfa. Wildman designed and described the agronomics for producing tobacco strictly for nutritional uses, including an extraction scheme for major plant components, each with directed end-uses. Even earlier, Kung et al. (1980) evaluated the nutritional content of tobacco leaf protein through analytical comparisons as well as animal feeding studies (rodent models). Kung fractionated total leaf protein from *N. tabacum* cv Maryland 609 into fraction I (FI), which contained ribulose 1,5-bis-phosphate carboxylase-oxygenase, and FII, comprised of soluble proteins from the cytoplasm and chloroplast. They compared the amino acid composition and nutritional value of FI and FII to milk-derived casein. Both *Nicotiana* protein fractions contained a full complement of essential amino acids, with only slight deficiencies in the sulfur-containing amino acids. Further, the protein efficiency ratio (PER; defined as animal weight gain per amount of protein consumed in feeding studies) of both FI and FII were comparable (within 10%) to the PER of casein.

In their discussion, Kung et al. (1980) cite similar nutritional studies with tobacco-derived FI and FII protein fractions, which showed that the *Nicotiana*-derived protein was similar in amino acid composition and nutritional value to soy protein (Kung 1978) and alfalfa leaf protein (Bickoff 1975), and better than other plant proteins, including rice, wheat and corn (Block 1951).

The above-cited studies did not report toxic effects as they were nutritional studies designed to assess animal weight gain. Conceivably, toxic metabolites from *Nicotiana* leaf preparations were removed in the extraction process or at least were reduced in concentration so as not to induce overt toxicity.

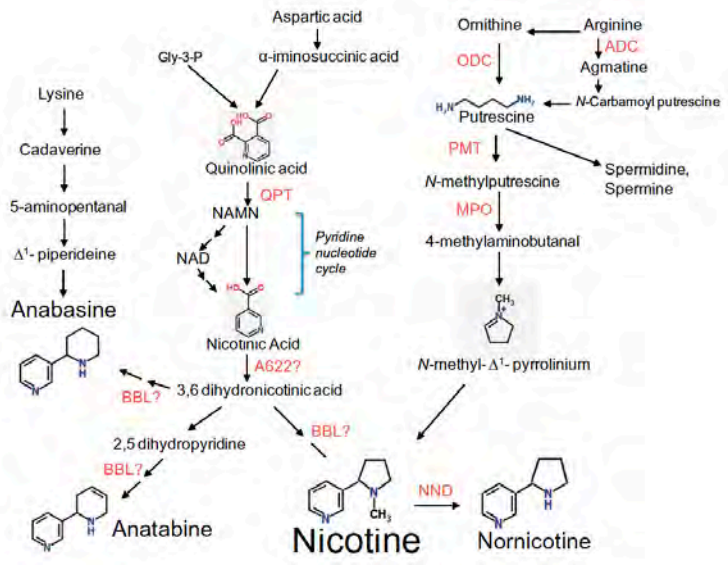
Notably, interest in *Nicotiana* as a source of nutritional protein occurred mainly in the 1970s and 1980s. The presence of toxicants and the availability of many other sources of vegetable protein may have discouraged additional research. This concept is supported by Siceloff (1981), who reviewed the impact of public policies on support for alternative uses of tobacco. However, Fu (2010) developed a modern tobacco purification process, as there may be renewed interest in low-cost plant proteins as meat analogs.

Of the minor constituents of *Nicotiana* species, it is the alkaloids that have the major impact on safety. Hence, 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.

Wang et al. (2015) reported on genetic factors in *Nicotiana* associated with nicotine content. In commercial tobacco cultivars, nicotine represents 90–95% of the total alkaloid pool and is synthesized exclusively in tobacco roots, transported to leaves, and stored in leaf cell vacuoles by a multidrug and toxic compound extrusion (MATE) transporter. They reviewed various factors that control nicotine biosynthesis and accumulation, including plant genetics, environmental conditions, insect predation, mechanical injury and agronomical management measures, such as topping (decapitation of the apical meristem at the early stage of flowering) and suckering (removing the axillary buds of plants activated by topping).

Nicotine has two ring moieties, a pyrrolidine ring and a pyridine ring, derived from two branched pathways. The pyrrolidine ring is formed from the amino acids arginine or ornithine via putrescine and methylputrescine. The pyridine ring is derived from nicotinic acid, an intermediate of the pyridine nucleotide cycle. Lysine gives rise to piperidine, which with nicotinic acid gives rise to anabasine. The biosynthetic pathway of nicotine, nornicotine, anatabine and anabasine is summarized in Figure 6-1.

Figure 6-1. Biosynthetic pathway of major alkaloids in *Nicotiana* species



Schematic diagram of alkaloid biosynthesis in *Nicotiana* spp. Enzymes identified for key metabolic conversions are shown. A622: isoflavone reductase-like protein; ADC: arginine decarboxylase; BBL: berberine bridge enzyme-like; MPO: N-methylputrescine oxidase; NND: nicotine N-demethylase; ODC: ornithine decarboxylase; PMT: putrescine methyltransferase; QPT: quinolinate phosphoribosyltransferase (Dewey 2013).

Because the biosynthetic pathways for these alkaloids have been largely defined (Katoh 2005), it may be possible to knock out or suppress key genes in the pathway to yield varieties of *Nicotiana* having either very low (e.g. RNAi) or no nicotine and related alkaloids (e.g. gene knock-out) (Gavilano 2006; Hibi 1994; Hildreth 2011; Hung 2013; Kajikawa 2009; Shoji 2013; Takizawa 2007; Todd 2010; Wang 2008). Interspecific *Nicotiana* hybrids are also under development with the same goal (e.g. Ling (2012)).

This opens the possibility of developing new varieties of *Nicotiana* that are devoid of potentially toxic constituents. These future hosts could be used to produce cruder (less purified) product formulations, akin to the purities currently acceptable when food species such as beet, spinach and lettuce are used to produce food additives and processing aids.

Notifier is also working on controlling host alkaloids "upstream" by interfering with alkaloid biosynthetic pathways. These studies will continue with the goal of further reducing alkaloid content to below the level found in vegetables. In the meantime, Notifier has successfully reduced the levels of host alkaloids by "downstream" process controls (APPENDIX B). This Notice focuses on colicins purified from non-transgenic *N. benthamiana* using only downstream process controls.

The need for purification is borne from the pharmacological activity of residual host alkaloids. These alkaloids exert neuroactive effects and gathering evidence support their role in protecting the plant from insect predation (Steppuhn 2004; Todd 2010). Tobacco (*N. tabacum*) has a long history of human use for its neurostimulant effects (nicotinic acetylcholine receptor agonist) derived from these alkaloids, principally nicotine, which represents more than 90-95% of the total alkaloid content (Wang 2015). While several species and varieties of “tobacco” have been cultivated, there is no evidence that *N. benthamiana* was ever cultivated for any such purposes, probably because of the plant’s morphology, its poor field performance, and the availability of hardier, larger species of tobacco with higher nicotine content.

The toxicities of *Nicotiana* alkaloids have been extensively studied, with variance due to multiple factors, including age (Okamoto 1994). For nicotine and its related analog nornicotine, the mouse and rat oral LD_{50} are in the range of 3-4 mg/kg and 50-60 mg/kg, respectively (see IPCS INCHEM 2012 http://www.inchem.org/documents/pims/chemical/nicotine.htm for comprehensive toxicity review). Human data from controlled toxicity studies are of course lacking, but the lower range of a human lethal dose is estimated to be 0.5 to 1.0 grams (~7-14 mg/kg), based on records of clinical overdoses (Mayer 2014). Table 6-1 from Baumung et al. (2016) summarizes some pharmacological threshold values for nicotine.

Table 6-1. Estimated pharmacological thresholds of nicotine

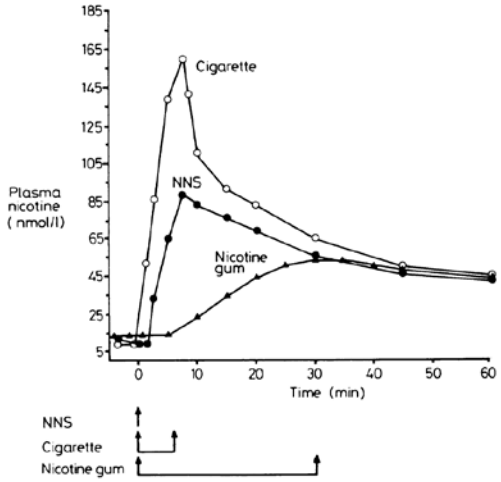
Species	Effect	Type of endpoint	Value [mg/kg bw]	Reference
Humans, i.v., acute	Heart rate acceleration	LOAEL	0.008[a]	EFSA[28] based on Lindgren *et al.*[26]
Humans, i.v., acute	Heart rate acceleration	BMDL for BMR = 1SD	0.013	Own modelling[c] based on data from Lindgren *et al.*[26]
Humans, chronic cigarette use	Addiction	Threshold	0.07[b]	Benowitz and Henningfield[29]
Humans (Children), dermal, acute	Various symptoms of intoxication	LOEL	0.01	EFSA[28] based on Woolf *et al.*[34]
Humans (Children), dermal, acute	Various symptoms of intoxication	BMDL10	0.004	Own modelling[c] based on data from Woolf *et al.*[34]
Various animal species, acute	Mortality (LD50 studies)	BMDL10[d]	3	Lachenmeier and Rehm[24]
Rats, 10-day study	Liver: fatty change	BMDL10	0.27	Own modelling[c] based on data from Yuen *et al.*[10]
Rats, 10-day study	Liver: focal necrosis	BMDL10	0.24	Own modelling[c] based on data from Yuen *et al.*[10]
Rats, 10-day study	Liver: dark cell change	BMDL10	0.21	Own modelling[c] based on data from Yuen *et al.*[10]
Rats, 10-day study	Pathological changes in liver	NOAEL	1.25	US EPA[61] based on data from Yuen *et al.*[10]

A key metric in Table 6-1 is the minimum dose of nicotine reported to induce a measurable pharmacologic effect in humans (increase in heart rate), considered to be the Lowest Observed Adverse Effect Level (**LOAEL**), which ranges from **8 to 13 µg/kg** (Baumung 2016). Presumably, the No Observed Adverse Effect Level (**NOAEL**) in humans via the i.v. route would be **<8 µg/kg.** However, and importantly, these values are for intravenous bolus injection of nicotine, and hence due to ADME differences the NOAEL and LOAEL are both expected to be higher values via the oral route relative to the i.v. route.

Uptake of **nicotine** after oral administration, as would occur from food, cannot be directly compared with the uptake from pulmonary exposure. Oral exposure to nicotine leads to molecules reaching the gastrointestinal tract and being absorbed through the gut mucosa. However, in the acidic pH of the stomach, little nicotine is absorbed because the molecule is in its ionized state (Andersson 2003; Karačonji 2005). Intestinally absorbed nicotine molecules are transported via the hepatic portal vein to the liver, where extensive metabolism occurs (60-80% to cotinine; Benowitz (1999)) before the alkaloid reaches the systemic circulation. Thus, nicotine will to some extent be metabolized by the bacteria and enzymes in the gastrointestinal tract, and to a large extent by the hepatic enzymes, before circulatory distribution. It has been calculated that 60-70 percent of orally supplied nicotine is metabolized by this type of first-pass metabolism before ever reaching the systemic circulation (Benowitz 1991).

Figure 6-2 is a pharmacokinetic profile showing nicotine bioavailability in human subjects through three different routes of administration, namely, inhalation via cigarette, intranasal spray (transmucosal), and orally from nicotine-containing chewing gum. The delay in absorption and the lower bioavailability of orally ingested nicotine is evident (Russell 1983).

Figure 6-2. Bioavailability of nicotine in humans as a function of route of administration



Average plasma nicotine concentrations of three subjects after smoking a cigarette, taking nasal nicotine solution (NNS), and chewing nicotine gum. Doses of nicotine were 2 mg for nasal nicotine solution and nicotine gum and averaged 1.97 mg for the cigarette. Conversion: SI to traditional units-Nicotine: 1 nmol/ ~0.16 ng/ml (from: Russell 1983).

There can be significant interindividual differences in the rates of nicotine metabolism in humans (Benowitz 1982). In studies with larger numbers of subjects, the half-life of systemically absorbed nicotine averaged 2-3 hours and that of cotinine, its major metabolite, about 17 hours (Benowitz 1996).

Because nicotine is rapidly metabolized and despite nicotine's physiological effects, it is unlikely that a person would overdose on nicotine through smoking, or nicotine replacement therapy (NRT) alone. The FDA stated in 2013 that *"There are no significant safety concerns associated with using more than one nicotine replacement therapy product (nicotine "patch" or nicotine gum) at the same time or using an NRT at the same time as another nicotine-containing product, including a cigarette"* (FDA 2013).

Anabasine (neonicotine) is a structural isomer (analog) of nicotine primarily found in the tree tobacco (*N. glauca*); it is present in *N. benthamiana* albeit at very low levels. It's acute oral LD_{50} is in the range of 200-300 mg/kg, rat (Toxnet: https://chem.nlm.nih.gov/chemidplus/rn/53912-89-3). Anabasine has been used medicinally to treat rectal prolapse in Russia, and as a urine biomarker for smoking cessation (Jacob 2002).

The concentration of nicotine and other alkaloids varies substantially among *Nicotiana* species and is impacted by genetics, cultivation practices, and biotic and abiotic stresses. The same is true for other species that biosynthesize these alkaloids. Several original articles and reviews have been published on the genetics and metabolism of alkaloid biosynthesis and accumulation. Saitoh et al. (1985) reported the alkaloid content of 60 *Nicotiana* species, including *N. benthamiana*. Similarly, Sisson and Severson (1990) reported on the alkaloid content across *Nicotiana* species.

Table 6-2 summarizes levels of nicotine and anabasine found in *N. benthamiana* by Notifier's own analyses compared to levels reported in the literature. 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 colicins 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 6-2. Levels of nicotine and anabasine in *N. benthamiana*

A	Notifier's Internal Data		Data From Literature	
Sample	**Nicotine** content ng/g fresh weight	**Nicotine** content ng/g dry weight	**Nicotine** content ng/g fresh weight	**Nicotine** content ng/g dry weight
N. benthamiana Buffer 1	**61,830 ± 29,590**	**690,000 ± 340,000**	~500,000 (Todd 2010)	~14,000,000 (Sisson 1984) ~3,000,000 (Saitoh 1985)
N. benthamiana Buffer 2	**103,330 ± 15,610**	**1,140,000 ± 190,000**		
B	**Notifier's Internal Data**		**Data From Literature**	
Sample	**Anabasine** content ng/g fresh weight	**Anabasine** content ng/g dry weight	**Anabasine** content ng/g fresh weight	**Anabasine** content ng/g dry weight
N. benthamiana Buffer 1	**7,070 ± 1,290**	**80,000 ± 20,000**		~1,300,000 (Sisson 1984) (9.3% of nicotine)
N. benthamiana Buffer 2	**12,930 ± 400**	**140,000 ± 10,000**		

Panels A and B show the levels of nicotine and anabasine, respectively, found by Notifier in analyses of *N. benthamiana* using two different buffer extraction systems, compared to results reported in the literature.

6.1.5 Process modification to lower host alkaloid content in COLICIN product

The downstream process modification to reduce the level of nicotine and anabasine in the purified colicin proteins expressed in *N. benthamiana* is described in Stephan (2017) and further detailed in APPENDIX B. The process successfully reduces the level of host alkaloids in the various colicin proteins by several hundred to over a thousand-fold relative to initial levels in the plant. Table 6-3 summarizes results from multiple manufacturing batches showing residual level of host alkaloids that are currently achievable. The process is subject to additional optimization that could further reduce alkaloid residues.

Table 6-3. Residual levels of *N. benthamiana* alkaloids in purified colicins

Colicin	Batch	Sample	Nicotine concentration (ng/mg colicin)	Anabasine concentration (ng/mg colicin)
colM	4	TSP extract	49 569.00	7 404.00
		Purified protein	**21.73**	**<1.54**
	5	TSP extract	47 738.00	4 095.00
		Purified protein	**39.77**	**<1.92**
colK	2	TSP extract	66 111.00	7 644.00
		Purified protein	**43.91**	**13.91**
	3	TSP extract	59 865.00	7 047.00
		Purified protein	**13.58**	**2.06**
colU	4	TSP extract	23 111.00	3 178.00
		Purified protein	**23.10**	**<2.50**
	5	TSP extract	56 699.00	7 647.00
		Purified protein	**19.71**	**2.87**
collb	2	TSP extract	47 843.00	7 569.00
		Purified protein	**128.46**	**43.59**
	3	TSP extract	57 487.00	8 637.00
		Purified protein	**171.84**	**39.18**

Averages from developmental batches

Average nicotine concentration

colM: 30.75 ng nicotine/mg colicin

colK: 28.75 ng nicotine/mg colicin

colU: 21.4 ng nicotine/mg colicin

collb: 150.15 ng nicotine/mg colicin

Average anabasine concentration

colM: <1.73 ng anabasine/mg colicin

colK: 7.98 ng anabasine/mg colicin

colU: <2.68 ng anabasine/mg colicin

collb: 41.38 ng anabasine/mg colicin

Compared to the levels of alkaloids in the host (Table 6-2) purification of ColM, ColK and ColU results in 1000x-2000x reduction of **nicotine** content and 500x-5000x reduction in **anabasine** content. Purification of ColIb results in 350x and 200-300x reductions in **nicotine** and **anabasine** content, respectively. Results from developmental batches 2-5 are shown.

For perspective, humans chronically ingest low levels of nicotine from their diet. Table 6-4 from Andersson (2003) summarizes the nicotine content of common vegetables as reported in multiple published studies.

Table 6-4. Average nicotine content in common vegetables

Sample	Data from literature		
	Nicotine content ng/g dry weight (Moldoveanu 2016)	Nicotine content ng/g dry weight	References
Cherry tomato, fruit	181.9 (tomato)	79-98 (a) 78-148 (b) 28-115 (c)	(a) Castro and Monji 1986 (d) Domino et al. 1993 (b) Davis et al. 1991 (e) Davis et al. 1986 (c) Liu et al. 2013 (f) Siegmund et al. 1999 (g) Andersson et al. 2003
Eggplant, fruit	174.3	1,000 (a) 52-150 (e)	
Potato (whole), tuber	42.6	71+/-59 (d) 76 (b) 45-123 (c)	
Cauliflower		3.8 (d)	
Sweet pepper		5.4 (g)	

Hence the natural dietary intake of nicotine can be calculated. Table 6-5 (Andersson 2003) summarizes the estimated food-borne nicotine exposure in Scandinavia; although daily consumption values for each vegetable sampled may differ from US consumption estimates, nicotine ingestion ranges from **900-1,300 ng/person-day** depending on dietary habits.

Table 6-5. Average daily consumption, average nicotine content and average daily dietary nicotine exposure from tomato, potato, eggplant and sweet pepper

Food Source		Average daily consumption (g/day)	Average nicotine content (μg/kg)	Average daily dietary nicotine exposure (ng/day)
TOMATO	- Sweden	21.6	4.4	95.0
	- Denmark	62		273
POTATO	- Sweden	168.6	5.8	977.9
	- Denmark	156		905
EGGPLANT	- Sweden	0.71[a]	34.4	24.4
	- Denmark	2.7		93
SWEET PEPPER	- Sweden	0.27*	5.4	1.5
	- Denmark	5.5**		30

[a] Swedish import of eggplant in 2000 (2 297 000 kg) divided with number of citizens in 2000 (8872294) and 365 days; * only green pepper; ** green, yellow or red pepper

In Section 3 of this Notice, Table 3-2, we presented the estimated potential intake values if all food was treated with *N. benthamiana*-produced COLICIN at the maximum application rate of 10 mg/kg and the product achieved 100% market penetration. The estimated per capita daily exposure to all host alkaloids in that "worst case" scenario was 330 ng/person-day. This value is about 1/3 the level of consumer exposure to food-borne nicotine from consumption of common vegetables (~1 microgram/person-day; Andersson (2003)). That is, treating all foods with COLICIN would only raise the alkaloid intake from ~1 μg/day to ~1.3 μg/day. For added perspective, 1 microgram of nicotine is also the amount a person would absorb in about 3 hours in a room with a minimal amount of residual tobacco smoke (Domino 1993).

6.1.6 Process impurities in *N. benthamiana*-based COLICIN manufacture

The process modification in the manufacture of colicin proteins 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). Biosynthesis of colicin 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 colicins 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.

6.1.7 Allergenic Potential of *N. benthamiana*-Produced COLICIN

The allergenic / hypersensitivity inducing potential of all colicins was assessed and reported to be "low to very low" for the various proteins (GRN 593 Section A.2.5 Low potential for development of allergenicity or immunogenicity; pp 34-37). The amino acid sequence and lack of or minor post-translational modifications are consistent for colicins produced in different hosts (APPENDIX C). Therefore, we assess that the allergenic / hypersensitivity potential of *N. benthamiana*-produced colicins will also be low to very low.

Although *N. benthamiana* has been used as a host for producing recombinant allergens as therapeutic products, we found no reports of endogenous allergens in the host plant itself. This lack of evidence, plus the low level of host-derived impurities reaching food from COLICIN application, suggest that non-colicin impurities in the product will present a minor to insignificant risk.

For perspective, two clinical studies of *N. benthamiana*-produced injectable vaccines, in which the minimum purity of the immunogens was set at only "≥90%" with concurrence by FDA, produced no adverse allergenic or hypersensitivity reactions after multiple administrations (McCormick 2008b; Tusé 2015).

6.2 Overall Safety Summary

In prior GRAS notices, Notifier had concluded using scientific procedures that colicin proteins produced in edible species of plants including spinach, beet and lettuce, are generally recognized as safe when applied singly or as mixtures at ≤10 mg colicin/kg food as antimicrobial food processing aids for fruits and vegetables (GRN 593) and meat products (GRN 676).

In the current Notice, Notifier has concluded using scientific procedures that colicin proteins produced in the host *Nicotiana benthamiana*, are generally recognized as safe when used at the same application rates and under the same conditions described in GRN 593 and GRN 676, provided that the levels of host-derived alkaloids are monitored and controlled to minimize risk. In our target Specification for COLICIN (Table 2-3 and Table B-1), we have set 75 ng/mg and 15 ng/mg as the maximum nicotine and anabasine levels, respectively, in a final blend of colicin proteins to allow for formulation flexibility. These higher alkaloid limits are still safe and are higher than what is actually present in the product. A COLICIN formulation (blend) containing 90 ng total alkaloid/mg (75+15) and applied at 10 mg/kg food would introduce 900 ng alkaloid/kg food, or <0.5 μg/person-day in a 0.560 kg/day diet and equivalent to the intake from ½ serving of vegetables per day at current US food intake rates.

In actuality, Colicin M is used at 3x the rate (w/w) as other colicins and contains lower levels of alkaloids; a 10-mg application of COLICIN at that ratio would introduce 575 ng alkaloids to treated foods, which, at current intake rates, translates to <330 ng/person-day or only 1/3 as much as from current dietary exposure (Table 3-2).

To elaborate, a total COLICIN-derived alkaloid intake of up to 330 ng/day would occur only in a worst-case scenario assuming that Notifier's product reaches 100% market penetration such that all foods (all fruits, all vegetables and all meats) are treated with Notifier's product and that no alkaloids are lost during cooking. Even in this highly unlikely scenario, the level of additional alkaloids reaching consumers would be a maximum of 1/3 of what consumers are currently ingesting from alkaloid-containing vegetables (e.g. potatoes, tomatoes, eggplant, peppers, cauliflower, etc.), and far less than their exposure from behavioral and environmental sources.

We present evidence from public sources that pyridine alkaloids are less well absorbed orally than they are via the pulmonary route and undergo metabolism prior to systemic distribution. Even if assuming 100% systemic absorption (worst case), COLICIN-derived alkaloids from treated food would constitute a dose of **4.71×10^{-3} μg/kg** in a 70-kg adult (330 ng/70 kg). These estimates are based on a full US adult daily diet but are expected to apply across all subpopulations. For example, for children, proportional scale-down of food intake by class and lower body mass still yields similarly low levels of exposure. A child weighing 1/3 as much as an adult (~23 kg or ~50 lbs) eating the same diet as US adults but consuming only 1/3 as much (190 g food/day) would ingest about 100 ng total COLICIN-derived alkaloids per day for a worst-case exposure to 4.35×10^{-3} μg/kg.

The threshold (first measurable) physiologic but non-toxic response to alkaloids in humans (elevation of heart rate; Table 6-1) occurs at 8-13 μg/kg (i.v.). Hence the **margin of safety** of our product even assuming 100% absorption of alkaloids ranges from **1,700 to 2,800** (i.e. 8/0.00471 μg/kg to 13/0.00471 μg/kg).

We present data from multiple public sources that these levels of pyridine alkaloids are below the threshold of pharmacologic or toxic activity even for intravenous administration. We reiterate that these low exposures from application of COLICIN to food are exaggerated by the assumption of 100% treatment of all food categories claimed at the maximum application rate of the product, without accounting for alkaloid loss upon food processing/cooking and prior to ingestion.

Notifier describes a process modification introduced into its manufacturing method when using *N. benthamiana* as the expression host for colicins (APPENDIX B). The modification involves using multi-modal chromatography followed by diafiltration. These downstream steps effectively reduce the levels of host alkaloids and other impurities in the purified colicin proteins.

There are no other identifiable toxic entities in *N. benthamiana* that we could find from extensive literature surveys. The protein, lipid, carbohydrate and mineral components of the plant have been studied and are found in the public record, and reveal no unusual properties of the plant that are not also present in solanaceous plant species consumed as food. Similarly, no allergenic or hypersensitivity inducing components have been reported for this species. In an average diet, exposure to residual *N. benthamiana* proteins would be ≤1.7 ppm/day; hence, host proteins are not a risk factor in the product.

With respect to antimicrobial, and especially antibacterial, activity of *N. benthamiana* host extracts, our data suggest that nothing in the background of impurities remaining in the COLICIN product contribute to product potency. Colicin-free plant extract solutions are used as controls and allow unimpeded growth of *E. coli* as determined in *in vitro* and on-matrix studies.

Aside from the safety of host-derived impurities, the methods for gene induction and the reagents used in manufacturing colicins with *N. benthamiana* are the same as those applied with food species hosts. Hence, no process-derived biotic or abiotic impurities or reagents used with any manufacturing host, including *N. benthamiana*, are expected to introduce an identifiable risk.

The level of heavy metals (APPENDIX C) appear quite low in purified, dried colicin samples analyzed to date, with levels of lead and cadmium, for example, of below 1 mg per kg of product (<1 ppm).

Conclusion

The totality of evidence, from Notifier's internal studies and from the extensive public record cited herein, suggests that consumer risks associated with ingestion of trace amounts of impurities derived from manufacturing colicin proteins in *N. benthamiana* is low. Consumers already ingest higher levels of the same impurities in their daily diet, and any contribution of additional alkaloids from COLICIN treatment is not expected to significantly raise dietary risk.

7 Supporting Data and Information

Multiple sources of information, both internal and external, were used to support the conclusion that the *N. benthamiana*-produced COLICIN product is GRAS for food safety applications. Table 7-1 lists the various data and other information discussed in this Notice and used in reaching this conclusion. Also listed in the table is whether the specific information cited was generated by Notifier and/or from databases or references in the public domain.

The COLICIN antibacterial product manufactured in *N. benthamiana* has the identical active ingredients (colicins) as the product made in food species hosts and differs only in the host- and process-derived impurities of the final formulation. Many of the properties of COLICIN have been presented in GRN 593 and GRN 676. Therefore, for brevity, reference is made to specific sections of prior GRNs that have already documented the safety attributes of colicin proteins.

Table 7-1. Information supporting COLICIN GRAS determination

Topic	Document	Location	Source	Availability
Colicins' mode of action; specificity	GRN 593	Table A-1, pg 20	(Yang 2014)	Public
History of human exposure to colicins	GRN 593	Section A.2, pp 21-25	Multiple references	Public
Natural human exposure from commensal and animal microflora	GRN 593	Section A.2.1, pp 21-23	Multiple references	Public
Natural human exposure from meat	GRN 593	Section A.2.1, pg 24	(Lange 2008; USDA ERS 2006)	Public
Natural human exposure from vegetables	GRN 593	Section A.2.1, pg 24	(Mukherjee 2004), (2006) (USDA ERS 2014a)	Public
Nature-identical composition of colicins, including those produced in *N. benthamiana*	GRN 593 and This Notice	Section A.2.2, pg 25 Section 2.3, pp 8-14 Appendix C, pp 75-76	Nomad Bioscience GmbH (Stephan 2017)	Public and to be made public through this GRN

Topic	Document	Location	Source	Availability
Uniform expression of colicins in food species hosts and in *N. benthamiana*	GRN 593 and This Notice	Appendix B, pp 51-57 Section 2.4, pp 15-17; Appendix B, pp 68	Nomad Bioscience GmbH (Schulz 2015)	Public and to be made public through this GRN
Safety of colicin production organism	GRN 593	Section A.2.2, pp 25-26 Appendix B, pp 51-57	Nomad Bioscience GmbH	Public
Safety of production food species hosts	GRN 593	Section A.2.2, pp 26-28	Multiple references	Public
Safety of production *N. benthamiana* host	This Notice	Section 6.1.3, pp 30-32 Section 6.1.4, pp 32-37 Section 6.1.5, pp 37-38 Section 6.1.6, pg 39	Multiple references	To be made public through this GRN
Safety of manufacturing process	GRN 593 and This Notice	Appendix B, pp 51-57 Section 6.1.5, pp 37-38 Section 6.1.6, pg 39 Section 6.1.7, pg 39	Nomad Bioscience GmbH	Public and to be made public through this GRN
Safety of food species hosts and process impurities	GRN 593	Section A.2.2, pp 27-28	Nomad Bioscience GmbH	Public
Safety of host and process impurities in *N. benthamiana*	This Notice	Section 6.1.2, pp 29-30 Section 6.1.4, pp 32-37 Section 6.1.6, pg 39	Nomad Bioscience GmbH (Stephan 2017)	To be made public through this GRN
Anticipated levels of exposure to colicin proteins from all food categories	GRN 593 GRN 676	Section A.2.3, pg 28 Section 3, pp 40-43	Nomad Bioscience GmbH	Public
Process modification to reduce the level of *N. benthamiana* impurities	(Stephan 2017) This Notice	Appendix B, pp 70-71	(Stephan 2017) Nomad Bioscience GmbH	Public and to be made public through this GRN
Calculation of potential intake of *N. benthamiana* impurities from foods treated with COLICIN	(Stephan 2017) This Notice	Section 3.3, pp 25-27	(Stephan 2017) Nomad Bioscience GmbH	Public and to be made public through this GRN
Digestibility of colicin proteins	GRN 593	Section A.2.3, pp 28-31 Table A-2, pg 30 Table A-3, pg 31	Nomad Bioscience GmbH	Public
Safety determined in feeding studies with monogastric animals	GRN 593	Section A.2.3, pg 32	(Cutler 2007a; 2007b)	Public

Topic	Document	Location	Source	Availability
Safety determined in human and primate cellular exposure studies *in vitro*	GRN 593	Section A.2.3, pp 32-33	(Farkas-Himsley 1976; 1995; Murinda 2003)	Public
Safe ingestion estimates of colicin proteins applied to meat products	GRN 676 and This Notice	Section 3.2, pp 42-43 Section 3, pp 24-27; Table 3-1, pg 25; Table 3-2, pg 26	Calculations of intake are based on colicin application rates and public data on meat consumption, including: (USDA ERS 2014c, 2015) CDC NHANES (Daniel 2011; DeBruicker 2011; Wang 2009))(Newport 2012) (USDA ERS 2014b) (USDA WASDE 2016) (USDA 2015 Dietary Guidelines)	Public and to be made public through this GRN
Low potential for development of resistance	GRN 593	Section A.2.4, pg 34	Nomad Bioscience GmbH	Public
Low potential for development of allergenicity or immunogenicity	GRN 593	Section A.2.5, pp 34-37 Table A-4, pg 35	(AllergenOnline 2015); calculated by Nomad Bioscience GmbH	Public
Safety from additive consumption of colicins from applied COLICIN product and from natural sources	GRN 593 and This Notice	Section A.2.1, pp 21-25 Section 3.2, pg 24-25; Table 3-1, pg 25; Section 3.3, pp 25-27; Table 3-2, pg 26	Nomad Bioscience GmbH	Public and to be made public through this GRN
Safety of colicin ingestion from all sources relative to colicin levels synthesized *in situ* by endogenous intestinal microflora	GRN 593 and This Notice	Section A.2.1, pp 21-25 Section 3.2, pp 24-25	Nomad Bioscience GmbH	Public and to be made public through this GRN
Non-interference of COLICIN application with pathogen detection methods	GRN 676 and This Notice	Section 2.6, pg 37 Section 2.6, pp 22-23	Nomad Bioscience GmbH	Public and to be made public through this GRN
Anticipated occupational safety of COLICIN usage	This Notice	Section 2.7, pg 23 Section 2.8, pg 23-24	Nomad Bioscience GmbH	To be made public through this GRN

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APPENDIX A. On Matrix Efficacy Determination SOP NMD 901-01

NOMAD	Standard Operating Procedure	NMD 901-01
	Determination of Efficacy and Duration of Bactericidal Effect of COLICIN (Colicin Mixtures) on Pathogenic Strains of *Escherichia coli* Applied to Meat Matrices	Page 1 of 10

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A.1 Purpose

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

A.2 Scope

Assays for COLICIN's efficacy and continued technical effect

Evaluation of efficacy encompasses the analysis of pathogenic *E. coli* (STEC, EHEC, EAEC) populations on contaminated meat samples subsequently treated with mixtures of various plant-made recombinant colicins, or a control carrier solution consisting of plant extract from the same production host but without colicins, and stored for various time periods at different temperatures.

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

Two levels of on-matrix (meat) microbial contamination are analyzed for efficacy determination:

Level I: A realistic or typical contamination level of 1-2 log CFU/g meat to substantiate the reduction of bacterial load with timed sampling and evaluation of re-growth; and

Level II: A contamination level of 4-5-log CFU/g of meat, using the same metrics.

The effect of COLICIN on the bacterial populations upon storage periods of 1-144 h are analyzed at two different temperatures (10 °C and 15 °C). The lower (10 °C) temperature may represent typical industrial processing environments while being suboptimal for bacterial growth. To analyze the duration of COLICIN's

technical effect under more stringent conditions, a higher temperature (15 °C) was studied for comparison, as higher temperatures are more favorable for bacterial growth.

Each treatment is performed in three replicates (2 replicates for pork meat with high contamination level and storage at 4 °C); each replicate is defined as an independent experiment starting with a new pathogen culture. In each replicate experiment, each sample is analyzed in 3 or 4 replicates.

A.3 Reference

FSIS/USDA Laboratory Guidebook: FSIS Procedure for the Use of *Escherichia coli* O157:H7 Screening Tests for Meat Products and Carcass and Environmental Sponges (MLG 5A.04; June 29, 2014)

A.4 Definitions

LB Luria Bertani medium

OD_{600} Optical density of bacterial solution at 600 nm

RT Room temperature

TSP Total soluble protein

CFU Colony forming unit

SMAC Sorbitol MacConkey Agar

A.5 Consumables

Culture vials (e.g. 20 mL plastic or glass vial) for cultivation of *E. coli* in liquid culture

Disposable plastic cuvettes for spectrophotometric measurement of OD_{600}

Sterile Petri dish, ~94x16 mm

Sterile Petri dish, quadratic, ~120x120x17 mm

Sterile 5 ml disposable plastic syringes with Luer lock connector

Sterile disposable 50 ml centrifuge (Falcon) tubes

Sterile disposable 15 ml centrifuge (Falcon) tubes

Sterile 2.0 ml disposable reaction tubes

Sterile disposable forceps 25 cm (RMP-med Steffen Roßberg, cat.# 720183)

Sterile disposable plastic spatulas

Sterile disposable 25 ml, 10 ml, 2 ml serological pipettes

Sterile lateral filter bags BagFilter®400 P (Interscience, cat.# 111 425)

Atomizer flasks (Carl Roth GmbH & Co. KG, cat.#N145.1)

Sterile tissue paper

A.6 Equipment

Sterile 100 ml wide neck Erlenmeyer flasks for cultivation of *E. coli* in liquid culture

Incubator shaker (150 rpm, 37 °C) for cultivation of EHEC strains

Spectrophotometer for measurement of OD_{600} of bacterial culture

Table top centrifuge

Pipetting aid for serological pipettes

100 ml sterile measuring cylinder

250 ml sterile measuring cylinder

500 ml sterile measuring cylinder

5 L sterile beaker

3 L sterile beaker

FW735 meat grinder (Beeketal Lebensmitteltechnik GmbH & Co. KG, Rastdorf, Germany)

Laminar flow cabinet

Microwave oven

Closing clip BagClip®400 (Interscience, cat.# 231 040)

Lab blender BagMixer® 400 CC® (Interscience, cat.# 024 230)

Incubator (37 °C)

Autoclave

Refrigerators (10 °C and 15 °C)

Freezer -80 °C

Freezer -20 °C

Personal protective equipment

A.7 Chemicals /Media/Solutions

LB medium (sterile, liquid): For cultivation of *E. coli* strains

1% (w/v) Bacto-tryptone (pancreatic digest of casein; Duchefa T1332)

0.5% (w/v) Yeast extract (Duchefa Biochemie, cat. #Y1333)

1% (w/v) NaCl (AppliChem GmbH, cat. #A4661)

pH 7.5, Autoclaved

LB medium (sterile, solid): For cultivation of *E. coli* strains

1% (w/v) Bacto-tryptone (pancreatic digest of casein; Duchefa T1332)

0.5% (w/v) Yeast extract (Duchefa Biochemie, cat. #Y1333)

1% (w/v) NaCl (AppliChem GmbH, cat. #A4661)

pH 7.5, Autoclaved

1.5% (w/v) Agar, bacteriology grade (AppliChem, cat. #0949)

0.8% (w/v) Agar, bacteriology grade (AppliChem, cat. #0949) for LB soft agar medium

Sorbitol MacConkey Agar (SMAC Agar, sterile solid): Selective medium for recovery of STEC/EHEC from food samples (Sifin GmbH, cat. #TN122)

48.5 g/l, Autoclaved

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

20 g/l, Autoclaved

Kanamycin (sterile): For selection of transformed *Agrobacterium* strain

50 mg/mL stock solution

Kanamycin sulfate (AppliChem GmbH, cat. #A1493) is dissolved in deionized water, sterile filtered, aliquoted and stored at -20 °C

Rifampicin: For selection for *Agrobacterium* strain

20 mg/mL Stock solution

Rifampicin (Duchefa Biochemie, cat. #R0146) is dissolved in DMSO, aliquoted and then stored at -20 °C

Nalidixic acid (sterile): For selection of resistant *E. coli* STEC mutant strains

25 mg/mL Stock solution

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

Cefixime (sterile): For selection for EHEC strains

50 µg/ml stock solution

Cefixime trihydrate (Sigma, cat. #18588-25MG) is dissolved in methanol to 0.5 mg/ml, diluted 1:10 with millipore water to 50 µg/ml, sterile filtered, aliquoted and stored at -20 °C

X-Gluc: detection of β- D-glucuronidase activity in *E. coli* strains

50 mg/ml stock solution

5-Bromo-4-chloro-3-indolyl-β-D-glucoronic acid, cyclohexylammonium salt (X-gluc.com) is dissolved in DMSO, aliquoted and stored at -20 °C

A.8 Biologicals

A.8.1 Bacterial tester strains used in efficacy and technical effect experiments

The *E. coli* strains used in the experiments conducted within this SOP are shown in Table A-1. These target strains are included because they represent the most predominant serotypes of *E. coli* responsible for food-borne outbreaks, including Shiga toxin producing (STEC), enterohemorrhagic (EHEC) and enteroaggregative (EAEC) strains, which vary in their mechanisms of pathogenesis.

Table A-1. *E. coli* pathotypes STEC/EHEC and STEC/EHEC/EAEC used in these studies

Strain	Culture Collection Reference #	Serotype	Sorbitol fermentation	β-D-Glucuronidase activity	Appearance on SMAC + 100 µg/ml X-Gluc + 0.05 µg/ml cefixime	Traits	Source
E. coli (STEC/EHEC)	CDC 03-3014	O26:H11	yes	yes	pink-red with purple center	positive for virulence genes *stx1* and/or *stx2* and *eae*	Big 7 STEC QC Set #5219, Microbiologics Inc., St. Cloud, MN, USA
E. coli (STEC/EHEC)	CDC 00-3039	O45:H2	yes	yes	pink-red with purple center		
E. coli (STEC/EHEC)	CDC 06-3008	O103:H11	yes	yes	pink-red with large purple center		
E. coli (STEC/EHEC)	CDC 2010C-3114	O111:H8	yes	no	pink-red		
E. coli (STEC/EHEC)	CDC 02-3211	O121:H19	yes	yes	purple with dark purple center		
E. coli (STEC/EHEC)	CDC 99-3311	O145:NM	yes	no	grey with pink center		
E. coli (STEC/EHEC)	ATCC® 35150™	O157:H7	yes	no	grey with pink center		
E. coli (STEC/EHEC/ EAEC)	ATCC® BAA-2326™	O104:H4	yes	yes	pink-purple with dark purple center	positive for *stx2*	QC strain #01104, Micro-biologics, Inc.

To better differentiate between pathogenic and naturally resident non-pathogenic strains, especially at low (1-2 log CFU/g meat) contamination level, nalidixic acid resistant derivatives of pathogenic strains are used. Nalidixic acid resistant spontaneous mutants are selected by cultivation of original isolates on LB agar plates supplemented with nalidixic acid concentrations of 25 µg/ml.

Comparable susceptibility of original strains and nalidixic acid resistant mutants thereof towards colicins are confirmed (Table A-2). These strains are stored in liquid nitrogen in LB broth supplemented with 15% glycerol and 25 µg/ml nalidixic acid. When using nalidixic acid resistant derivatives of STEC/EHEC strains, all media employed for bacterial growth are supplemented with 25 µg/ml nalidixic acid.

Table A-2. Relative sensitivity to colicins of wild type and nalidixic acid resistant mutants of corresponding *E. coli* strains

Colicin	*Escherichia coli* Serotype and Accession Number															
	O26:H11		O45:H2		O103:H11		O111:H8		O121:H19		O145:NM		O157:H7		O104:H4	
	CDC 03-3014	CDC 03-3014 nalR#11	CDC 00-3039	CDC 00-3039 nalR#1	CDC 06-3008	CDC 06-3008 nalR#5	CDC 2010C-3114	CDC 2010C-3114 nalR#3	CDC 02-3211	CDC 02-3211 nalR#9	CDC 99-3311	CDC 99-3311 nalR#7	ATCC 35150	ATCC 35150 nalR#1	ATCC BAA-2326	ATCC BAA-2326 nalR#3
Col E7	(64)	(64)	(64)	(64)	(4)	0	0	0	4096	8192	(128)	(64)	(64)	(32)	1048576	1048576
Col K	(32)	(64)	(64)	(64)	0	0	0	0	8192	8192	(128)	(64)	128	128	32768	32768
Col 5	(64)	(64)	(128)	(64)	0	0	0	0	4096	4096	(128)	(64)	64	64	32768	32768
Col U	0	0	0	0	0	0	0	0	16384	16384	0	0	0	0	0	0
Col Ia	128	128	2048	1024	(32)	(32)	(64)	(32)	16384	16384	(1024)	(256)	64	64	16384	16384
Col M	128	64	64	64	(64)	128	2048	2048	(2)	0	(8)	(8)	4096	4096	16384	16384

Nalidixic acid resistant mutants were selected based on analyses of growth *in vitro* on LB and SMAC media and colicin sensitivity. These mutants are used in antibacterial experiments on matrices inoculated with low densities (e.g. 1-2 log CFU/g) of *E. coli* pathogen mix.

Colicin activity is detected by soft agar overlay assay. Colicin activity in AU/mg FW plant material is calculated from the highest colicin dilution showing growth inhibition (incubation for 16 h at 37 °C).

Selected nalidixic acid resistant mutants (gray background) show comparable sensitivity to their corresponding wild type strains (white background) when exposed to various plant-made colicins. Concentrations and exposure protocols are described in the text.

A.9 Precautions

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

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

A.10.1 Colicins

Colicin proteins are produced in plants as generally described in GRN 593. The intended product contains individual plant-made colicins or a blend of several colicin proteins selected from the list that includes colicins E2 (#AAA23068.1), E7 (#AAA98054.1), K (#Q47502.1), 5 (#CAA61102.1), Ia (#WP_001283344.1), M (#AAA23589.1), E3 (#AAA88416), E6 (#AAA23080.1), D (#P17998.1), N (#P08083.1), U (#CAA72509.1), and B (#P05819.3). If additional colicins are added to this list, they will be screened to comply with identity, purity and other quality criteria before being considered candidates for product formulation.

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

Before use, the supplied colicin formulations should be diluted/dissolved in the appropriate volume of deionized water and stored at low temperature (4 °C).

A.10.1.1 Verification of basic functionality of colicin (blend) solution

The antimicrobial activity of the prepared COLICIN solution is analyzed semi-quantitatively by spot-on-lawn soft agar overlay assay. LB plates are prepared in advance by pouring ~25 ml melted LB solid medium in sterile Petri dishes (quadratic, ~120x120x17 mm). On the day of experiment, plates are incubated at 50 °C, LB soft agar medium is melted, aliquoted to 25 ml in 50 ml Falcon tubes and incubated at 50 °C.

Saturated liquid cultures of *E. coli* bacterial strains described in 10.3. are diluted to OD_{600}=1.0 and mixed in equal proportions (1:1:1:1:1:1:1:1). 25 ml soft agar at 50 °C is supplemented with 250 µl bacterial culture of OD_{600}=1.0, mixed and immediately poured on the pre-poured LB plate (final bacterial concentration in soft agar is about $1x10^6$ CFU/ml).

A 1:1 dilution series starting from undiluted with 17 dilution steps of colicin (blend) solutions is prepared with a dilution buffer or deionized water. A volume of 5 µl of each dilution, to include undiluted and solutions from the 17 dilution steps, are applied to the surface of the soft agar medium using a pipette, plates are kept open in a sterile cabinet until drops are dry, and incubated at 37 °C for 18-24 h to allow bacterial growth to occur.

Antimicrobial activity is recorded visually using a background light source. Activity is expressed as AU/mg fresh weight (FW) plant material at the highest dilution showing a difference in opacity (growth) between COLICIN-treated and plate background.

A.10.1.2 Preparation of devices for spray application of colicin blend or carrier solution

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

A.10.2 Preparation of meat test matrix

A.10.2.1 Purchase of meat in local retail outlets

No special sourcing of meat samples is used to ensure that COLICIN activity is evaluated in representative consumer products. Raw pork fillet or raw untrimmed beef round roast are purchased at retail outlets (for these studies, ALDI or Kaufland supermarkets, respectively, Halle, Germany), one day before the experiment.

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 A-3.

Table A-3. Matrix for preparation of beef and pork meat cuts

Test matrix	**Whole cuts**		**Ground meat**
	Raw pork steak	Raw beef steak	Raw beef prior to grinding
Initial meat block	Pork fillet steak ~90-110 g	Untrimmed round roast	Untrimmed round roast
Preparation	A part of pork fillet steaks is trimmed using a sterile scalpel to obtain steaks of ~85 g weight (~10x6x1.2 cm)	The meat block is cut into steak pieces using a knife cleaned with Bacillol to obtain steaks of ~85 g weight (~10x6x1.2 cm)	The meat block is cut into cubic pieces using a knife cleaned with Bacillol to obtain cubes of ~100 g weight (~5x5x5 cm)

A.10.3 Preparation of bacterial cultures used to experimentally contaminate meat

The meat test matrices are experimentally contaminated with a 1:1:1:1:1:1:1:1 mixture of 8 *E. coli* strains representing the Big 7 strains, plus O104:H4, which was responsible for the outbreak in Europe in 2011 (CDC 03-3014, O26:H11; CDC 00-3039, O45:H2; CDC 06-3008, O103:H11; CDC 2010C-3114, O111:H8; CDC 02-3211, O121:H19; CDC 99-3311, O145:NM; ATCC® 35150™, O157:H7; and ATCC® BAA-2326™, O104:H4), as shown in Table in 8-1.

Before inoculation the strains are thawed, individually grown on LB agar medium, and individually inoculated to LB broth. Individual saturated LB broth overnight cultures (37 °C, 150 rpm) are diluted to OD_{600}=0.05 with fresh LB broth and grown to OD_{600}~0.3 which corresponds to ~$3x10^7$ CFU/ml (~7.5 log CFU/ml). Individual cultures are diluted with LB broth to OD_{600}=0.3 and mixed 1:1:1:1:1:1:1:1. The strain mix is further diluted to the desired cell number (see Table A-4) with LB broth for use as meat contamination suspension.

Subsequently, 100 µl aliquots of serial dilutions of the bacterial suspensions are plated on SMAC supplemented with 0.05 µg/ml cefixime and 100 µg/ml X-Gluc for STEC WT strains or on SMAC supplemented with 25 µg/ml nalidixic acid and 100 µg/ml X-Gluc for nalidixic acid resistant mutants of pathogenic *E. coli* strains in order to determine the cell density.

A.10.4 Contamination of meat

Steaks (whole cuts) are dipped individually into each 12 ml of bacterial suspension and inverted and dipped again to inoculate both sides. Meat cubes are supplemented with 10 ml/kg bacterial contamination suspension while being tumbled and hand kneaded to ensure uniform exposure.

Contaminated meat and bacteria are allowed to dry and colonize matrix samples, respectively, for 30 min at RT, during which time steaks are inverted and meat cubes are tumbled every 15 min.

A summary matrix of the process is shown in Table A-4.

Table A-4. Matrix for experimental contamination of meats with mixed pathogen suspension

Test matrix - general	**Whole cuts**		**Ground meat**
	Raw pork steak	Raw beef steak	Raw beef prior to grinding
Test matrix - specific	Individual steaks of ~85 g weight (~10x6x1.2 cm)	Individual steaks of ~85 g weight (~10x6x1.2 cm)	Pool of meat cubes of ~100 g weight (~5x5x5 cm)
Containment	Sterile Petri dishes, ~120x120x17 mm	Sterile Petri dishes, ~120x120x17 mm	5 L Beaker, sterile
Density of pathogenic *E. coli* suspension for colicin efficacy tests - contamination **Level I**	Not analyzed	~$1x10^4$-$1x10^3$ CFU/ml (OD_{600}=0.0001-0.00001; 4-3 log/ml) nalidixic acid resistant derivatives of STEC/EHEC strains	~$2.5\text{-}5x10^3$ CFU/ml (OD_{600}= 0.00005-0.000025; 3.4-3.7 log/ml) nalidixic acid resistant derivatives of STEC/EHEC strains
Density of pathogenic *E. coli* suspension for colicin efficacy tests - contamination **Level II**	~$5x10^5$ CFU/ml (OD_{600}= 0.005; 5.7 logs/ml) STEC/EHEC WT strains	~$1x10^7$-$5x10^6$ CFU/ml (OD_{600}=0.1-0.05; 7-6.7 logs/ml) STEC/EHEC WT strains	~$1x10^7$ CFU/ml (OD_{600}=0.1; 7 logs/ml) STEC/EHEC WT strains
Application of *E. coli* suspension	Dipping from both sides	Dipping from both sides	Equal distribution, tumbling, hand mixing
Dose of *E. coli* suspension	12 ml per steak	12 ml per steak	10 ml/kg
Expected bacterial load of contaminated meat for colicin efficacy tests - contamination **Level I**	1-$5x10^1$ CFU/g	1-$5x10^1$ CFU/g	1-$5x10^1$ CFU/g
Expected bacterial load of contaminated meat for colicin efficacy tests - contamination **Level II**	0.1-$1x10^5$ CFU/g	0.1-$1x10^5$ CFU/g	0.1-$1x10^5$ CFU/g

A.10.5 Application of colicin (blend) solution

Contaminated meat is either treated with carrier or colicin blend solution by low-pressure spraying (2-4 bar) using atomizer flasks, as shown in Table A-5. Reasonably even coverage of the entire surface is ensured. Proposed application rates are 3 mg/kg for colicin M and 1 mg/kg for any other colicin used in the blend.

The meat is further incubated for 30 min at RT while steaks are inverted and meat cubes are tumbled every 15 min.

Table A-5. Matrix for application of COLICIN to meat samples

Test matrix - general	**Whole cuts**		**Ground meat**
	Raw pork steak	Raw beef steak	Raw beef prior to grinding
Test matrix - specific	Individual steaks of ~85 g weight (~10x6x1.2 cm)	Individual steaks of ~85 g weight (~10x6x1.2 cm)	Pool of meat cubes of ~100 g weight (~5x5x5 cm)
Containment	Sterile Petri dishes, ~120x120x17 mm	Sterile Petri dishes, ~120x120x17 mm	5 L beaker, sterile
Application of COLICIN/carrier solution	Spraying from both sides	Spraying from both sides	Spraying, equal distribution by tumbling, hand mixing
Application rate	~47 ml/kg ~56.5 mg total protein/kg 3+1 up to 3+1+1+1+1+1+1+1 mg colicin/kg of Col M, E7, Ia, K, E6, E2, 5 and U	~42 ml/kg ~50.8 mg total protein/kg 3+1+1+1+1+1 mg colicin/kg of Col M, E7, Ia, K, 5 and U	~42 ml/kg ~50.8 mg total protein/kg 3+1+1+1+1+1 mg colicin/kg of Col M, E7, Ia, K, 5 and U

A.10.6 Aliquoting and packaging of meat samples

Thirty (30) min after COLICIN application, whole meat is cut into aliquots and beef cubes are ground. Replicate meat samples are placed into sterile sample bags (BagFilter®400 P), the exact weight of each sample is recorded, and sample bags are closed using a closing clip (BagClip®400). A summary matrix of these steps in the process is shown in Table A-6.

Table A-6. Matrix for aliquoting and packaging of COLICIN-treated meat samples

Test matrix - general	**whole cuts**		**ground meat**
	Raw pork steak	Raw beef steak	Raw beef prior to grinding
Test matrix - specific	Individual steaks of ~85 g weight (~10x6x1.2 cm)	Individual steaks of ~85 g weight (~10x6x1.2 cm)	Pool of meat cubes of ~100 g weight (~5x5x5 cm)
Containment	Sterile Petri dishes, ~120x120x17 mm	Sterile Petri dishes, ~120x120x17 mm	5 L Beaker, sterile
Aliquoting	Cut each steak into 4 pieces of similar size (~20 g weight) using a sterile scalpel	Cut each steak into 4 pieces of similar size (~20 g weight) using a sterile scalpel	Grind meat using FW735 meat grinder (Beeketal Lebensmitteltechnik GmbH & Co. KG, Rastdorf, Germany) – first with a ∅ 6 mm then with a ∅ 3 mm die, hand mix ground meat
Packaging	One steak piece of ~20 g placed into a sterile bag BagFilter® 400 P using sterile forceps; for each	Each one steak piece of ~20 g from 2 steaks placed into a sterile bag BagFilter® 400 P using sterile forceps;	~40 g ground meat is placed into a sterile bag BagFilter® 400 P using a sterile spoon; for each

	treatment group, 4 replicates are prepared	for each treatment group, 4 replicates are prepared	treatment group, 3 replicates are prepared
Incubation at RT upon COLICIN application	1 h (including time for aliquoting and packaging)	1 h (including time for aliquoting and packaging)	2 h (including time for grinding, aliquoting and packaging)

A.10.7 Storage of meat samples

The sealed meat samples from Step 10.6 are then stored at one of several controlled temperatures. Beef meat samples are stored at either at 10 °C, or 15 °C and sampled at 1 h, 24 h, 48 h, 72 h and 144 h. Pork meat samples are stored at 4 °C and sampled at 1 h, 24 h and 72 h.

A.10.8 Analysis of viable populations of pathogenic *E. coli* on meat samples

A.10.8.1 Preparation of sample homogenates

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

A.10.8.2 Quantification of pathogenic E. coli population density on meat samples by dilution plating and CFU enumeration

In COLICIN efficacy tests with beef meat at low contamination Level I, 25 ml microbial suspension from the filtered part of the storage bag resulting from sample homogenization is transferred into a 50 ml Falcon tube using a serological pipet. The microbial suspensions are concentrated 10-fold by centrifugation: 25 ml aliquots are centrifuged for 10 min. at 4000 rpm, RT, the supernatant is removed and the pellet is resuspended in 2500 µl peptone water. A 1:10 dilution series of concentrated microbial suspension (100 µl microbial suspension + 900 µl peptone water) is prepared. 100 µl aliquots of undiluted or diluted microbial suspensions are plated on Sorbitol-MacConkey agar (SMAC) supplemented with 25 µg/ml nalidixic acid and 100 µg/ml X-Gluc. For samples without COLICIN treatment, 10 aliquots are plated. For samples with COLICIN treatment, 20 aliquots are plated. The plates are incubated for 18-24 h at 37 °C and the CFU are enumerated.

In COLICIN efficacy tests with beef meat at high contamination Level II, 15 ml microbial suspension from the filtered part of the storage bag resulting from sample homogenization is transferred into a 15 ml Falcon tube using a serological pipet. The microbial suspensions are concentrated 5-fold by centrifugation: 1.5 ml aliquots are centrifuged for 2 min at 13000 rpm, RT, the supernatant is removed and the pellet is resuspended in 300 µl peptone water. A 1:10 dilution series of concentrated microbial suspension (100 µl microbial suspension + 900 µl peptone water) is prepared. Subsequently, 100 µl aliquots of undiluted and diluted microbial suspensions are plated on Sorbitol-MacConkey agar (SMAC) supplemented with 0.05 µg/ml cefixime and 100 µg/ml X-Gluc. For control samples without COLICIN treatment, 5 aliquots are plated. For samples with COLICIN treatment, 10 aliquots are plated. The plates are incubated for 18-24 h at 37 °C and the CFU are enumerated.

For pork meat at high contamination Level II, microbial suspensions are not concentrated; instead, a 1:10 dilution series of microbial suspension from the filtered part of the storage bag (100 µl microbial suspension + 900 µl peptone water) is prepared. 100 µl aliquots of undiluted or diluted microbial suspensions are plated on Sorbitol-MacConkey agar (SMAC) supplemented with 0.05 µg/ml cefixime and 100 µg/ml X-Gluc.

For samples without COLICIN treatment, 5 aliquots are plated. For samples with COLICIN treatment, 10 aliquots are plated. The plates are incubated for 18-24 h at 37 °C and the CFU are enumerated.

The CFU number per g sample is calculated as follows:

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

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

A.10.9 Statistical analysis

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

APPENDIX B. COLICIN Manufacturing Process

B.1 Introduction and Rationale

Notifier produces colicin proteins recombinantly using a plant-based manufacturing process. This approach minimizes concerns over toxicity of colicins to the producing host and offers a more scalable and cost-effective manufacturing option relative to fermentation. In Notifier's process, leaf tissue of several plant species, including *Beta vulgaris* (beet), *Spinacia oleracea* (spinach), lettuce (*Lactuca sativa*) or *Nicotiana benthamiana* can be transduced to express colicins 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 components of the expression system and host plants are prepared independently and subsequently combined.

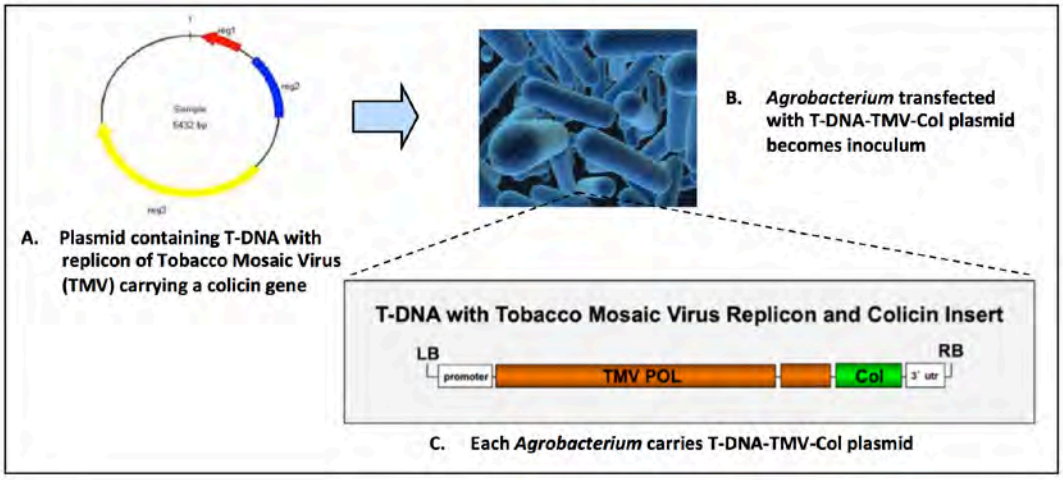
Alternatively, colicins can be produced in the same plant hosts carrying transgenically the colicin gene and an ethanol-inducible promoter, with induction by dilute ethanol. After induction with either method, colicin protein is allowed to accumulate in leaf tissues for several days. Plants are subsequently harvested, and the protein is extracted and concentrated from the plant material. Notifier's COLICIN product may be formulated to contain a single colicin protein or blended as a mixture of two or more colicins that act synergistically to control targeted pathogens.

COLICIN 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 colicins and other antimicrobials. The process was described in detail in GRN 593 and key parts are summarized below for convenience. When using *N. benthamiana* as the host plant, additional downstream purification is employed to reduce the level of host alkaloids.

B.2 Organism Used and Gene Expression Cassette

In the agroinduction method, the production organism *Agrobacterium tumefaciens* harboring the binary plasmid vector pNMD10220 containing a TMV replicon with inserted colicin 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-Col replicon is transfected into *A. tumefaciens* to prepare the inoculum (Figure B-1-**B**). Each bacterium in the inoculum contains the T-DNA-TMV-Col plasmid (Figure B-1-**C**).

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



B.3 Procedure

A flow diagram summarizing the overall process for producing colicin proteins is shown in Figure B-2 (from GRN 593). Summary descriptions of key process steps follow; step numbers correspond to the steps indicated in Figure B-2. A **process modification** applied when using *N. benthamiana* as the host is described following definition of the overall process.

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 colicins using native cultivars of *N. benthamiana* as the host plant, chromatographic purification of the process stream is necessary to remove host alkaloids. Specific steps in downstream purification that are employed to reduce alkaloids in *N. benthamiana*-produced colicins (process modification) are described in more detail.

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

A proprietary industrial strain of *Agrobacterium tumefaciens* harboring the binary plasmid vector pNMD10220 containing a TMV replicon with inserted colicin gene is 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 by ultra- and dia-filtration during plant biomass extraction and processing. All raw materials and processing aids are food grade. A multi-vial Master Vector Bank of the vector is prepared and stored at -80°C, from which aliquots are removed as Working Vector Banks of the inoculum for each manufacturing batch.

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

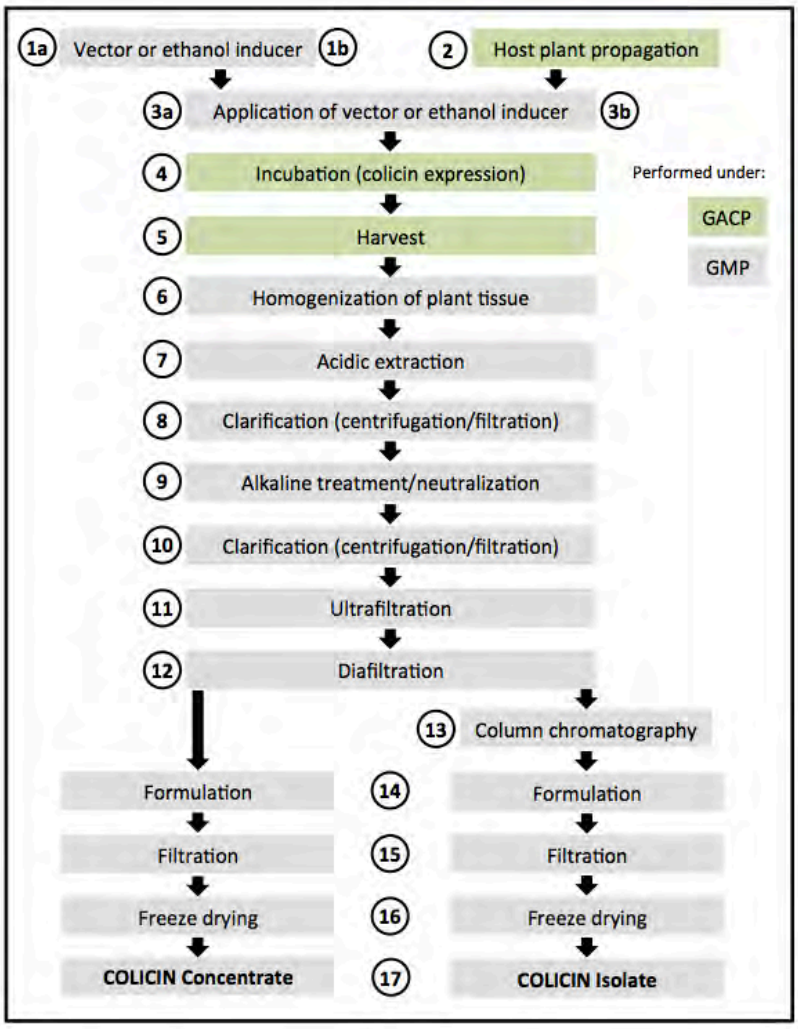
Step 1b. Ethanol induction of transgenic plants

In this variation of the method, transgenic plants carrying an ethanol-inducible promoter are used. The procedure was developed by Notifier and described by Werner et al. (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 colicin protein.

Step 2. Host plant 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 colicin driven by an ethanol-inducible promoter. With either method of induction, plants are propagated in trays using a food-crop compatible soil-based substrate or neutral substrate for hydroponic irrigation, 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 COLICIN production in plants



Step 3a. Inoculation of host plants with agrobacterial vector

The *A. tumefaciens* inoculum carrying the selected colicin 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 point after seeding the plants are treated with a defined concentration of *Agrobacterium* in dilution buffer.

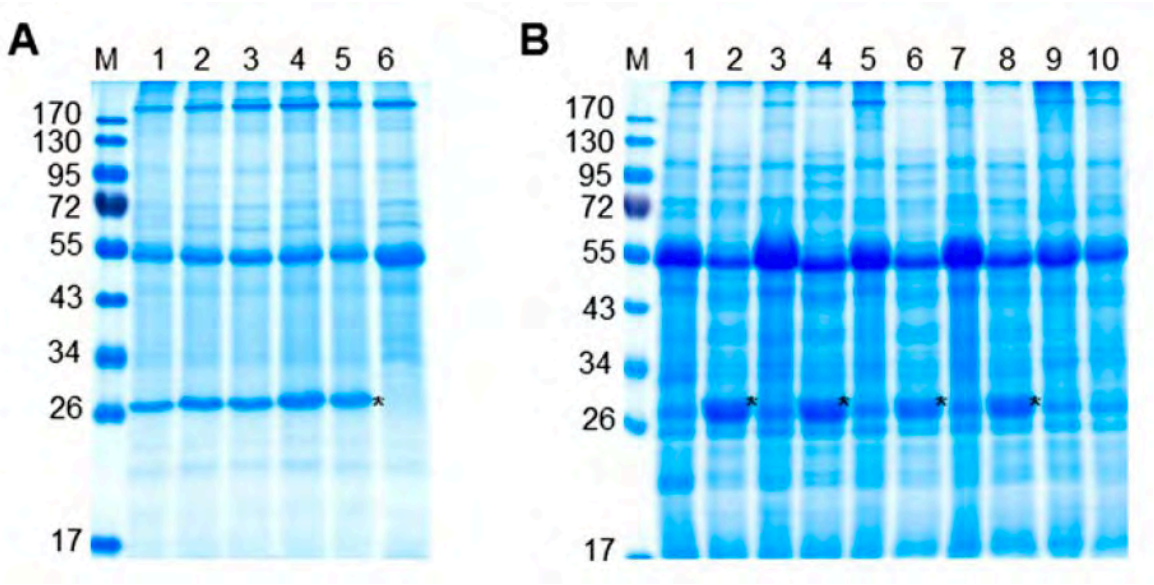
Inoculation of plants is accomplished by either vacuum-mediated infiltration after dipping the plant leaves in a suspension of the inoculum, or via a procedure wherein the inoculum is sprayed onto plant leaves mixed with a surfactant (Gleba 2014; Hahn 2015); the latter approach potentially providing economic advantages (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 colicin-encoding RNA transcripts. Amplification of the transcript and translation of the colicin RNA message into colicin occurs in the cytoplasm of each plant cell. Neither the vector nor colicin 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.

Step 3b. Ethanol induction

In this variation of the method, a simple treatment of the transgenic plants carrying the colicin gene with dilute ethanol (2.5% v/v) releases the replicon leading to RNA amplification and high-level colicin 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, colicin 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.

Figure B-3 shows comparative results for one model colicin, colicin M, produced in *N. benthamiana* host plants. The left panel (**A**) shows transient expression of ColM at various times post inoculation with *Agrobacterium*, whereas the right panel (**B**) shows expression of ColM at various times post induction of transgenic plants with ethanol (Werner 2011). Full description of these studies is found in Schulz (2015).

Figure B-3. Comparative expression of Colicin M in *N. benthamiana* with *Agrobacterium* inoculation of non-recombinant hosts and with ethanol induction of transgenic hosts



Images and caption adapted from Figure S2 in Schulz et al. (2015). Expression of colicins in *Nicotiana benthamiana* plants. **Panel A** – **Transient expression** of colicin M after spraying with *Agrobacterium tumefaciens* carrying TMV-based vector. Coomassie-stained SDS protein gel loaded with TSP extracts corresponding to 5 µg protein from plant material expressing colM harvested at (lane 1) 5, (lane 2) 6, (lane 3) 7, (lane 4) 8, and (lane 5) 9 dpi or from (lane 6) non-transfected leaf tissue.
Panel B – Inducible expression of colM in stable transgenic plants. Coomassie-stained SDS protein gel loaded with crude extracts corresponding to 1.5 mg fresh weight prepared with 2x Laemmli buffer from (lanes 1, 3, 5, 7, and 9) non-induced and (lanes 2, 4, 6, 8, and 10) plant material 4 dp induction with ethanol (lanes 9 and 10). *N. benthamiana* WT plants (lanes 1 and 2), (lanes 3 and 4), (lanes 5 and 6), and (lanes 7 and 8) indicate different transgenic plants of T0 generation (12, 20, 29, and 42, respectively). M, molecular weight marker with weights (kDa) shown (Left). Asterisks indicate recombinant proteins.

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 colicin protein are harvested typically 8-9 days post inoculation/induction. Samples of plant biomass are taken for analyses of colicin 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 using an extraction buffer; 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. Acidic extraction

The complex stream from Step 6 is subjected to low pH treatment to help precipitate major host cell proteins, resulting in a partially purified stream enriched for the colicin protein.

Step 8. First clarification

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

Step 9. Neutralization

After clarification in Step 8, the process stream is pH-adjusted with alkali for further processing.

Step 10. Second clarification

The solution from Step 9 is further clarified by centrifugation and/or filtration.

Step 11 and Step 12. Ultrafiltration / diafiltration

Additional impurities are removed by ultrafiltration and diafiltration; typically, impurities that are less than 5-10 kDa in mass, including non-bound host alkaloids, are eliminated at this step.

Step 13. Chromatography

At this stage, the product-enriched solution can be subjected to one of two additional purification steps. If a relatively pure colicin product is desired, as it is when using *N. benthamiana* as the production host, the solution is subjected to ion-exchange chromatography, which removes additional host-cell proteins and plant metabolites such as polyphenols, resulting in a clarified, enriched product. One or more colicin proteins prepared by this method can be dried and blended as **COLICIN Isolate**.

For colicins produced in non-food species plant hosts, such as ***N. benthamiana***, a specific **process modification** is applied, as described in Section B.4 of this Notice. The modification ensures isolation of colicins with low levels of plant host impurities, including host alkaloids.

When using food species plant hosts, the less purified bulk product **COLICIN Concentrate** can be prepared by omitting the chromatography step.

Steps 14 – 17. Formulation, fill and finish

The final *N. benthamiana*-derived **COLICIN Isolate** precursor solution 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 for refrigerated storage, or freeze or spray dried to produce a dry, off-white to light tan powdered product. Prior to release, the bulk product is tested to ensure compliance with the product specification for COLICIN Isolate.

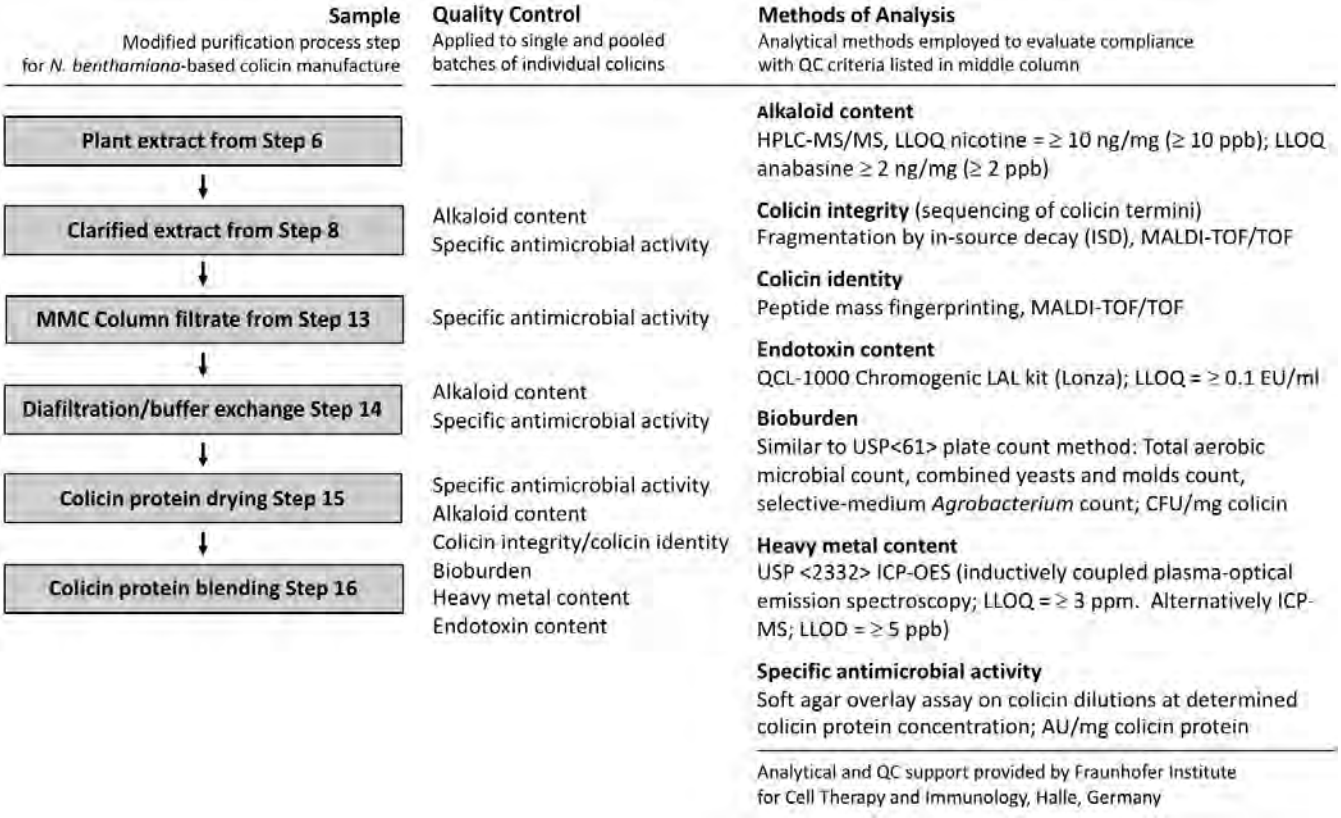
In-Process controls and quality assurance

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 rigorous 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 Process Modification

The main goal of more stringent downstream purification of the *N. benthamiana*-derived process stream is to lower the content of host alkaloids in the final colicin proteins. The modified process and the QC steps implemented to monitor protein quality are summarized in Figure B-4.

Figure B-4. Downstream process modifications and QC of *N. benthamiana*-produced colicin proteins



The upstream parts of the process defined in Sections B.2 and B.3 and depicted in Figure B-2 of this Notice are the same regardless of which plant host is used and regardless of which gene induction system is applied. Beginning at Step 7, the homogenized biomass suspension from *N. benthamiana* is subjected to selective acidic extraction of colicins in 20 mM citric acid, 20 mM NaH_2PO_4, 30 mM NaCl, pH 4-5.5.

Neutralization/clarification in Steps 8 and 9 follow procedures described in Section B.3. The next step (**Step 13**) involves a single-step (1-step) purification of colicins using CaptoMMC (GE HealthCare) multi-modal chromatography using one of several elution buffers depending on the properties of the colicin being purified (a typical buffer consists of 50 mM Na_2HPO_4 pH 7.84, 10 mM citric acid, 1 M NaCl).

Modifications to **Step 14** and **Step 15** in the process are now implemented. The purified column filtrate from Step 13 is diafiltered against 20 mM Na_2HPO_4 pH 7.5, 10 mM citric acid, 50 mM NaCl overnight at 4°C and filter-sterilized (**New Step 14**). The purified colicin protein concentrate is then dried (lyophilization or spray drying) into a stable protein (**New Step 15**). Colicins purified via this procedure can be **blended** as dry powders (**New Step 16**) at prescribed ratios into the final COLICIN product, which, for *N. benthamiana*-produced proteins, constitutes **COLICIN Isolate** (**Step 17**). The individual colicin proteins as well as the final blended product should be ≥70% purity with respect to colicin protein.

B.5 Manufacturing Facilities

Notifier can manufacture COLICIN from any plant species host, including edible species and *N. benthamiana*, 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 colicin 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 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 COLICIN 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, supplements, additives or treatment aids.

B.7 Specification

The **target specification** for *N. benthamiana*-derived **COLICIN Isolate** purified by the more stringent process described in Section B.4 of this Notice is shown in Table B-1.

Table B-1. Target specification of COLICIN (mixed colicins) product

COLICIN Produced in *N. benthamiana*			
Parameter	**Method**	**Specification limit**	**Results of analyses***
Appearance	Visual	Powder, white to beige	Conforms
Specific Activity (colicin protein basis)	Viability inhibition; *E. coli* DH10B strain	$\geq 2.5 \times 10^6$ AU/g	$2.15 \pm 0.89 \times 10^9$ AU/g
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	$\leq$ 30 ppm	In progress
Heavy metals: Lead	USP38<233> ICP-OES	$\leq$ 5 ppm	< 1 ppm
Heavy metals: Cadmium	USP38<233> ICP-OES	$\leq$ 5 ppm	< 1 ppm
Nicotine (per total colicin blend)	HPLC/MS	$\leq$ 75 ng/mg colicin	Average 48.76 ng/mg
Anabasine (per total colicin blend)	HPLC/MS	$\leq$ 15 ng/mg colicin	Average 9.54 ng/mg
Bioburden	USP32<61>	$\leq$ 10 CFU/25 g sample	0 (absent)
Agrobacterium (CFU/10 g sample)	Selective plate-based assay	0 (absent)	0 (absent)
Undesirable microorganisms: *Escherichia coli, 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	> 8 months

*Results of analyses for a dry, 100% *N. benthamiana*-derived COLICIN (mixed-colicin) product are based on average results obtained from analyses of individual colicin proteins blended at their optimal ratio (dwb), typically 3x colM, and 1x each of colicins K, U and Ib (w/w).

APPENDIX C. Methodology

The methods employed to assess the properties and characteristics of plant-made colicin proteins that are candidate components of the COLICIN product were described in detail in past notices. Descriptions included results from antibacterial studies *in vitro*, as well as Notifier's results of efficacy and suitability studies on-matrix with various vegetables and fruits (GRN 593) and various meat products (GRN 676). Data provided in past notices included full digestibility of colicins in simulated gastric and intestinal fluids, low allergenic potential, and low probability for development of bacterial resistance.

In Schulz (2015), GRN 593 and GRN 676, we detailed methods and results of on-matrix studies with colicins. The physicochemical properties of purified colicins are consistent regardless of which plant host is used for gene expression. Here we present representative results of studies that show equivalence and comparability of *N. benthamiana*-produced colicins to colicins produced in food plant species (GRN 593 and GRN 676), and describe special methods used to characterize *N. benthamiana*-produced colicins in particular.

Our summary of methods employed in this current Notice includes:

- Purification of *N. benthamiana*-made colicins by single-step chromatography;
- Confirmation of amino acid sequences of *N. benthamiana*-produced colicins by mass spectrometry
- Analysis of alkaloid content in the host plant and its reduction in colicin proteins through the purification method described in APPENDIX B of this Notice;
- Analysis of heavy metal content in purified colicins;
- Batch-to-batch consistency of *N. benthamiana*-produced colicin proteins as determined in analyses of non-sequential developmental production batches; and
- Stability of *N. benthamiana*-produced colicins stored as dry powders and as aqueous solutions.

C.1 Purification of *N. benthamiana*-made colicins using single-step chromatography

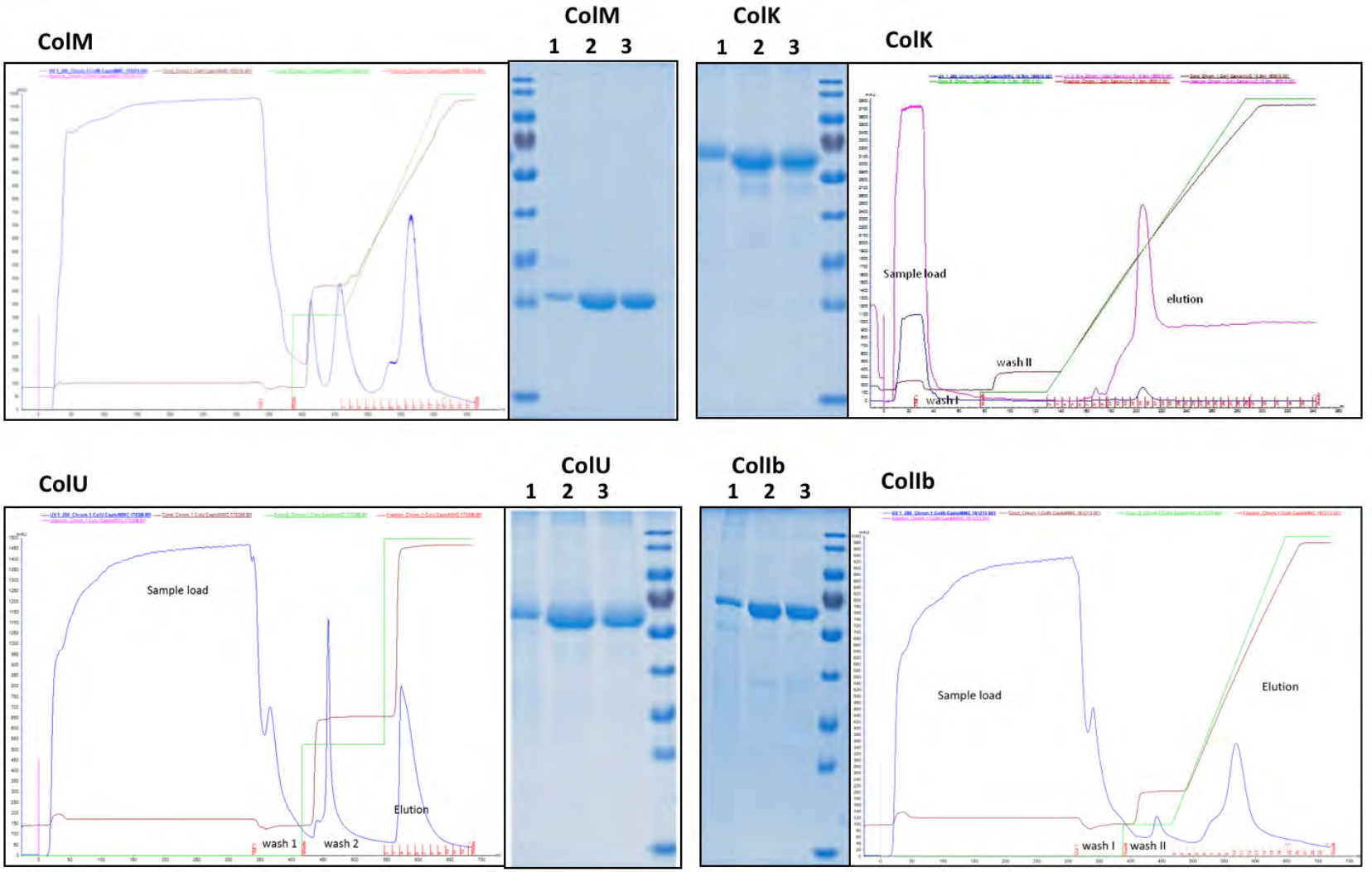
Recently we described a simple and efficient method for reducing the host alkaloid content in *N. benthamiana*-produced colicins (Stephan 2017). The one-step multi-modal chromatography employed in our improved downstream process can efficiently separate colicin proteins from host impurities. Purity of model colicins and example chromatographs are shown in Figure C-1. Representative recovery yields are shown in Table C-1.

The results shown in Figure C-1 are from several non-sequential developmental lots of colicins using *N. benthamiana* as the expression host. Lanes marked 1 show Coomassie stains of total soluble protein extract, lane 2 shows multi-sample pooled column filtrate, and lane 3 shows colicin purity after dialysis.

As shown in Table C-1, some colicins are more efficiently purified than others by the one-step chromatographic procedure. Nevertheless, even the lower yielding colicins like ColIb can be purified at >0.5 g/kg (fresh weight) of plant host biomass, hence maintaining the economic advantages of the process.

The chromatography step does result in reduction in the level of host alkaloids in the final product by several hundred- to several thousand-fold relative to the level of alkaloids in *N. benthamiana* host plant (see Section C.3).

Figure C-1. Single-step purification of 4 colicins expressed in *N. benthamiana*



Lanes marked 1 show Coomassie stains of total soluble protein extract, Lanes 2 show multi-sample pooled column filtrates, and Lanes 3 show colicin purity after dialysis. MW marker shown in first or last lanes in gels.

Table C-1. Representative colicin recoveries using one-step chromatography

Colicin	Plant biomass (g)	Colicin recovery (%)	Yield lyophilized colicin (mg)	Colicin yield (mg/g FW)
ColM	486	87	653	1.3
ColK	203	76	469	2.3
ColU	420	81	652	1.6
ColIb	713	55	458	0.6

C.2 Confirmation of colicin amino acid sequences by MALDI-MS

Methods

Nicotiana benthamiana host plants are inoculated with *Agrobacterium* vectors carrying inserts for individual colicins (see APPENDIX B for colicin expression methods). The gene sequences for the colicin inserts (GRN 593; Section 2, pp 7-14) in the expression vectors are verified by DNA sequencing.

Plants are extracted to yield total soluble protein (TSP) extracts containing colicins; each TSP is run on SDS-PAGE gels and stained with Coomassie for visualization or maintained in appropriate buffers for further processing.

The sequences of the colicins are verified by 2 to 3 complementary mass spectrometry methods, including peptide mass (PMF) and peptide fragment fingerprinting (PFF), or by in-source decay (ISD):

1. MALDI-TOF MS verification of sequence by peptide mass fingerprinting (PMF). Each colicin protein is digested with 3 proteases, namely pepsin, trypsin and chymotrypsin for maximum coverage of the full protein sequence with peptides of suitable mass (500-3,500 Da range) for detection (Methods: in-source decay (ISD); RP_Proteomic detection, reflector-positive mode, PMF);

2. MALDI-TOF MS/MS amino acid sequence determination by analysis of peptides at the N- and C-termini of the protein (peptide fragment fingerprinting; PFF). Peptides detected by the methods in "1" above for PMF are fragmented and the mass of each amino acid is measured. Predicted and actual masses of the sequences are analyzed are then compared;

3. MALDI-ISD is a mass spectrometry method that is used for the simultaneous sequencing of protein N- and C-termini. The sequencing occurs without cleaving the sample with chemicals or enzymes. MALDI-ISD also determines post-translational modifications and their localization within the protein.

Sequence coverage and results

When combined, the methods described above give sufficiently high sequence coverage for verification of the amino acid sequences of the plant-made colicins (e.g. 89-100% coverage). All *N. benthamiana*-made colicin proteins conform to their predicted composition and match the consensus amino acid sequences of the bacterially produced native colicin proteins.

Table C-2 summarizes the results of amino acid sequence analyses by MALDI MS for 4 colicins extracted from *N. benthamiana*. The table shows results of Notifier's analysis of plant-made colicins and published results of analyses with bacterial proteins, where available.

Post-translational processing of the plant-made and bacterial polypeptides, if any, is also shown. Note that N-terminal sequences are identical regardless of source host.

Table C-2. Identity confirmation of *N. benthamiana*-produced colicins by mass spectrometry

Colicin	*N. benthamiana*-produced (determined by Notifier)			Bacterial (literature values)
	N-terminus	C-terminus	Full-length coverage	N-terminus (method). Reference
M	Confirmed. N-terminal met is present; thr3 is acetylated	Confirmed	100%	N-terminal met verified by Edman degradation (Dreher 1985)
K	Confirmed. N-terminal met is present	Confirmed	95%	(Pilsl 1995)
U	Confirmed. N-terminal met is cleaved off; mature protein starts with N-terminal pro	Confirmed	89%	(Šmajs 1997)
Ib	Confirmed. N-terminal met is cleaved off; mature protein starts with N-terminal ser; ser2 is acetylated	Confirmed	MALDI-TOF MS/MS and MALDI-ISD confirm intact protein	N-terminal met is cleaved off; mature protein starts with N-terminal ser (DNSC method; Edman degradation). (Konisky 1972; Mankovich 1986)

C.3 Alkaloid content of *N. benthamiana*-produced colicins

Downstream reduction of alkaloids

Using more stringent downstream purification methods, Notifier successfully reduced the level of host impurities in *N. benthamiana*-produced colicin proteins. These results were used by Notifier to assess safety. The multi-modal chromatography step employed for purifying colicins produced in *N. benthamiana* successfully reduces host-derived alkaloids and other impurities (Stephan 2017). The process modification is described in APPENDIX B, Section B-4 of this Notice. Alkaloid content was determined by HPLC/MS analysis and was conducted on behalf of Notifier by the Fraunhofer Institute of Cell Therapy and Immunology (Halle, Germany). The method has a LLOQ of ~20 ng/mL (20 ppb) and linearity of 20-1,500 ng/mL (20-1,500 ppb).

Table C-3 is a summary of representative results from developmental batches of various colicins, showing the original alkaloid content of the host (**Panel A**, nicotine; **Panel B**, anabasine, converted to ng alkaloid/mg sample, and where the range was determined from public literature and by Notifier), and the nicotine and anabasine content in total soluble protein (TSP) extracts of *N. benthamiana*, in purified multi-modal chromatography/diafiltered solution, and in dried purified colicin protein powder (**Panel C**). Results of two production batches are shown as examples. Compared to the levels of alkaloids in the host, purification of ColM, ColK and ColU results in 1000x-2000x reduction of **nicotine** content and 500x-5000x reduction in **anabasine** content. Purification of ColIb results in 350x and 200-300x reductions in **nicotine** and **anabasine** content, respectively. Results from developmental batches 2-5 are shown.

Table C-3. Reductions in alkaloid content currently achievable by downstream purification

A	**Notifier's Internal Data**		**Data From Literature**	
Sample	**Nicotine** content ng/mg fresh weight	**Nicotine** content ng/mg dry weight	**Nicotine** content ng/mg fresh weight	**Nicotine** content ng/mg dry weight
N. benthamiana Buffer 1	**61.8 ± 29.5**	**690.0 ± 340.0**	~500.0 (Todd 2010)	~14,000.0 (Sisson 1984) ~3,000.0 (Saitoh 1985)
N. benthamiana Buffer 2	**103.3 ± 15.6**	**1,140.0 ± 190.0**		
B	**Notifier's Internal Data**		**Data From Literature**	
Sample	**Anabasine** content ng/mg fresh weight	**Anabasine** content ng/mg dry weight	**Anabasine** content ng/mg fresh weight	**Anabasine** content ng/mg dry weight
N. benthamiana Buffer 1	**7.1 ± 1.3**	**80.0 ± 20.0**		~1,300.0 (Sisson 1984) (9.3% of nicotine)
N. benthamiana Buffer 2	**12.9 ± 4.0**	**140.0 ± 10.0**		

C

Nicotine content (ng/mg protein)

Colicin	Batch	TSP extract	Purification and Dialysis	Lyophilization
ColM	4	49569	15.02	21.73
	5	47738	21.61	39.77
ColK	2	66111	30.66	43.91
	3	59865	8.61	13.58
ColU	4	23111	54.11	23.10
	5	56699	12.03	19.71
ColIb	2	47843	145.53	128.46
	3	57487	129.07	171.84

Anabasine content (ng/mg protein)

Colicin	Batch	TSP extract	Purification and Dialysis	Lyophilization
ColM	4	7404	<1.21	<1.54
	5	4095	2.83	<1.92
ColK	2	7644	<1.75	13.91
	3	7047	2.44	2.06
ColU	4	3178	<2.34	<2.5
	5	7647	3.67	2.87
ColIb	2	7569	23.61	43.59
	3	8637	39.26	39.18

Reduction in host alkaloid content in purified colicins. Compared to the levels of alkaloids in the host plant (**Panel A**) purification of ColM, ColK and ColU results in 1000x-2000x reduction of **nicotine** content and 500x-5000x reduction in **anabasine** content. Purification of ColIb results in 350x and 200-300x reductions in **nicotine** and **anabasine** content, respectively. Results from developmental batches 2-5 are shown. Literature values reported in Panels A and B reflect procedural variabilities and accuracy of detection methods. Notifier uses HPLC/MS-based analysis.

C.4 Heavy metal analysis of *N. benthamiana*-produced colicins

Residual heavy metals were analyzed for sample colicins 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 colicins were analyzed by ICP-OES for lead and cadmium content first. 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. If higher sensitivity is required, the alternative detection method ICP-MS can be used, with LOD of ppb (FSIS CLG-TM3 2016). Results for lead and cadmium are shown in Table C-4.

Table C-4. Summary of lead and cadmium residues in dried colicins produced in *N. benthamiana*

Colicin	Powder analyzed (g colicin per g powder)	Lead (mg/kg powder)	Cadmium (mg/kg powder)
colM Batch 9	**0.21 / 0.13**	**< 1 ppm**	**< 1 ppm**
colK Batch 4	**0.49 / 0.13**	**< 1 ppm**	**< 1 ppm**
colU Batch 8	**0.336 / 0.12**	**< 1 ppm**	**< 1 ppm**
colIb Batch 11	**0.464 / 0.08**	**< 1 ppm**	**< 1 ppm**

Multi-batch (non-sequential) results of lead and cadmium content in dried powders of sample colicins expressed in *N. benthamiana* and purified by single-step chromatography per method in APPENDIX B. Both heavy metals are below limits of detection by ICP-OES.

As indicated by the results summarized in Table C-4, purified colicin powders have very low residues of lead and cadmium. A daily intake of 0.560 kg food treated with 10 mg/kg colicin containing 1 ppm levels of Lb and Cd 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, colicin-derived Lb and Cd intake is insignificant compared to other sources of these elements in the diet.

Analysis of total metal content (e.g. sum of [Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, Sn]) is in progress and will be reported separately. The hydroponic plant culture medium is controlled for inorganic content and therefore we expect the total heavy metal content remaining in colicins to be at or below the limit of detection.

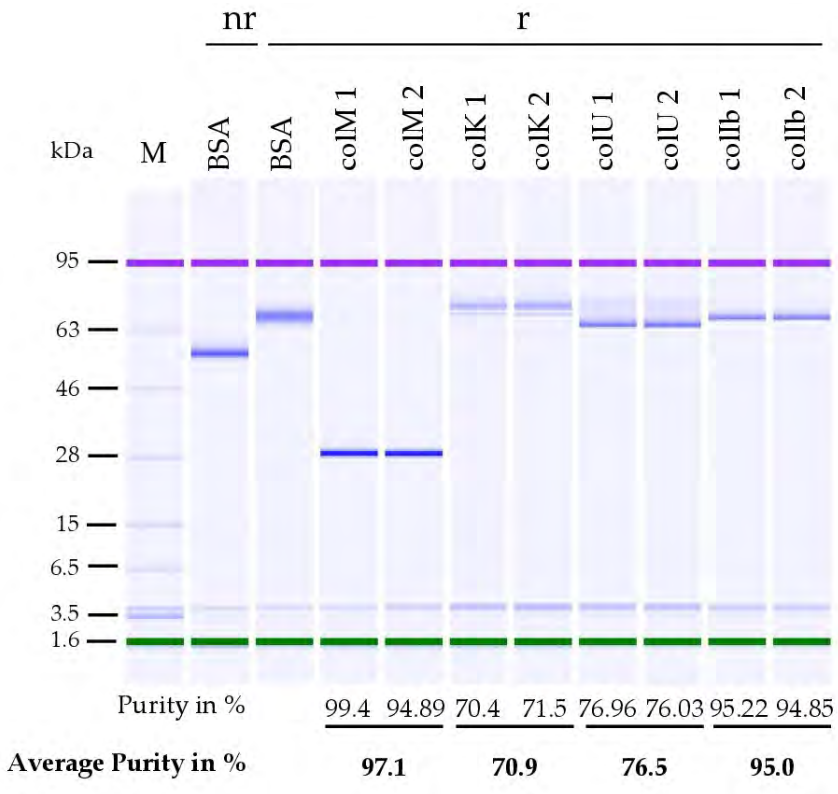
C.5 Batch-to-batch manufacturing consistency of *N. benthamiana*-produced colicins

Batch-to-batch consistency of *N. benthamiana*-produced colicins was determined using dry (lyophilized) powders. Multiple, non-sequential batches were used to assess consistency of the manufacturing method. Yield of colicins, recovery efficiencies and specific activities were determined. Table C-5 and Figure C-2 published in Stephan et al. (2017) summarize results for 4 colicins.

Table C-5. Batch-to-batch consistency of *N. benthamiana*-produced dry colicin powders

Colicin	Number of Batches Analyzed	Average Fresh Weight Plant Material (g)	Average Recovery of Colicin After Purification (%)	Average Specific Activity (AU/mg Colicin)	Average Yield of Lyophilized Colicin (mg)
Col M	9	54	88 ± 6.1	$2.47x10^6 \pm 2.11x10^6$	72.6 ± 17.8
Col K	4	50.7	76 ± 8.9	$1.89x10^6 \pm 7.56x10^5$	117.3 ± 19.9
Col U	8	58.8	81 ± 10	$1.08x10^6 \pm 9.34x10^5$	81.6 ± 16.4
Col Ib	13	59.7	55 ± 9.9	$3.19x10^6 \pm 2.67x10^6$	35.2 ± 14

Figure C-2. Purity consistency of *N. benthamiana*-produced colicins



Purity of ColM, ColK, ColU and ColIb expressed in *N. benthamiana* and purified to dry powders in two independent production batches. Capillary gel electrophoresis was used to analyze 1 mg/mL solutions of each colicin. Purity of each colicin is expressed as a percent of total soluble protein obtained upon dissolution of the dried colicin powders (Stephan 2017).

Table C-5 and Figure C-2 present currently achievable results. Notifier's manufacturing process for colicins from *N. benthamiana* biomass is the subject of continued optimization to further enhance product recovery while lowering the residual levels of impurities. As shown in Table C-5, Colicin K results were obtained from 4 different and non-sequential production batches, whereas Colicins U, M and Ib results were obtained from 8, 9 and 13 different and non-sequential batches. These multiple runs are allowing us to optimize conditions for more efficient recovery and purification of each colicin. For product release, analyses of 3 independent and non-sequential cGMP-compliant batches will be evaluated to verify conformance with the final product's specification.

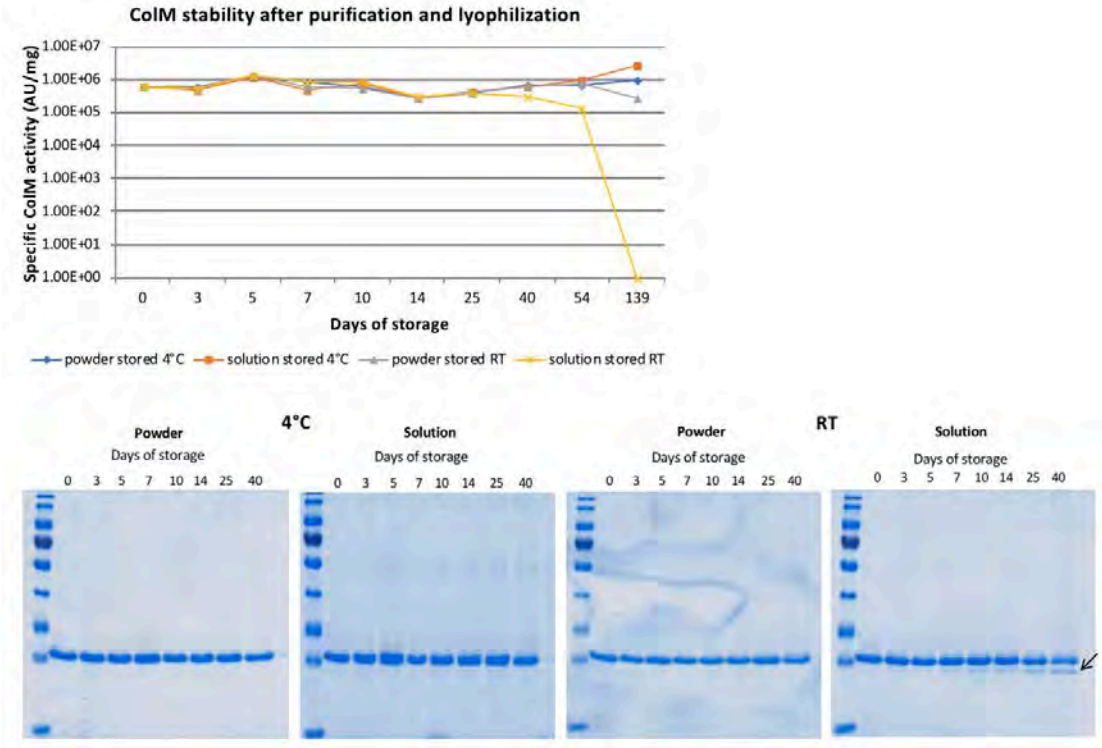
C.6 Stability of *N. benthamiana*-produced colicins

To assess the stability of *N. benthamiana*-produced colicins upon storage, samples of each purified colicin shown in Table C-5 were analyzed under different storage conditions for various durations of storage. The samples were stored as (a) dry (lyophilized) powders or as (b) 1% solutions in buffer at either 4°C or at room temperature (RT). Stability was determined by quantifying specific activity by soft-agar overlay assay using *E. coli* tester strain DH10B, and by SDS-PAGE analysis of the proteins to detect potential degradation under each storage condition.

Figure C-3 shows results of analysis of Colicin M dry powder and solution prepared from the dry powder at the two storage temperatures for antimicrobial activity and by SDS-PAGE. ColM is very stable when stored dry at either 4°C or at room temperature. The ColM solution was equally stable at 4°C but showed eventual degradation when stored at RT (arrow in figure shows degradant band), but significant loss of protein integrity occurred only at the 139-day (4.6 month) sampling point.

Because the solutions were maintained sterile during storage, we presume that the loss of activity in the colicin solution held at RT was due to physicochemical events. Hence, solutions of ColM that are prepared and used within ≤14 days of application are expected to be stable and active. If stored dry or if stored as a solution at 4°C, the projected stability of ColM is >6 months.

Figure C-3. Stability of *N. benthamiana*-produced Colicin M



Similar stability studies under the same storage conditions with representative colicins Col Ib, Col U and Col K powders and solutions made from the powders produced the results shown in Figure C-4, Figure C-5, and Figure C-6, respectively. Degradation bands of proteins in solutions are indicated by the arrows.

Figure C-4. Stability of *N. benthamiana*-produced Colicin Ib

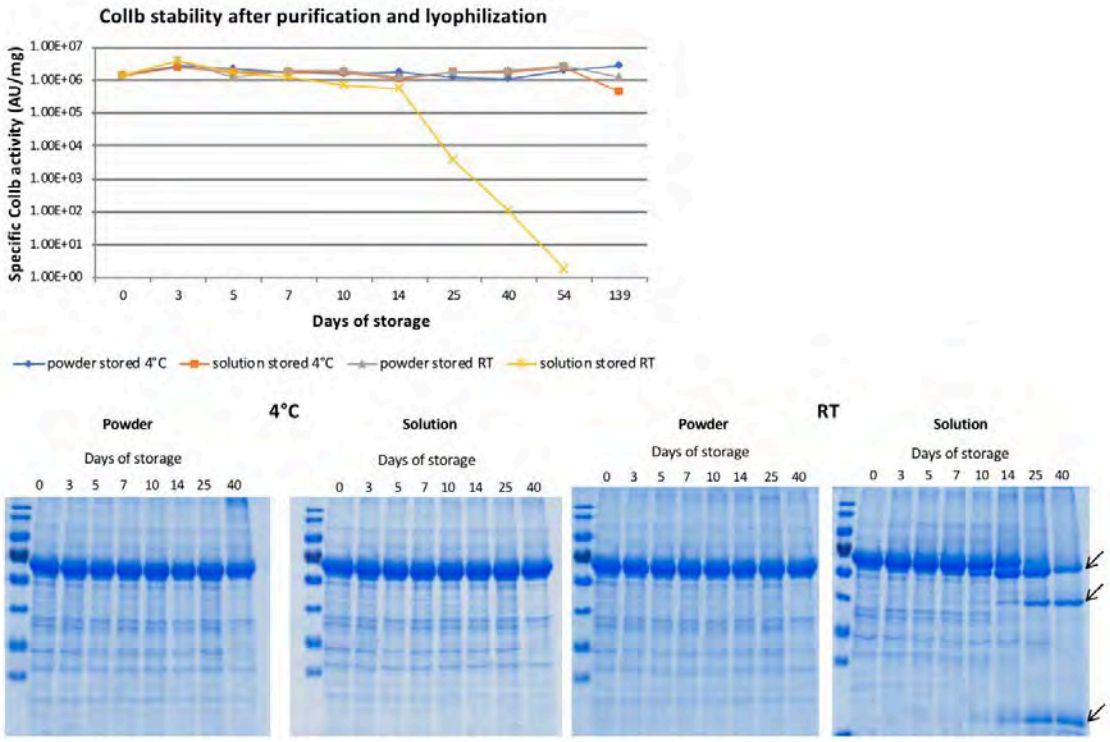


Figure C-5. Stability of *N. benthamiana*-produced Colicin U

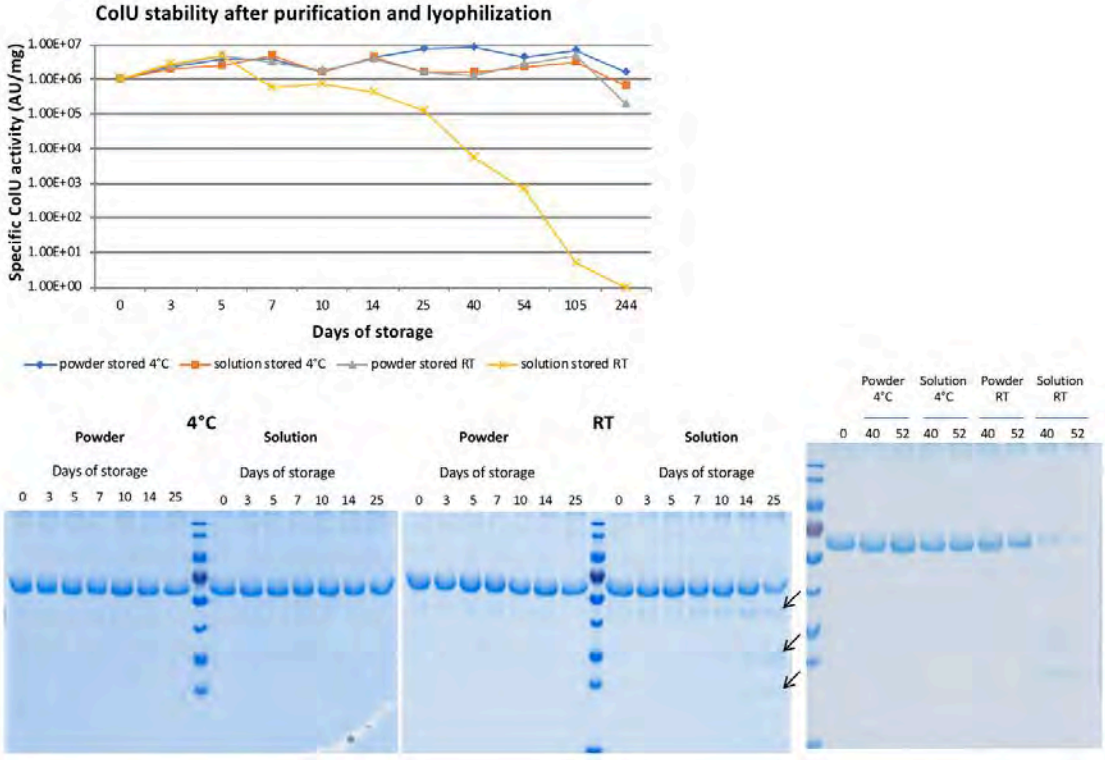
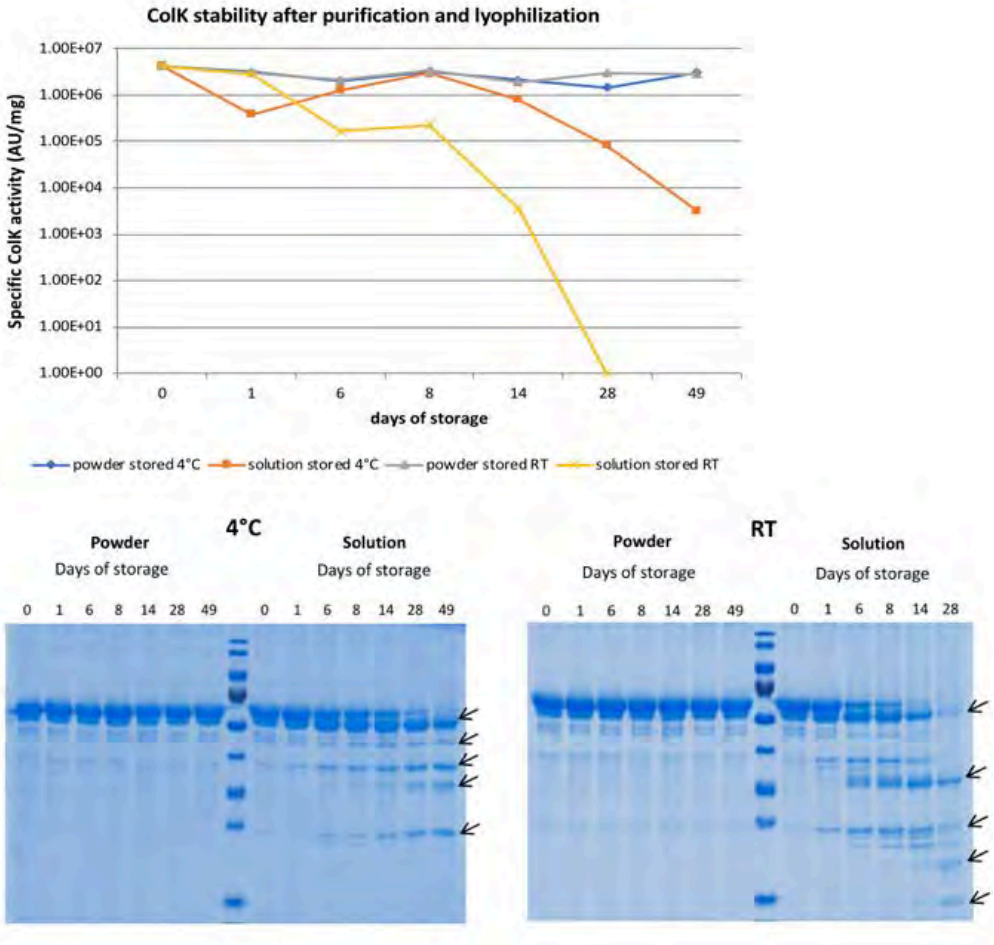


Figure C-6. Stability of *N. benthamiana*-produced Colicin K



The specific activity plots on the top portions of these figures show that for these four sample colicins antibacterial activity is maintained if dry powders are stored at 4°C or at RT. While the stability program is on-going, based on results to date the extrapolated shelf-life of ColM, ColIb, ColU and ColK exceed **6 months** in storage. Dry powder storage is the preferred preservation modality for these proteins.

Solutions prepared from dry colicin proteins and stored at 4°C were also stable for several months, with the possible exception of Colicin K, which showed a significant loss of activity after 2 months. Solutions stored at room temperature have an even shorter shelf life and should be used within 14 days of preparation.

Several new bands appear in SDS-PAGE gels with prolonged storage of solutions kept at RT (arrows in figures). For Colicin M, low-temperature storage showed no visible difference in appearance on the gel over time and this observation was consistent with measured specific activity. At RT storage of ColM solution, 1 degradation fragment was visible at 7 days of storage, which increased until the 40-day sampling point. However, there was no loss of activity observed beyond that time, suggesting that the fragment too may have antibacterial activity.

There was some loss of activity of ColIb solution stored at RT and this loss corresponded with visible new fragments on the gel. ColIb is otherwise highly stable dry or in solution at low temperature.

Loss of activity of ColU solution stored at RT coincided with appearance of degradation fragments. ColU is stable as dry powder at low temperature or RT storage, or in solution if stored at low temperature. There was a slight decrease if ColU stored at RT as powder after 244 days (>8 months).

Colicin K solution was the least stable at RT and showed instability even at 4°C. Many degradation bands can be seen in gels over time, which may be related to storage temperature. Dry ColK was very stable at RT and in cold storage.

The other candidate colicins that could be formulated into a blended COLICIN product have been placed in a stability program, as have blends of colicins to determine if mixed-protein storage impacts stability and activity. These results will be reported once they become available.

The final specification for a blended COLICIN product will assure **stability of ≥6 months prior to release**.

End of Notice