ORIGINAL SUBMISSION

Since 1973
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Center for Regulatory Services, Inc.

5200 Wolf Run Shoals Road
Woodbridge, VA 22192-5755
703 590 7337 (Fax 703 580 8637)
CFR@cfr-services.com

July 24, 2015

Mr. Richard E. Bonnette
Division of Biotechnology and GRAS Notification Review, HFS-255
U.S. FDA, Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740

Dear Mr. Bonnette:

SUBJECT: Transmittal of the NOMAD BIOSCIENCE GmbH - GRAS Notification for Colicin Antimicrobial Product

Enclosed you will find the GRAS notification for colicin antimicrobial product as submitted by NOMAD BIOSCIENCE GmbH.

This is the revised submission to replace that notification made on June 19, 2015. Based on our telephone conversation of July 16, 2015, the only outstanding issue was the request to consider some of the manufacturing information confidential, as such, since the request for confidentiality has been removed, the GRN will be filed promptly.

Should you have any questions on this notification, please contact me directly, so that I can quickly remedy any issues. We have appreciated working your careful review.

Sincerely,

(b) (6)

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

KOS:gbt
KOS-Nomad-07-24-15

Attachments:
FDA Form 3667 (Hard Copy and CD-Copy)
COLICIN GRN COMPLETE Notification (Hard Copy and CD-Copy)
All References (CD-Copy)

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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)*: 2014/12/11

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 Yuri Gleba, Ph.D.	Position Chief Executive Officer		
	Company *(if applicable)* Nomad Bioscience GmbH			
	Mailing Address *(number and street)* Biozentrum Halle, Weinbergweg 22			
City Halle/Saale	State or Province	Zip Code/Postal Code D-06120	Country Germany	
Telephone Number 49 345 555 9887	Fax Number 49 345 1314 2601	E-Mail Address gleba@nomadbioscience.com		
1b. Agent or Attorney *(if applicable)*	Name of Contact Person Kristi O. Smedley, Ph.D.	Position Sponsor's US Regulatory Representative		
	Company *(if applicable)* Center for Regulatory Services, Inc.			
	Mailing Address *(number and street)* 5200 Wolf Run Shoals Rd.			
City Woodbridge	State or Province Virginia	Zip Code/Postal Code 22192	Country United States of America	
Telephone Number 703-590-7337	Fax Number 703-580-8637	E-Mail Address smedley@cfr-services.com		

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance
COLICIN

2. Submission Format: *(Check appropriate box(es))*
☐ Electronic Submission Gateway
☐ Paper
☒ Electronic files on physical media with paper signature page
If applicable give number and type of physical media
One (1) paper file copy plus (3) CDs containing electronic files of Notification

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 _____
☐ b) GRAS Affirmation Petition No. GRP _____
☐ c) Food Additive Petition No. FAP _____
☐ d) Food Master File No. FMF _____
☐ e) Other or Additional *(describe or enter information as above)* _____

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

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

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*
☐ Yes, see attached Designation of Confidential Information
☐ Yes, information is designated at the place where it occurs in the submission
☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*
☐ Yes, a redacted copy of the complete submission
☐ Yes, a redacted copy of part(s) of the submission
☐ No

PART IV – INTENDED USE

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

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 intended to be used as a spray, solution, dip or package additive to control *E. coli* on fresh or processed vegetables and fruits, including ready to eat produce, at an application rate of 1-10 mg COLICIN (total colicin protein) per kg of vegetable or fruit product (approximately 0.5-5 mg/lb).

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 E1 Registry No = GenBank entry nos. Colicin E7 Colicin Ia		AAA87379.1 CAA45164.1 ADW79574.1	Plant, recombinant Plant, recombinant Plant, recombinant	
2	Colicin M Colicin N Colicin K		AAA23589.1 CAA68592.1 AAB41288.1	Plant, recombinant Plant, recombinant Plant, recombinant	
3	Colicin U Colicin 5 Colicin B		CAA72509.1 CAA61102.1 AAA98063	Plant, recombinant Plant, recombinant Plant, recombinant	

[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 Notification documents provided.

3. Synonyms

Provide as available or relevant:

1	
2	
3	

Add Continuation Page

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 on 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.	07/24/2015

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 as an Antimicrobial in Food Processing (PDF of Notification)	
	COLICIN GRN References - For FDA Internal Review Only (PDFs of All Cited References - Not for Republication)	

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

24 July 2015

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

Re: **GRAS Notification for COLICIN as an Antimicrobial in Food Processing**

Dear Dr. Mattia,

Nomad Bioscience GmbH ("NOMAD") is submitting printed copies and a CD containing duplicate electronic files of this GRAS Notification for its **COLICIN antimicrobial** product. NOMAD has determined that the naturally occurring proteins comprising COLICIN are Generally Recognized as Safe (GRAS) for use as an antimicrobial treatment to reduce the levels of *E. coli* bacteria (bactericidal). The product is intended for use on raw and processed produce, including vegetables and fruits, and can be delivered via spray, wash, dip or as a package additive.

NOMAD has determined that COLICIN is GRAS based on scientific procedures as described in the following sections of this notification. COLICIN and its proposed uses are therefore exempt from pre-market approval requirements as specified in Section 201(s) of the Federal Food, Drug, and Cosmetic Act (U.S. FDA, 2010a).

The findings supporting COLICIN as GRAS include:

Safety

- Colicins are naturally occurring antibacterial proteins produced endogenously by commensal enteric bacteria in the human gut;
- There is an extensive documented history of colicin exposure in humans and other animals;
- Colicin exposure occurs naturally from commensal bacteria as well as from food;
- There is no documented etiologic relationship between colicins and disease;
- Colicins are notoriously unstable to heat, acid and proteolytic digestion;
- There are no reports of colicins being allergenic or posing a hypersensitivity risk;
- The manufacturing process described herein utilizes food plants to produce recombinant colicins;
- The compositions of the plant-made recombinant colicins match those of the bacterial colicins; and
- The recombinant plant-made colicins are not glycosylated, just as the native bacterial colicins.

Suitability

- The target specificities of plant-made colicins match those reported for the bacterial colicins;
- The specific activities (potency) of plant-made colicins are in the range of those reported for bacterial colicins;
- Colicins (plant-made as well as native bacterial) exhibit complementary and synergistic activities and can be used singly or as mixtures depending on the intended use and target pathogen(s);
- Notifier's product, COLICIN, is comprised of single colicins or mixtures thereof;
- COLICIN can be formulated at different purities depending on the intended end use;
- COLICIN is active against target food pathogens at use rates not to exceed 10 mg COLICIN/kg food;
- COLICIN is bactericidal on fruits and vegetables, and can be used to treat bulk raw produce (wash), processed/cut produce (wash, dip or spray), or included as a package additive in ready-to-eat fruits and vegetables.

Our Notification includes the following documents.

Hard copies

FDA Form 3667 Nomad Bioscience COLICIN GRN
COLICIN GRN Nomad Bioscience

Electronic files provided in enclosed CD

FDA Form 3667 Nomad Bioscience COLICIN GRN (PDF)
COLICIN GRN Nomad Bioscience (PDF)
Full set of literature references cited (References folder containing PDFs)

For the Agency's convenience, the electronic version of this Notification has been fully bookmarked and hyperlinked for on-screen review.

If the Agency has any questions or requires additional information to aid its review of NOMAD's conclusions, please contact us at the address listed above. For convenience, you may also contact our regulatory representative in the USA, Dr. Kristi Smedley at Center for Regulatory Services Inc., Woodbridge, VA (Tel 703-590-7337; Email smedley@cfr-services.com).

Sincerely,

(b) (6)

Yuri Gleba, Ph.D.
Chief Executive Officer

Table of Contents

1 General Introduction and Claim of Exemption from Premarket Approval Requirements

COLICIN is comprised of one or more naturally occurring proteins of the Colicin family that are produced in the gastrointestinal tract of humans and other animals and in other natural environments by strains of the commensal (indigenous) bacterium *Escherichia coli*. Notifier's COLICIN product is produced recombinantly using a plant-based manufacturing process to match the amino acid composition of the naturally occurring proteins. COLICIN has been determined to be generally recognized as safe, and therefore exempt from the requirement of premarket approval under proposed 21 CFR 170.36(a)(I), under the conditions of intended use as described herein. The basis of the finding is presented in the following sections.

1.1 Name and Address of Notifier

NOMAD BIOSCIENCE GmbH
Biozentrum Halle
Weinbergweg 22
D-06120 Halle/Saale
Germany
Tel. 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
Tel. 703-590-7337
Cell. 703-786-7674
Fax. 703-580-8637
eMail. smedley@cfr-services.com

1.2 Common or Usual Name of the Notified Substance

COLICIN

1.3 Background on Notified Substance

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 by colicinogenic strains of the commensal (indigenous) bacterium *Escherichia coli* (*E. coli*), and act against non-colicin-producing strains of *E. coli*, including human enteropathogenic strains (EHEC/STEC/ETEC). These food-borne pathogens are responsible for a significant number of annual disease outbreaks and consequently numerous food safety recalls globally.

All colicins provide the producing strain with a survival (competitive) advantage in the complex environment of the human gut, and hence these proteins have been conserved throughout evolution. Colicins may naturally protect the host (i.e. humans) from microbial pathogenesis by controlling the growth of ingested pathogenic strains of coliform bacteria. Their safety and activity profiles make colicins prime candidates for development as novel antibacterials to control food-borne pathogens.

Many variations of colicins exist and exhibit different and complementary mechanisms of antibacterial action. Published studies have verified that a common feature exhibited by various natural colicins is their high safety profile. Studies have also shown that colicin variants exhibit target strain specificities; these findings have been verified by Notifier. Further, Notifier's research has found that colicins can be used singly or as mixtures to confer the desired spectrum of antimicrobial activity against pathogenic strains of *E. coli*. Using colicins individually or as mixtures mimics their synthesis and activity in the gut's natural environment.

The product COLICIN can be formulated to contain one or a mixture of two or more individual colicin proteins, depending on the breadth of application needed in various food products. Because individual colicins have been proven safe by scientific methods, and humans are naturally exposed to mixtures of colicins endogenously, the use of colicins individually or as mixtures should not alter the molecule's or the mixture's safety profile. The record of extensive human exposure to colicins without an identified causal relationship to pathology underscores the high safety profile of these antimicrobial proteins.

1.4 Conditions of Use

COLICIN is intended to prevent or minimize contamination of food products by pathogenic strains of *E. coli*. Specifically, the intended use of COLICIN is as a spray, solution, dip or package additive to control enteropathogenic strains of *Escherichia coli* on fresh or processed vegetables and fruits, at an application rate of 1-10 mg COLICIN per kg of vegetable or fruit product (approximately 0.5-5 mg/lb). This notification covers two grades of COLICIN, a purer **COLICIN ISOLATE**, and a cruder **COLICIN Concentrate**, which were developed in parallel to offer cost-effective suitability in various intended applications, for example:

COLICIN ISOLATE (higher purity): Package additive for processed fruits or vegetables

COLICIN CONCENTRATE (lower purity): Spray, dip or wash of bulk fresh produce

1.5 Basis for Notifier's GRAS Determination

COLICIN has not been used in food before. COLICIN has been determined to be GRAS by scientific procedures as defined by 21 CFR 170.30(b). A comprehensive review of the literature, in conjunction with Notifier's own data generated to verify published results, support our assertion of safety and suitability for the intended end use. The basis of the safety determination can be found in APPENDIX A.

1.6 Availability of Information for FDA Review

The data and all information that serve as a basis for the GRAS determination are available for the Food and Drug Administration's review from Notifier's US regulatory representative at the following address:

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192
Tel. 703-590-7337
Cell. 703-786-7674
Fax. 703-580-8637

2 Detailed Information about the Identity of the Substance

2.1 Identity

COLICIN is a recombinant version of one or more of the naturally occurring proteins belonging to the colicin family of bacteriocins. The bacteriocins superfamily of non-antibiotic antimicrobial proteins and peptides includes the commercial product nisin, although nisin and colicins are in different classes. A colicin protein may act individually or in concert with other colicin-family proteins, and can be used singly or in combination as an antimicrobial product to maintain food safety.

Table 2-1 lists the components of COLICIN product that may be used singly or in combination to achieve the desired antibacterial suitability.

Table 2-1. Active components of COLICIN product formulation

Colicin	GenBank No.	Mode of Action	Receptor / Translocator	Targets
E1	AAA87379.1	Pore-forming	BtuB / TolC, TolAQ	ETEC/EHEC/STEC
E7	CAA45164.1	DNase	BtuB / OmpF, TolABQR	ETEC/EHEC/STEC
Ia	ADW79574.1	Pore-forming	Cir / TonB-ExbBD, Cir	ETEC/EHEC/STEC
M	AAA23589.1	Peptidoglycanase	FhuA / TonB-ExbBD	ETEC/EHEC/STEC
N	CAA68592.1	Pore-forming	OmpF / TolAQR	ETEC/EHEC/STEC
K	AAB41288.1	Pore-forming	Tsx / OmpAF, TolABQR	ETEC/EHEC/STEC
U	CAA72509.1	Pore-forming	OmpA / OmpF, TolABQR	ETEC/EHEC/STEC
5	CAA61102.1	Pore-forming	Tsx / TolC, TonB-ExbBD	ETEC/EHEC/STEC
B	AAA98063	Pore-forming	FepA / TonB-ExbBD	ETEC/EHEC/STEC

Universally, all colicins are produced naturally in the human gut by commensal (indigenous) strains of *E. coli* or closely related species and demonstrate complementary modes of action (Table A-1; Yang 2014). Of the various colicins studied for these applications, **colicin E1**, **colicin E7**, **colicin Ia, colicin M, colicin N, colicin K**, **colicin U**, **colicin 5** and **colicin B** are particularly effective for food protection against pathogenic strains of *E. coli*. Importantly, all colicins studied share a high safety profile.

2.2 Mode of Action

Colicins' mode 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. For example, colicin U, colicin K and colicin 5 are pore formers, while colicin M and colicin E7 enzymatically attack cellular components. The identity and biology of colicins have been extensively reviewed by Cascales (Cascales 2007) and others. *In vivo*, the mode of action of colicins is complex. Their activity is regulated by a multi-partite system of receptors, transporters and translocators, and in some cases even immunity proteins because the colicin-producing strains are not necessarily impervious to the effects of their own antimicrobials (Cascales 2007). These features ensure specificity as well as safety.

The COLICIN product consists of isolated colicin proteins (i.e. no bacteria or accessory molecules), and may consist of single colicins or mixtures to take advantage of complementary modes of pathogen attack. For example, colicin M acts by enzymatic degradation of target cell macromolecules; specifically, the protein interferes with development and integrity of the bacterial cell wall component peptidoglycan (Yang 2014). Colicin M enzymatically degrades lipid I and lipid II peptidoglycan (murein) intermediates. The cleavage occurs between the lipid moiety and the pyrophosphoryl groups. This then leads to the arrest of peptidoglycan polymerisation (Harkness 1989; El Ghachi 2006) rather than the direct degradation of the peptidoglycan (Schaller 1981). The result is a rapid weakening of the bacterial cell wall leading to hydrostatic instability and cell death.

Colicin E7 also enzymatically degrades target cell macromolecules, in this case DNA (Chak 1991; Cursino 2002). The DNase catalytically destroys the target's ability to reproduce and encode new protein, leading to a rapid halting of cell division and eventual cell death.

Although differing in molecular structure, colicin E1, colicin U, colicin K and colicin 5 are pore formers. These and other pore-forming proteins share the same mode of action, namely, they embed themselves into the target cell's lipid bilayer and produce physical pores, destroying hydrostatic stability and causing ion leakage that leads to membrane depolarization and cell death (Smajs 1997; Pilsl 1995b, c).

Colicin mixtures show additive potency because they attack the pathogen via different targets. Because Notifier's recombinant colicins are identical to the naturally occurring proteins, their mode of action and specificity against enteropathogenic strains of *E. coli* match those reported for the native proteins.

2.3 Structural Formula of COLICIN components

Gene and amino acid sequence information of colicins comprising COLICIN product

Nine (9) recombinant colicins are included as components of the COLICIN product, to be used either singly or in combination. Structural information is provided for **colicin E1**, **colicin E7, colicin Ia, colicin M, colicin N, colicin K, colicin U, colicin 5** and **colicin B**. Notifier's COLICIN component proteins are produced recombinantly using a plant-based manufacturing process. Because there are differences in the way plant, microbial and mammalian cells express genes, the codon sequence at the gene level can be optimized for stable, high-yield expression in plants. In some cases protein maturation among hosts can result in slight differences (e.g. 1 or 2 amino acids) in the final protein post translation, such as in retention or deletion of an N-terminal methionine. Such maturation events are well known, are found in approved pharmaceuticals produced in heterologous hosts, and are not impactful on protein safety, as reviewed by Kamionka (2011). Comparisons of maturation events between plant and bacterial colicins (when information for the latter was available) revealed that the mature plant and bacterial proteins are equivalent (see Table C-1).

Importantly, the full-length amino acid consensus sequences responsible for the structure, activity and safety of the colicin proteins have been retained in every case. Therefore, the present proteins produced recombinantly in plants share the amino acid sequences of naturally occurring bacterial colicins, as reported in the literature and as verified from published gene and protein accession databases.

Lack of glycosylation

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

Structural information

Colicin E1. Colicin E1 is a recombinant version of the naturally occurring *E. coli*-produced colicin E1 protein (CAS 11032-88-5; GenBank AAA87379.1 deposited Feb 8, 1996; UniProt Entry P02978, last modified Jul 21, 1996). The protein is a pore-former that targets the BtuB receptor in susceptible cells, resulting in bacteriocidal effects (Yamada 1982). Structure-activity information is provided in Section A.3.1. Notifier's plant-produced colicin E1 has the identical amino acid sequence as naturally occurring colicin E1, as reported in the literature (Yamada 1982; see below). The bacterially produced protein has a MW of 57.280 kDa and consists of 522 amino acids, with the following amino acid sequence as filed with the GenBank database (CAA61102.1; Yamada 1982, submitted Feb 8, 1996; UniProt P02978, last modified Jul 21, 1986). Notifier's recombinant version has the same amino acid composition as the bacterial protein.

```
  1  METAVAYYKD GVPYDDKGQV IITLLNGTPD GSGSGGGGGK GGSKSESSAA IHATAKWSTA QLKKTQAEQA
 71  ARAKAAAEAQ AKAKANRDAL TQRLKDIVNE ALRHNASRTP SATELAHANN AAMQAEDERL RLAKAEEKAR
141  KEAEAAEKAF QEAEQRRKEI EREKAETERQ LKLAEAEEKR LAALSEEAKA VEIAQKKLSA AQSEVVKMDG
211  EIKTLNSRLS SSIHARDAEM KTLAGKRNEL AQASAKYKEL DELVKKLSPR ANDPLQNRPF FEATRRRVGA
281  GKIREEKQKQ VTASETRINR INADITQIQK AISQVSNNRN AGIARVHEAE ENLKKAQNNL LNSQIKDAVD
351  ATVSFYQTLT EKYGEKYSKM AQELADKSKG KKIGNVNEAL AAFEKYKDVL NKKFSKADRD AIFNALASVK
421  YDDWAKHLDQ FAKYLKITGH VSFGYDVVSD ILKIKDTGDW KPLFLTLEKK AADAGVSYVV ALLFSLLAGT
491  TLGIWGIAIV TGILCSYIDK NKLNTINEVL GI
```

Using current standard software to calculate atomic mass and key characteristics from the protein's amino acid sequence (e.g. ProtParam analytical tools on the ExPASy Server, Gasteiger 2005), a comparison of the native bacterial and recombinant plant-produced colicin E1 yields the following information.

Property of colicin E1	**Native *E. coli***	**Plant-produced**
Molecular Weight:	57.280 kDa	57.280 kDa
Isoelectric Point:	9.31	9.31
Extinction Coefficient:	41370 M^{-1} cm^{-1}	41370 M^{-1} cm^{-1}
Absorbance (0.1% sol)	0.722	0.722

Notifier confirms the composition of plant-produced colicins at the gene level by DNA sequencing and at the protein level by peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF) using MALDI-MS with bioinformatic fragment alignment. The identity of the recombinant protein is thus verified.

Colicin E7. Colicin E7 is a recombinant version of the naturally occurring *E. coli*-produced colicin E7 protein (GenBank CAA45164.1, deposit date 2000-04-26; UniProt Q47112). The protein is a DNase that targets the BtuB receptor in susceptible cells, resulting in bacteriocidal effects (Chak 1991; Cursino 2002). Structure-activity information is provided in Section A.3.1. Notifier's plant-produced colicin E7 has the identical amino acid sequence as naturally occurring colicin E7, as reported in the literature (Chak 1991; see below). The bacterially produced protein has a MW of 61.350 kDa and consists of 576 amino acids, with the following amino acid sequence as filed with the GenBank database (AAA23072.1; Chak 1991). Notifier's recombinant version has the same amino acid composition as the bacterial protein.

```
  1  MSGGDGRGHN SGAHNTGGNI NGGPTGLGGN GGASDGSGWS SENNPWGGGS GSGVHWGGGS GHGNGGGNSN
 71  SGGGSNSSVA APMAFGFPAL AAPGAGTLGI SVSGEALSAA IADIFAALKG PFKFSAWGIA LYGILPSEIA
141  KDDPNMMSKI VTSLPAETVT NVQVSTLPLD QATVSVTKRV TDVVKDTRQH IAVVAGVPMS VPVVNAKPTR
211  TPGVFHASFP GVPSLTVSTV KGLPVSTTLP RGITEDKGRT AVPAGFTFGG GSHEAVIRFP KESGQKPVYV
281  SVTDVLTPAQ VKQRQDEEKR LQQEWNDAHP VEVAERNYEQ ARAELNQANK DVARNQERQA KAVQVYNSRK
351  SELDAANKTL ADAKAEIKQF ERFAREPMAA GHRMWQMAGL KAQRAQTDVN NKKAAFDAAA KEKSDADVAL
421  SSALERRKQK ENKEKDAKAK LDKESKRNKP GKATGKGKPV NNKWLNNAGK DLGSPVPDRI ANKLRDKEFK
491  SFDDFRKKFW EEVSKDPELS KQFSRNNNDR MKVGKAPKTR TQDVSGKRTS FELHHEKPIS QNGGVYDMDN
561  ISVVTPKRHI DIHRGK
```

Using current standard software to calculate atomic mass and key characteristics from the protein's amino acid sequence (e.g. ProtParam analytical tools on the ExPASy Server, Gasteiger 2005), a comparison of the native bacterial and recombinant plant-produced colicin E7 yields the following information.

Property of colicin E7	**Native *E. coli***	**Plant-produced**
Molecular Weight:	61.350 kDa	61.350 kDa
Isoelectric Point:	9.69	9.69
Extinction Coefficient:	51450 M^{-1} cm^{-1}	51450 M^{-1} cm^{-1}
Absorbance (0.1% sol)	0.839	0.839

Notifier confirms the composition of plant-produced colicins at the gene level by DNA sequencing and at the protein level by peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF) using MALDI-MS with bioinformatic fragment alignment. The identity of the recombinant protein is thus verified.

Colicin Ia. Colicin Ia is a recombinant version of the naturally occurring *E. coli*-produced colicin Ia protein (CAS 37217-77-9; GenBank ADW79574.1 deposited Feb 27, 2015; UniProt Entry E9LLU1, last modified May 27, 2015). The protein is a pore-former that targets the Cir receptor in susceptible cells, resulting in bactericidal effects (Tokuda 1979; Jakes 2010). Structure-activity information is provided in Section A.3.1. Notifier's plant-produced colicin Ia has the identical amino acid sequence as naturally occurring colicin Ia, as reported in the literature (Johnson 2011; see below).

The bacterially produced protein has a MW of 69.427 kDa and consists of 626 amino acids, with the following amino acid sequence as filed with the GenBank database (ADW79574.1; submitted Feb 27, 2015; UniProt E9LLU1, last modified May 27, 2015). Notifier's recombinant version has the same amino acid composition as the bacterial protein.

```
  1  MSDPVRITNP GAESLGYDSD GHEIMAVDIY VNPPRVDVFH GTPPAWSSFG NKTIWGGNEW VDDSPTRSDI
 71  EKRDKEITAY KNTLSAQQKE NENKRTEAGK RLSAAIAARE KDENTLKTLR AGNADAADIT RQEFRLLQAE
141  LREYGFRTEI AGYDALRLHT ESRMLFADAD SLRISPREAR SLIEQAEKRQ KDAQNADKKA ADMLAEYERR
211  KGILDTRLSE LEKNGGAALA VLDAQQARLL GQQTRNDRAI SEARNKLSSV TESLNTARNA LTRAEQQLTQ
281  QKNTPDGKTI VSPEKFPGRS STNHSIVVSG DPRFAGTIKI TTSAVIDNRA NLNYLLTHSG LDYKRNILND
351  RNPVVTEDVE GDKKIYNAEV AEWDKLRQRL LDARNKITSA ESAVNSARNN LSARTNEQKH ANDALNALLK
421  EKENIRNQLA GINQKIAEEK RKQDELKATK DAINFTTEFL KSVSEKYGAK AEQLAREMAG QAKGKKIRNV
491  EEALKTYEKY RADINKKINA KDRAAIAAAL ESVKLSDISS NLNRFSRGLG YAGKFTSLAD WITEFGKAVR
561  TENWRPLFVK TETIIAGNAA TALVALVFSI LTGSALGIIG YGLLMAVTGA LIDESLVEKA NKFWGI
```

Using current standard software to calculate atomic mass and key characteristics from the protein's amino acid sequence (e.g. ProtParam analytical tools on the ExPASy Server, Gasteiger 2005), a comparison of the native bacterial and recombinant plant-produced colicin Ia yields the following information.

Property of colicin Ia	**Native *E. coli***	**Plant-produced**
Molecular Weight:	69.427 kDa	69.427 kDa
Isoelectric Point:	9.15	9.15
Extinction Coefficient:	59360 M^{-1} cm^{-1}	59360 M^{-1} cm^{-1}
Absorbance (0.1% sol)	0.855	0.855

Notifier confirms the composition of plant-produced colicins at the gene level by DNA sequencing and at the protein level by peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF) using MALDI-MS with bioinformatic fragment alignment. The identity of the recombinant protein is thus verified.

Colicin M. Colicin M is a recombinant version of the naturally occurring *E. coli*-produced colicin M protein (CAS 39386-24-8; Swiss-Prot Entry PO5820; SID 135305941, deposit date 2012-03-21). The protein is a peptidoglycanase that targets the FhuA receptor in susceptible cells, resulting in bacteriocidal and bacteriostatic effects (Köck 1987). Structure-activity information is provided in Section A.3.1. Notifier's plant-produced colicin M has the identical amino acid sequence as naturally occurring colicin M, as reported in the literature (Köck 1987; see below).

In the 1980s the bacterially produced protein was originally described as having a MW of 29.45 kDa and consisting of 271 amino acids, with the following amino acid sequence as filed with the GenBank database (AAA23589.1; Köck 1987). Notifier's recombinant version has the same amino acid composition as the bacterial protein.

```
  1  METLTVHAPS PSTNLPSYGN GAFSLSAPHV PGAGPLLVQV VYSFFQSPNM CLQALTQLED YIKKHGASNP
 71  LTLQIISTNI GYFCNADRNL VLHPGISVYD AYHFAKPAPS QYDYRSMNMK QMSGNVTTPI VALAHYLWGN
141  GAERSVNIAN IGLKISPMKI NQIKDIIKSG VVGTFPVSTK FTHATGDYNV ITGAYLGNIT LKTEGTLTIS
211  ANGSWTYNGV VRSYDDKYDF NASTHRGIIG ESLTRLGAMF SGKEYQILLP GEIHIKESGK R
```

Using current standard software to calculate atomic mass and key characteristics from the protein's amino acid sequence (e.g. ProtParam analytical tools on the ExPASy Server, Gasteiger 2005), a comparison of the native bacterial and recombinant plant-produced colicin M yields the following information.

Property of colicin M	**Native *E. coli***	**Plant-produced**
Molecular Weight:	29.48 kDa	29.48 kDa
Isoelectric Point:	8.9	8.9
Extinction Coefficient:	33475 M^{-1} cm^{-1}	33475 M^{-1} cm^{-1}
Absorbance (0.1% sol)	1.135	1.135

Notifier confirms the composition of plant-produced colicins at the gene level by DNA sequencing and at the protein level by peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF) using MALDI-MS with bioinformatic fragment alignment. The identity of the recombinant protein is thus verified.

Colicin N. Colicin N is a recombinant version of the naturally occurring *E. coli*-produced colicin N protein (GenBank CAA68592.1; UniProt Entry P08083, modified Aug 1 1998). The protein is a pore-former that targets the OmpF receptor in susceptible cells, resulting in bacteriocidal effects (Pugsley 1987; Vetter 1998). Structure-activity information is provided in Section A.3.1. Notifier's plant-produced colicin N has the identical amino acid sequence as naturally occurring colicin N, as reported in the literature (Pugsley 1987; see below).

The bacterially produced protein has a MW of 41.743 kDa and consists of 387 amino acids, with the following amino acid sequence as filed with the GenBank database (CAA68592.1; Pugsley 1987, submitted Sep 12, 1993). Notifier's recombinant version has the same amino acid composition as the bacterial protein.

```
  1  MGSNGADNAH NNAFGGGKNP GIGNTSGAGS NGSASSNRGN SNGWSWSNKP HKNDGFHSDG SYHITFHGDN
 71  NSKPKPGGNS GNRGNNGDGA SAKVGEITIT PDNSKPGRYI SSNPEYSLLA KLIDAESIKG TEVYTFHTRK
141  GQYVKVTVPD SNIDKMRVDY VNWKGPKYNN KLVKRFVSQF LLFRKEEKEK NEKEALLKAS ELVSGMGDKL
211  GEYLGVKYKN VAKEVANDIK NFHGRNIRSY NEAMASLNKV LANPKMKVNK SDKDAIVNAW KQVNAKDMAN
281  KIGNLGKAFK VADLAIKVEK IREKSIEGYN TGNWGPLLLE VESWIIGGVV AGVAISLFGA VLSFLPISGL
351  AVTALGVIGI MTISYLSSFI DANRVSNINN IISSVIR
```

Using current standard software to calculate atomic mass and key characteristics from the protein's amino acid sequence (e.g. ProtParam analytical tools on the ExPASy Server, Gasteiger 2005), a comparison of the native bacterial and recombinant plant-produced colicin N yields the following information.

Property of colicin N	**Native *E. coli***	**Plant-produced**
Molecular Weight:	41.743 kDa	41.743 kDa
Isoelectric Point:	9.62	9.62
Extinction Coefficient:	50880 M^{-1} cm^{-1}	50880 M^{-1} cm^{-1}
Absorbance (0.1% sol)	1.219	1.219

Notifier confirms the composition of plant-produced colicins at the gene level by DNA sequencing and at the protein level by peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF) using MALDI-MS with bioinformatic fragment alignment. The identity of the recombinant protein is thus verified.

Colicin K. Colicin K is a recombinant version of the naturally occurring *E. coli*-produced colicin K protein (CAS 1403-40-3; GenBank AAB41288.1, deposit date May 22, 1995; UniProt Entry Q47502, revised Nov 1 1996). The protein is a pore-former that targets the Tsk receptor in susceptible cells, resulting in bacteriocidal effects (Pilsl 1995b, c). Structure-activity information is provided in Section A.3.1. Notifier's plant-produced colicin K has the identical amino acid sequence as naturally occurring colicin K, as reported in the literature (Pilsl 1995c; Izard 1995; see below).

The bacterially produced protein has a MW of 59.661 kDa and consists of 548 amino acids, with the following amino acid sequence as filed with the GenBank database (AAB41288.1; submitted 22 May 1995, Izard 1995). Notifier's recombinant version has the same amino acid composition as the bacterial protein.

```
  1  MAKELSGYGP TAGESMGGTG ANLNQQGGNN NSNSGVHWGG GSGHGNNGGQ GNSNSSGSTS TVMKTGESYL
 71  TPWGDVVINN DGLPVMNGIV MTEENSTLVD NPFGGVSRVL NSLISDMPSL FAESSGNNNN NTASVNTAPT
141  NAQVSDMDKS SKVVSNVINE KQKQKNKIAT QISEKQKKIE EMKKVFKHHS YHGITDLERD VDELQKKSNQ
211  LDADISKLNS YKNTLQSKIG DVNKQKEAEE KARENAEVAE HETLNEEKQA VAEAEKRLAE AKAELAKAES
281  DVQSKQATVS RVAGELENAQ KSVDVKVTGF PGWRDVQKKL QRQLEAKQAE YSAVENELKN AVSFRDGKAA
351  EVKEAEQKLK EAQDALEKSQ IKDAVDTMVG FYQYITEQYG EKYAKIAQDL AEKSKGKKIQ GVDEALAAFE
421  KYKNVLDKKF SKVDRDAIFN ALESVNYDEL SKNLTKISKS LKITSRVSFL YDVGSDFKNA IETGNWRPLF
491  VTLEKSAVDV GVAKIVALMF SFIVGVPLGF WGIAIVTGIV SSYIGDDELS KLNELLGI
```

Using current standard software to calculate atomic mass and key characteristics from the protein's amino acid sequence (e.g. ProtParam analytical tools on the ExPASy Server, Gasteiger 2005), a comparison of the native bacterial and recombinant plant-produced colicin K yields the following information.

Property of colicin K	**Native *E. coli***	**Plant-produced**
Molecular Weight:	59.661 kDa	59.661 kDa
Isoelectric Point:	5.53	5.53
Extinction Coefficient:	46870 M^{-1} cm^{-1}	46870 M^{-1} cm^{-1}
Absorbance (0.1% sol)	0.786	0.786

Notifier confirms the composition of plant-produced colicins at the gene level by DNA sequencing and at the protein level by peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF) using MALDI-MS with bioinformatic fragment alignment. The identity of the recombinant protein is thus verified.

Colicin U. Colicin U is a recombinant version of the naturally occurring bacterial colicin U protein natively produced by *Shigella boydii* (GenBank CAA72509.1; UniProt Entry O24681, Jan 1 1998). The protein is a pore-former that targets the OmpA receptor in susceptible cells, resulting in bacteriocidal effects (Smajs 1997). Structure-activity information is provided in Section A.3.1. Notifier's plant-produced colicin U has the identical amino acid sequence as naturally occurring colicin U, as reported in the literature (Smajs 1997; see below).

The bacterially produced protein has a MW of 66.289 kDa and consists of 619 amino acids, with the following amino acid sequence as filed with the GenBank database (CAA72509.1; Smajs 1997, last updated Feb 4, 2015). Notifier's recombinant version has the same amino acid composition as the bacterial protein.

```
  1  MPGFNYGGHG DGTGWSSERG DGPAPGGGMQ GNGGGHSGNN DSGSNSVSQQ ISAIQNDQKL KQKVVNMLIA
 71  ARKMNPDAKM ILGSIAPSGV MQVTIEGVTS TQARQLGLGG LVMGYNASGV IGAVGEIDTG HRLNASGAST
141  PGSETSVDSF VNGQKPAEEW HAVAKDSWTG AGPVNTGLVN NAIKSVRIIK KGYVTGVLTP EEVMNKAEYK
211  AMRQAFDSLP LAKQGEAVRQ IVAAWSLAYQ DFPVNLKKEM GRVTERIVDA INLALILNQT ESRLSESQKN
281  VDVANQIISD TVKAINDVNK KIAEKRNQQV SLTDLMNKKQ KEVEDLKKIF KNHSYHRIRD AQREYDDARN
351  KYALLASDIN ALQAQVSGLT ARKQQAEQNK AAAEKAKADA AAKAAAEKAA AEAKAKAEAE KARKEAEEKA
421  NDEKAVLTKA SEIIISVGDK AGEYLGDKYK VLSREIADNI KNFQGKTIRS YDEAMASVNK LMANPDLKIN
491  AADRDAIVNA WKAFDAEDMG NKFAALGKTF KAADYVMKAN NVREKSIEGY QTGNWGPLML EIESWVLSGI
561  ASAVALSFFS AIFGTFAMLG VFSTSLAGIL AVILAGLVGA LIDDNFVDKL NNEIIRPAY
```

Using current standard software to calculate atomic mass and key characteristics from the protein's amino acid sequence (e.g. ProtParam analytical tools on the ExPASy Server, Gasteiger 2005), a comparison of the native bacterial and recombinant plant-produced colicin U yields the following information.

Property of colicin U	**Native *S. boydii***	**Plant-produced**
Molecular Weight:	66.289 kDa	66.289 kDa
Isoelectric Point:	8.49	8.49
Extinction Coefficient:	59360 M^{-1} cm^{-1}	59360 M^{-1} cm^{-1}
Absorbance (0.1% sol)	0.895	0.895

Notifier confirms the composition of plant-produced colicins at the gene level by DNA sequencing and at the protein level by peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF) using MALDI-MS with bioinformatic fragment alignment. The identity of the recombinant protein is thus verified.

Colicin 5. Colicin 5 is a recombinant version of the naturally occurring *E. coli*-produced colicin 5 protein (GenBank CAA61102.1; UniProt Entry Q47500, Nov 1 1996). The protein is a pore-former that targets the Tsx receptor in susceptible cells, resulting in bactericidal effects (Pilsl 1995a, b, c). Structure-activity information is provided in Section A.3.1. Notifier's plant-produced colicin 5 has the identical amino acid sequence as naturally occurring colicin 5, as reported in the literature (Pilsl 1995a, b, c). The bacterially produced protein has a MW of 53.137 kDa and consists of 490 amino acids, with the following amino acid sequence as filed with the GenBank database (CAA61102.1; Pilsl 1995a, b, c; submitted Jun 7, 1995). Notifier's recombinant version has the same amino acid composition as the bacterial protein.

```
  1  MDKVTDNSPD VESTESTEGS FPTVGVDTGD TITATLATGT ENVGGGGGAF GGASESSAAI HATAKWSTAQ
 71  LKKHQAEQAA RAAAAEAALA KAKSQRDALT QRLKDIVNDA LRANAARSPS VTDLAHANNM AMQAEAERLR
141  LAKAEQKARE EAEAAEKALR EAERQRDEIA RQQAETAHLL AMAEAAEAEK NRQDSLDEEH RAVEVAEKKL
211  AEAKAELAKA ESDVQSKQAI VSRVAGELEN AQKSVDVKVT GFPGWRDVQK KLERQLQDKK NEYSSVTNAL
281  NSAVSIRDAK KTDVQNAEIK LKEAKDALEK SQVKDSVDTM VGFYQYITEQ YGEKYSRIAQ DLAEKAKGSK
351  FSSVDEALAA FEKYKNVLDK KISKVDRDAI FNALESVNYD ELSKNLTKIS KSLKITSRVS FLYDVGSDFK
421  NAIETGNWRP LFVTLEKSAV DVGVAKIVAL MFSFIVGVPL GFWGIAIVTG IVSSYIGDDE LNKLNELLGI
```

Using current standard software to calculate atomic mass and key characteristics from the protein's amino acid sequence (e.g. ProtParam analytical tools on the ExPASy Server, Gasteiger 2005), a comparison of the native bacterial and recombinant plant-produced colicin 5 yields the following information.

Property of colicin 5	**Native *E. coli***	**Plant-produced**
Molecular Weight:	53.137 kDa	53.137 kDa
Isoelectric Point:	5.40	5.40
Extinction Coefficient:	35410 M^{-1} cm^{-1}	35410 M^{-1} cm^{-1}
Absorbance (0.1% sol)	0.666	0.666

Notifier confirms the composition of plant-produced colicins at the gene level by DNA sequencing and at the protein level by peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF) using MALDI-MS with bioinformatic fragment alignment. The identity of the recombinant protein is thus verified.

Colicin B. Colicin B is a recombinant version of the naturally occurring *E. coli*-produced colicin B protein (CAS 11038-10-1; GenBank AAA98063; UniProt Entry Q47500, Nov 1 1996). The protein is a pore-former that targets the FepA receptor in susceptible cells, resulting in bacteriocidal effects (Schramm 1987). Structure-activity information is provided in Section A.3.1. Notifier's plant-produced colicin B has the identical amino acid sequence as naturally occurring colicin B, as reported in the literature (Schramm 1987; see below).

The bacterially produced protein has a MW of 54.86 kDa and consisting of 511 amino acids, with the following amino acid sequence as filed with the GenBank database (AAA98063; Schramm 1987). Notifier's recombinant version has the same amino acid composition as the bacterial protein.

```
  1  MSDNEGSVPT EGIDYGDTMV VWPSTGRIPG GDVKPGGSSG LAPSMPPGWG DYSPQGIALV QSVLFPGIIR
 71  RIILDKELEE GDWSGWSVSV HSPWGNEKVS AARTVLENGL RGGLPEPSRP AAVSFARLEP ASGNEQKIIR
141  LMVTQQLEQV TDIPASQLPA AGNNVPVKYR LTDLMQNGTQ YMAIIGGIPM TVPVVDAVPV PDRSRPGTNI
211  KDVYSAPVSP NLPDLVLSVG QMNTPVRSNP EIQEDGVISE TGNYVEAGYT MSSNNHDVIV RFPEGSGVSP
281  LYISAVEILD SNSLSQRQEA ENNAKDDFRV KKEQENDEKT VLTKTSEVII SVGDKVGEYL GDKYKALSRE
351  IAENINNFQG KTIRSYDDAM SSINKLMANP SLKINATDKE AIVNAWKAFN AEDMGNKFAA LGKTFKAADY
421  AIKANNIREK SIEGYQTGNW GPLMLEVESW VISGMASAVA LSLFSLTLGS ALIAFGLSAT VVGFVGVVIA
491  GAIGAFIDDK FVDELNHKII K
```

Using current standard software to calculate atomic mass and key characteristics from the protein's amino acid sequence (e.g. ProtParam analytical tools on the ExPASy Server, Gasteiger 2005), a comparison of the native bacterial and recombinant plant-produced colicin B yields the following information.

Property of colicin B	**Native *E. coli***	**Plant-produced**
Molecular Weight:	54.86 kDa	54.86 kDa
Isoelectric Point:	4.82	4.82
Extinction Coefficient:	63370 M^{-1} cm^{-1}	63370 M^{-1} cm^{-1}
Absorbance (0.1% sol)	1.155	1.155

Notifier confirms the composition of plant-produced colicins at the gene level by DNA sequencing and at the protein level by peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF) using MALDI-MS with bioinformatic fragment alignment. The identity of the recombinant protein is thus verified.

Confirmation of colicin molecular mass and amino acid sequence by MALDI-MS

The gene sequences for the colicin inserts in the expression vector are verified by DNA sequencing. For verification, the composition of the plant-expressed proteins is analyzed by 2 complementary methods: (1) MALDI-TOF after digestion of the colicin with pepsin, trypsin and chymotrypsin and analysis of resultant peptide fragments, and (2) MALDI-TOF MS/MS amino acid sequence determination by analysis of peptides at the N- and C-termini of the protein. Predicted and actual masses of the sequences analyzed are then compared.

A description of the peptide mass and peptide fragment fingerprinting methods used for protein sequence verification is presented in APPENDIX C, Section C.2 Methods for confirming colicin amino acid sequences by MALDI-MS.

All plant-made colicins conform to their predicted compositions and share the amino acid sequences of the bacterially produced native colicin proteins.

2.4 Quantitative Composition

COLICIN is prepared in bulk as a concentrated solution or as a dry powder at two different purities as described in APPENDIX B. It is dissolved/diluted in water to a concentration of 0.05 mg/mL (50 mg/L) for spray application at a rate not to exceed 20 mL solution/kg (9 mL/lb) of product. Alternatively, fresh vegetables and fruits 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 at a rate not to exceed 10 mg/kg (<4.6 mg/lb).

Colicin proteins can be prepared singly or in combination with other colicins. If mixtures are produced for blending, 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.

2.5 Method of Manufacture

Notifier uses a **food plant-based manufacturing process** for producing COLICIN components. The production method is an adaptation of the process used to manufacture pharmaceutical proteins and vaccines under cGMP guidelines, said proteins having been administered in clinical trials under FDA IND.

The colicin proteins are produced using recombinant technology to yield concentrated extracts at two different purities. Host plants are the food species **spinach** (*Spinacia oleracea*), **red beet** (*Beta vulgaris*) or **lettuce** (*Lactuca sativa*). Expression of a colicin gene in a food host plant is achieved by one of two methods:

1. Agrobacterial vector induction. Plant-viral vectors with the selected colicin gene are introduced into a non-recombinant plant host using *Agrobacterium tumefaciens*-mediated inoculation (see review by Gleba 2014); or

2. Ethanol induction in transgenic plants. Transgenic plant hosts carrying the selected colicin gene linked to an ethanol-inducible promoter are induced to synthesize colicin protein upon foliar exposure to dilute ethanol (Werner 2011).

The *Agrobacterium* vector in the first method carries the gene encoding the colicin protein but is otherwise derived from, and is very similar to, the *Agrobacterium* strains that are found naturally in many vegetable crops. The dilute ethanol used in the second method is metabolized or evaporated prior to protein purification and therefore absent in the final product.

In both processes, induced plants are grown indoors in clean rooms under environmentally controlled conditions. A summary of the colicin protein manufacturing process is presented in APPENDIX B.

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.

2.6 Composition and Specification

2.6.1 Characteristic Properties

Colicins recombinantly produced in plants consist of water-soluble antimicrobial proteins of the same composition as their natural counterparts produced in the human and animal gastrointestinal tract, and in other natural environments, by commensal enteric bacteria. Colicins exert antibacterial effects on target pathogens either by destroying lipid membrane integrity or via enzymatic degradation of macromolecules in the cell wall or nucleic acids (Yang 2014). These effects are highly selective for susceptible strains of bacteria and colicins do not impact humans or animals exposed to these proteins. The characteristic molecular properties of colicins are defined in Section 2.3 of this notification.

Colicins, by definition, are unstable to proteolytic digestion (Murinda 1996; Zhang 1992; Cascales 2007). Colicins are either denatured by heat (cooking) or stomach acid and are degraded rapidly upon exposure to gastric and intestinal enzymes. Using candidate plant-produced colicins, Notifier has confirmed the rapid degradation of colicins in simulated gastric fluid and simulated intestinal fluid (see Section A.2.3 and APPENDIX C).

A property of particular importance to safety is the potential for colicins to induce allergic or hypersensitive reactions. Notifier studied at the molecular level the allergenic potential of candidate colicins for use in food (Section A.2.5) using sequence searches and bioinformatics from public databases of known allergens.

Of the 9 colicins screened representing thousands of amino acids, only three 8-aa sequences correlated with proteins known to be allergenic. However, those sequences were not allergenic epitopes but rather sequences in common with other proteins that possess allergenic epitopes. The native bacterial colicins that are produced in the human gut are not known to be immunogenic or allergenic (no literature reports on the topic). Based on bioinformatics, the plant-produced recombinant versions that match the natural compositions can therefore also be considered non-immunogenic and non-allergenic.

2.6.2 Formulation

COLICIN is provided as a concentrated solution or as a dry powder at two purity levels (cruder **COLICIN Concentrate** and purer **COLICIN Isolate**). The product is dissolved and/or diluted in water according to instructions and applied as a wash, spray, dip or package fill depending on the intended end-use.

2.6.3 Content of Potential Human Toxicants in COLICIN

None.

2.6.4 Specifications

Specifications for two formulations of COLICIN are presented in Table 2-2 (cruder **COLICIN Concentrate**) and Table 2-3 (purer **COLICIN Isolate**).

The process used to manufacture products with these draft specifications is presented in APPENDIX B (Section B.3 and Section B.4).

Table 2-2. Specification of COLICIN Concentrate Product

COLICIN Concentrate		
Parameter	**Specification limit**	**Method**
Appearance	Powder, beige to brownish	Visual
Specific Activity	>10,000 AU/g	Serial-dilution based assay
pH of a 1% solution	6.5-8.5	Potentiometric
Heavy metals (sum of Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, Sn)	≤30 ppm	USP38<233>
Lead	≤5 ppm	USP38<233>
Bioburden	≤5,000 CFU total per g	USP32<61>
Agrobacterium per 10 g sample	0 (absent)	Selective plate-based assay
Undesirable microorganisms, including *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp.* or coagulase-positive *Staphylococcus spp.*, per 25 g	0 (absent)	USP32<1111>
Stability (dry concentrate; 0-10°C)	>6 months	Specific activity by serial dilution-based assay

Table 2-3. Specification of COLICIN Isolate Product

COLICIN Isolate		
Parameter	**Specification limit**	**Method**
Appearance	Powder, white to beige	Visual
Specific Activity	>25,000 AU/g	Serial-dilution based assay
pH of a 1% solution	6.5-8.5	Potentiometric
Heavy metals (sum of Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, Sn)	≤30 ppm	USP38<233>
Lead	≤5 ppm	USP38<233>
Bioburden	≤10 CFU total per 25 g sample	USP32<61>
Agrobacterium per 10 g sample	0 (absent)	Selective plate-based assay
Undesirable microorganisms, including *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp.* or coagulase-positive *Staphylococcus spp.*, per 25 g	0 (absent)	USP32<1111>
Stability (dry concentrate; 0-10°C)	>6 months	Specific activity by serial dilution-based assay

3 Information on Any Self-Limiting Levels of Use

None.

4 Basis for GRAS Determination (Proposed 170.36(c)(4))

The detailed summary of the basis for the GRAS determination is provided in **APPENDIX A**, which includes the suitability summary (link below).

APPENDIX A: Information to Establish the Safety of COLICIN and the Suitability of Use

Manufacturing information is provided in **APPENDIX B** (link below).

APPENDIX B: COLICIN Manufacturing Process

Methodology used to analyze the properties of plant-produced colicins and assess their suitability are summarized in **APPENDIX C** (link below).

APPENDIX C: Methodology

APPENDIX A. Information to Establish the Safety of COLICIN and the Suitability of Use

A.1 Introduction and Background

Colicins are a group of bacteriocin-class antimicrobial proteins produced by and effective against *Escherichia coli* and closely related bacteria. Research on colicins began with their initial discovery and classification over fifty years ago, with reports by Gratia (1925; 1945) and Gratia and Fredericq (1946) documenting the discovery and first characterization of these molecules, as documented in Cascales' extensive review of colicins (Cascales 2007).

Colicins are classified based on their mode of bactericidal activity (either enzymatic or pore-forming), their membrane receptor, and by the mechanism they utilize for translocation through the outer membrane and across the periplasmic space of Gram-negative bacteria. Universally, colicins are produced naturally in many environments, including the human and animal gastrointestinal tract by commensal (indigenous) strains of enteric bacteria, and demonstrate complementary modes of action Table A-1; Yang 2014). For example, **Colicin E1** is a pore-forming colicin that utilizes BtuB as its receptor and the TolCAQ translocation system (reviewed by James 1996). **Colicin N** is also a pore-forming colicin, which utilizes OmpF as its receptor and the OmpF and TolARQ translocation system (Pugsley 1987). Pore-forming colicins such as E1 or N function as potent bactericidal agents via formation of depolarizing ion channels in the cytoplasmic membrane (Lazdunski 1988; Pugsley 1985).

Colicin M and **Colicin E7** are also naturally produced by *E. coli* strains in the human gut but they exert their bactericidal activity through a different mode of action than pore forming colicins. Instead of attacking the cell membrane, E7 and M degrade and/or inhibit formation of essential macromolecular structures in target strains. Like E1, E7 uses BtuB as its receptor, but employs the OmpF and Tol ABQR translocation system (Chak 1991; Cursino 2002). E7 is a 61.35 kDa protein that exerts its effects intracellularly as a DNase (Chak 1991). Colicin M, a 29.45 kDa protein, is a peptidoglycanase that uses the FhuA receptor and the TonB-ExbBD translocation system to gain entry into susceptible cells (Köck 1987).

The mode of action of other colicins falls into these two main categories (pore forming or enzymatic) and individual colicins make use of different receptors and translocation systems. This property enables **additive or synergistic effects** among colicins and suggests that blends or mixtures of different colicins might have preferential potency at lower doses against certain pathogens. An additional advantage of using blends of colicins over individual colicins is that the practice would act to further reduce the chance for development of resistance, as the probability of simultaneous mutations for resistance to multiple colicins would be highly unlikely.

From a safety standpoint, it bears mentioning that the target structures underlying the mode of action of colicins, namely, the cholesterol-free bacterial cell membrane and the peptidoglycan (murein) layer of the Gram-negative bacterial cell wall, are structures not present in humans or animal cells. In addition other bactericidal activities of certain colicins, such as nucleolytic activity, require entry of the proteins into susceptible cells via specific receptors and transporters that are not found in human or animal cells.

Colicins are typically expressed by native producers under conditions such as nutrient depletion, overcrowding, osmotic shock, UV light, and/or DNA damaging antibiotics (Riley 1999; Spangler 1985). When faced with these conditions, the bacteria react to the stress by initiating the SOS response (van

den Elzen 1982). The LexA protein is one of the primary transcriptional repressors of the SOS response and colicin synthesis. Under colicinogenic conditions, LexA degradation stimulates cAMP and the transcription of the colicin protein (van den Elzen 1982; Eraso 1992; Eraso 1996).

Table A-1. Classification of colicins studied to date by different translocator systems (Tol- and Ton-dependent) in *Escherichia coli* (from: Yang 2014).

Colicins	Antibacterial activity	Receptor	Translocators	Molecular weight (Da)	Producing strain	References
GROUP A						
A	Pore-forming	BtuB	OmpF, TolABQR	62989	*Citrobacter freudii*	Varenne et al., 1981; Morlon et al., 1983
E1	Pore-forming	BtuB	TolC, TolAQ	57279	*Escherichia coli*	Yamada et al., 1982
K	Pore-forming	Tsx	OmpAF, TolABQR	59611	*Escherichia coli*	Pilsl and Braun, 1995a,b,c
N	Pore-forming	OmpF	OmpF, TolAQR	41696	*Escherichia coli*	Pugsley, 1987
S4	Pore-forming	OmpW	OmpF, TolABQR	54085	*Escherichia coli*	Pilsl et al., 1999
U	Pore-forming	OmpA	OmpF, TolABQR	66289	*Shigella boydii*	Smajs et al., 1997
28b	Pore-forming	OmpA	OmpF, TolABQR	47505	*Serratia marcescens*	Guasch et al., 1995 GenBank: CAA44310.1
E2	DNase	BtuB	OmpF, TolABQR	61561	*Escherichia coli* *Shigella sonnei*	Herschman and Helinski, 1967; Cursino et al., 2002
E7	DNase	BtuB	OmpF, TolABQR	61349	*Escherichia coli*	Chak et al., 1991; Cursino et al., 2002
E8	DNase	BtuB	OmpF, TolABQR	~70000	*Escherichia coli*	Toba et al., 1988
E9	DNase	BtuB	OmpF, TolABQR	61587	*Escherichia coli*	Chak et al., 1991; Macdonald et al., 2004
E3	16S rRNase	BtuB	OmpF, TolABQR	57960	*Escherichia coli*	Herschman and Helinski, 1967; Cursino et al., 2002
E4	16S rRNase	BtuB	OmpF, TolABQR	ND	*Escherichia coli*	Males and Stocker, 1982
E6	16S rRNase	BtuB	OmpF, TolABQR	58011	*Escherichia coli*	Akutsu et al., 1989; Cursino et al., 2002
DF13	16S rRNase	IutA	OmpF, TolAQR	59293	*Escherichia coli*	van den Elzen et al., 1983
E5	tRNase	BtuB	OmpF, TolABQR	58254	*Escherichia coli* *Shigella sonnei*	Males and Stocker, 1982 GenBank: KF925332.1
GROUP B						
B	Pore-forming	FepA	TonB-ExbBD	54742	*Escherichia coli*	Schramm et al., 1987
Ia	Pore-forming	Cir	TonB-ExbBD	69429	*Escherichia coli*	Konisky and Richards, 1970 GenBank: AAA23182.2
Ib	Pore-forming	Cir	TonB-ExbBD	69923	*Escherichia coli* *Shigella sonnei*	Konisky and Richards, 1970 GenBank: AAA23188.1
5	Pore-forming	Tsx	TolC, TonB-ExbBD	53137	*Escherichia coli*	Pilsl and Braun, 1995a
10	Pore-forming	Tsx	TolC, TonB-ExbBD	53342	*Escherichia coli*	Pilsl and Braun, 1995b
D	tRNase	FepA	TonB-ExbBD	74683	*Escherichia coli*	Roos et al., 1989
M	Peptidoglycanase	FhuA	TonB-ExbBD	29453	*Escherichia coli*	Kock et al., 1987

ND, not determined.

The production of colicins is currently thought of as a mechanism to promote the survival and proliferation of the colicin-producing *E. coli* in a mixed microbial niche, such as that found in the human intestinal tract. Interestingly, the production and release of colicin actually damages the producing cell and will therefore only provide a survival benefit for other cells containing the same colicin plasmid, which in addition to the colicin gene also contains a constitutively expressed immunity protein. When induced, the colicin producers release colicin into the extracellular environment by increasing the permeability of their own cell membrane, resulting in cell damage (Cao 2002). The colicin producer is not damaged by the action of the colicin itself but rather by the increase in cellular permeability needed to release the protein into the environment (Cao 2002). Other bacterial cells that possess the plasmid to make the colicin also have the immunity protein, so they survive in the event of colicin secretion by

another cell (Alonso 2000). The immunity protein is part of the colicin plasmid and is constitutively produced by the cell in the bacterial lipid bilayer (Espesset 1996). With pore forming colicins such as colicin E1, the immunity protein protects the cell from exogenous colicin by binding to the colicin and preventing the C terminus from entry into the cell membrane; thereby preventing voltage gating and subsequent loss of ionic gradient potential in the cell (Lindeberg 2000). Colicin immunity proteins range in size from 85 to 178 amino acids and are highly specific for individual colicins (Alonso 2000).

Ecologically, colicinogenic strains of *E. coli* could provide a survival advantage to the animal/human host. Colicinogenic strains have been found in abundance in food crops. By producing colicins that inhibit pathogenic strains of *E. coli in situ*, the pathogen load in certain foods would be reduced prior to food ingestion. Studies by Schamberger (Schamberger 2002, 2004) showed that of 23 natural *E. coli* isolates, 14 produced colicins that could inhibit *E. coli* O157:H7, and of those, 6 strains produced one or more colicins that inhibited 10 non-O157 pathogenic strains of *E. coli*. Results of the study suggest that the number of contaminating cells of pathogenic *E. coli* in foods could be higher without natural control by colicinogenic strains of the same species.

Because colicins have different specificities against pathogenic strains of *E. coli* and related pathogenic bacteria and can act individually as well as in concert, colicins are natural candidates as active ingredients in products designed for food protection and preservation. Colicin-containing products can be designed with targeted activities against relevant pathogens using one or more natural colicin proteins that have a long history of human safety from commensal and environmental exposure.

A.2 Support for GRAS Status of COLICIN

A.2.1 Long history of human exposure

Natural exposure from human commensal and domestic animal microflora

It is widely accepted that humans have a very long history of exposure to colicins. From the original studies that isolated and characterized colicins (Gratia 1945, 1946 as reviewed in Cascales 2007), colicin-producing *E. coli* have been isolated from fecal samples of healthy humans, animals and multiple environmental samples (Obi 1978; Riley 1992; Riley 1994; Schamberger 2002; Hossneara 2007; Smarda 2001; Smarda 2007; Cascales 2007). Estimates for the number of colicin producing *E. coli* in the colon of healthy humans have ranged from as low as 9% of the total number of *E. coli* isolated (Lorkiewicz 1964 as reviewed in Smarda 2001) to as high as 83% of recovered isolates (Hossneara 2007).

Therefore it is likely that humans have been exposed to colicins endogenously for as long as the human gut has carried *E. coli* as a commensal organism. Battistuzzi (Battistuzzi 2004) provided evidence that the genera *Escherichia* and *Salmonella* diverged around 102 million years ago (mya), which coincides with the divergence of their hosts: the former being found in mammals and the latter in birds and reptiles. This was followed by a split of the escherichian ancestor into five species (*E. albertii, E. coli, E. fergusonii, E. hermannii* and *E. vulneris*). The last *E. coli* ancestor split between 20 and 30 million years ago and would have co-existed with hominids, which originated 15-20 mya (Lecointre 1998). Humans (genus *Homo*), which appeared as a species less than 2 mya, would have had a long history of exposure to and intestinal commensalism with *E. coli* as would have other mammals, including primates at that time.

In 1885, the German-Austrian pediatrician Theodor Escherich, discovered the organism that would eventually be named after him in the feces of healthy individuals and called it *Bacterium coli commune,*

due to the fact it is found in the colon and early classifications of Prokaryotes placed these in a handful of genera based on their shape and motility. Following a taxonomic revision of the genus *Bacterium* in 1895 (Migula 1895), it was reclassified as *Bacillus coli* and later reclassified in the newly created genus *Escherichia*, named after its original discoverer (Castellani 1919). Subsequently, Gratia (1945, 1946) discovered that certain strains of *E. coli* present in the human intestinal microflora produced protein antibacterials that acted selectively against strains of the same genus. Because the genes of almost all colicins are carried by *E. coli* in plasmids (Cascales 2007), the transmission of the colicinogenic trait can be assumed to be widely dispersed as well as highly conserved through the bacterium's evolution with mammalian hosts because of the selective advantage afforded by colicin production.

While very few studies have examined the production of specific colicins by commensal *E. coli* in healthy humans, Gordon (Gordon 2006) examined 266 *E. coli* isolates and found that 24% produced at least one colicin, and that one half of those colicinogenic strains produced more than one colicin. These studies corroborated that colicin-producing *E. coli* are prevalent in the gastrointestinal tract of healthy humans. In addition, due to the highly competitive microbial environment of the gastrointestinal tract, we would expect that these *E. coli* are producing and secreting colicins through survival pressure. This further supports the presumption that humans have always been exposed to colicins throughout evolution.

Although the percentage of colicinogenic strains in the human gut has been reported (cited studies above), no reports have appeared in the public literature quantifying the amount of colicin protein present in the human gastrointestinal tract. This figure would be nearly impossible to calculate accurately without the conduct of large clinical studies, due to the high variation in the human diet, subject age, digestive profile, medications, immune status and other factors that would impact the gut's microflora and its collective synthesis of colicins. What is clear is the high percentage of strains isolated from human fecal samples that are colicinogenic.

Estimated natural exposure from biosynthesis by commensal microflora in the human gut

Analyzing the literature on record, an estimate of the daily exposure of humans to colicins from natural commensal microflora can be developed. Assuming that bacteria comprise 60% dry weight of human feces (Stephen 1980), that the average human fecal output is 150 g wet weight (30 g dry weight basis, dwb) per day (Stephen 1980), that the bacterial content is $\geq 10^{11}$ cells/g of stool dwb (Berg 1996; Gorbach 1996; so 3 x10^{12} bacteria dwb/daily stool), that *E. coli* represents 0.1-1% of the gut microflora (Eckburg 2005; so 3 x 10^{10} to 3 x 10^{11} *E. coli* dwb/daily stool), and that colicinogenic strains make up 50% of the *E. coli* component (range: 9-83% reported by Lorkiewicz 1964 in Smarda 2001, and Hossneara 2007, respectively), then the "steady state" colicinogenic bacterial population could be estimated as 10^{10} to 10^{11} cells/day (i.e. chronic colicin production/exposure). From colicin induction studies it has been reported that >30 μg colicin (35-45 μg range reported) can be obtained from 10^9 cells (i.e. 35-45 μg colicin/ml culture at a density of 10^9 cells/ml; Gökçe 2003), suggesting that the endogenous colicin content of the human lower intestinal tract on an average day might be 3 mg. Given the published fecal output of 30 g per day dwb, each person could be exposed to 100 ppm (3 mg colicin/0.030 kg feces dwb), chronically, from commensal synthesis.

Natural exposure from vegetables and soil

While coliform bacteria have been historically linked with the intestinal microflora of humans and other animals, colicinogenic strains of *E. coli* have been found in plants, soil and the rhizosphere (plant root zone). Humans and herbivores (domesticated and wild animals) would have been exposed to colicins through plant intake. The colonization of multiple habitats by coliforms and other microorganisms is no

longer surprising. There is now ample evidence documenting cross-kingdom jumps by non-pathogenic as well as pathogenic strains of enteric bacteria, as well as viruses and protozoans (reviewed by van Baarlen 2007). For example, *E. coli* O157:H7 proliferates quite extensively in the mouth parts of the common house fly (Kobayashi 1999); the plant pathogens *Dickeya dadantii* and *Pantoea ananatis* also cause diseases in pea aphids and humans, respectively (Grenier 2006), while the endophyte/plant pathogen *Pantoea agglomerans* and endophyte *K. pneumoniae* have both been associated with opportunistic infections in animals, including humans (Garcia de la Torre 1985; Dong 2001; De Baere 2004; Cruz 2007; Morales-Valenzuela 2007; Mano 2008).

The ability of animal pathogenic enterobacteria to contaminate non-animal (i.e. non-meat) products is an accepted fact (Beuchat 1996). Foodstuffs as diverse as unpasteurized fruit juice, dairy products, dried herbs and spices all act as vehicles for transmission. Fresh fruit and vegetables are now recognized to be a major route of entry for animal pathogenic enterobacteria into the food chain. One of the largest outbreaks of VTEC-derived gastroenteritis occurred in Japan in 1996 as a result of contamination of radish sprouts with *E. coli* O157:H7 (Michino 1999; Watanabe 1999). It has been reported that almost one quarter of all documented cases of VTEC in the United States were associated with fruits and vegetables (Rangel 2005). And one of the most serious cases of *E. coli* contamination of vegetables occurred in Northern Germany in 2011, when fenugreek seeds contaminated with O104:H4 (a Shigatoxigenic enteroaggregative [EAEC] strain) in an organic sprouts farm afflicted nearly 4,000 people, ultimately causing 53 deaths (51 in Germany, with one third of casualties from HUS complications; WHO/EFSA 2011).

While these outbreaks command public attention, the vast majority of enteric bacteria present in fruits and vegetables are not pathogenic. Several studies have documented the level of enteric bacteria found in produce, in soil and in the rhizosphere of various plants. *E. coli* is naturally present and capable of growing in certain soil environments (Byappanahalli 2004; Ishii 2006). Ishii (2006) reported that the population of natural *E. coli* in the rhizosphere could reach 10^5 CFU/g of soil. Mukherjee (Mukherjee 2006) surveyed bacterial loads in fruits and vegetables from various standard, semi-organic and organic farms during the 2003 and 2004 growing seasons, and reported that fruits and vegetables that tested positive for *E. coli* had average counts that ranged from 2 to 2.4 log most-probable number (MPN)/g of produce. A prior and similar study by Mukherjee (Mukherjee 2004) reported an average *E. coli* count of 3.1 log MPN/g in preharvest produce samples. In another similar study, coliform counts ranging from 1.5 to 3.0 log CFU/g of produce were found in preharvest samples of spinach, cilantro, mustard greens, parsley and cantaloupe (Johnston 2005). These and other studies have reported that up to 92% of fresh vegetables and fruits may contain non-pathogenic strains of coliform bacteria, with between 5% and 50% of the coliforms being represented by non-pathogenic strains of *E. coli* (Mukherjee 2006).

The high prevalence of coliform bacteria in fruits and vegetables is not so surprising when one considers that domesticated animals are mainly herbivores, and that the human-animal-plant co-dependence includes in part adaptation of our multispecies microflora. This hypothesis has gained support with new evidence from recent studies that have documented the molecular and physiologic mechanisms used by coliforms to adhere to and even colonize the tissues of plants (see, for example, Wright 2013, and reviews by Holden 2009; Holden 2014; Martínez-Vaz 2014). Just as colicins in the gut of humans and animals confer a competitive advantage to colicinogenic strains of *E. coli*, one can envision colicins also playing a selective role in the competitive environment of the rhizosphere (Mukherjee 2004; Mukherjee 2006). Humans, therefore, would have been exposed to colicins and colicinogenic strains of *E. coli* from animal, plants and soil sources, and from the animal meat, fruits and vegetables consumed as food.

Natural exposure from colicinogenic bacteria in food

Colicin-producing *E. coli* have been found as commensal organisms in the gastrointestinal tract of many food animals, including cattle, pigs, chickens, sheep, goats, ducks and deer (Obi 1978; Schamberger 2002). It is therefore highly likely that the food products produced from these animals would also have colicin-producing *E. coli* present at some level. Additionally, because the feces from these production animals are frequently used as fertilizer (manure), ingestion of crops and produce would have resulted in ingestion of resident colicins. Due to the virtually ubiquitous nature of *E. coli* in food products and the frequency of colicinogenicity seen among *E. coli* isolated from multiple environments (Smarda 2001; Gordon 2006; Hossneara 2007), it is expected that there has been a very long history of exposure to colicins in food.

While no research to date has systematically examined food products for colicins, it is likely that they are present in multiple food sources. Two estimates are provided here to illustrate potential exposure levels from food sources carrying colicinogenic strains.

Exposure from meat (beef). The level of naturally occurring colicins present in beef can be estimated based on the following assumptions. Assuming high expression of colicins in the bacterium's natural habitat in the colon (equivalent to laboratory/production induction in a culture of 10 logs of *E. coli* per ml), 10,000 *E. coli* cells per g of beef (82% producing colicins; Lange 2008) and an average of 77.9 g of meat consumed per day (based on the USDA ERS 2006 Food Disappearance Database, data for beef), it is estimated that 0.02 μg colicins/person-day could be ingested from natural beef consumption alone. Total consumption would be higher if other meats and meat products are included.

Exposure from produce. Similarly, one can estimate the natural exposure to colicins from the consumption of produce. Using the ranges of microbial counts reported for produce in various studies, non-pathogenic strains of *E. coli* would be present in various fruits and vegetables at levels of 1.5-3 log CFU (Johnston 2005) or 2-3.1 log MPN (Mukherjee 2004; Mukherjee 2006) per gram of produce. Considering a per capita consumption of approximately 330 lbs of fresh vegetables and fruits per year (~410 g/person-day; USDA ERS 2014 Food Disappearance Database, data for vegetables), a microbial load of 2 to 3 log CFU/g, would equate to >40,000 (10^2 cells/g x 410 g produce/day) to >400,000 (10^3 cells/g x 410 g produce/day) *E. coli* cells per day. Assuming that 82% of those cells represent colicinogenic strains (Lange 2008) and the same rate of colicin synthesis as in beef, it is estimated that an individual could ingest 0.01-0.02 μg colicins/person-day from consumption of fresh fruits and vegetables.

Conclusion

It is estimated that humans are chronically exposed to low levels of colicins from foods as well as to colicins naturally produced in the gastrointestinal tract by commensal colicinogenic strains of *E. coli* and other enteric bacteria. Through traditional practices used in food cultivation, preparation and consumption, humans have likely been chronically exposed to colicins from food sources for millenia. The above calculations suggest that the level of human exposure to colicins from various foods, while small (parts per million levels ingested per day), is nevertheless consistent due to dietary and cultural habits.

No reports have appeared in the literature linking colicin consumption (ingestion) with onset of disease, morbidity or mortality. In large part, this can be explained by evolutionary adaptation by humans and animals to colicin protein exposure, including immune tolerance. But importantly, the physicochemical

properties of colicins, notably their instability in the gastric and upper intestinal environments, contribute significantly to their safety profile and support the use of colicins as food preservatives and food processing aids (see Section A.2.3).

A.2.2 Physical properties of colicins and manufacturing process features supporting the safety of colicins for use in food

Identity of colicin proteins in COLICIN product

Recombinant, plant-produced colicin proteins are identical to the *E. coli*-produced antimicrobial proteins described in the literature, the sequences for which were further described in the corresponding accession numbers and used in the construction of the plant-viral vectors (see Section 2.3). The natural bacterial and recombinant plant-produced colicins are thus identical in amino acid sequence and no deletions, substitutions or additions have been introduced into the product. Therefore, the recombinant colicins produced by our process are identical to the proteins produced by *E. coli* or other enteric bacteria in the human intestine.

Safety of colicin production organism and manufacturing process

Colicins can be produced using either of two similar plant-based manufacturing processes, as described in APPENDIX B. The overall processes applied to produce the antimicrobials are very similar to the plant-based processes used to manufacture human therapeutic proteins and vaccines. One method entails propagation of a plant-viral vector encoding the gene for a protein of interest, in this case a chosen colicin, in a non-transgenic host plant. Accumulation of the colicin in plant tissue is followed by grinding and extraction of the protein, purification, fill and finish. All manufacturing steps are monitored and practiced under strict quality control and quality assurance procedures leading to batch-by-batch final product release. A second method utilizes transgenic plants carrying the gene encoding the selected colicin that is induced to synthesize the protein upon exposure to dilute ethanol. These two approaches are presented in more detail in APPENDIX B and are summarized below from the standpoint of safety.

Transient gene expression using viral vectors. The biosynthesis of a colicin protein using the plant-viral induction method is initiated by application of a non-pathogenic RNA-containing plant viral vector (e.g. Tobacco Mosaic Virus (TMV) or Potato Virus X (PVX)). Processes employing these expression vectors have met regulatory scrutiny in the production of injectable therapeutic proteins for clinical evaluation under FDA IND (e.g. McCormick 2008; Bendandi 2010; Tusé 2011) and should therefore be safe for production of food additives. The vector encoding the structural gene for a selected colicin is introduced into a non-recombinant (i.e. non-transgenic) green plant, in this case the food plants *Beta vulgaris* (beet), *Spinacia oleracea* (spinach), or *Lactuca sativa* (lettuce), which enables rapid viral replication and accumulation of the colicin protein in the cytosol of plant leaves. The RNA vector cannot integrate into the host plant chromosomes (plants do not possess reverse transcriptase) nor into plant seeds, and therefore expression of the product in the plant is considered a transient process.

For efficiency, the viral vector is introduced into the plant as a replicon within a plasmid carried by the bacterium *Agrobacterium tumefaciens.* A specialized industrial strain of *A. tumefaciens* is used that has been adapted to ensure environmental safety and compatibility, even though the colicin production process is practiced indoors in contained manufacturing environments. The agrobacterial vector and the viral replicon are inactivated in the process of colicin protein purification and thus do not present a health risk from use of the product. No viable vector remains after colicin protein purification and the

waste stream remaining from the process is disposed of according to local regulations. No part of the waste stream is used in any human or animal food product, additive or supplement.

Gene expression using transgenic host plants. Alternatively, the colicin structural gene and nuclear expression elements encoding RNA viral replicons can be stably integrated into the same food plant hosts used in the method described above. One of the elements introduced into the expression cassette in these transgenic host plants is an ethanol-inducible promoter. These plants are not exposed to *Agrobacterium* vectors; rather, mature plants are sprayed with a dilute (~2.5% v/v) ethanol solution. This activates the promoter and initiates synthesis of colicin protein throughout the plant. Colicin protein is extracted using the same downstream process as above. The dilute ethanol is metabolized or evaporated prior to protein purification and is therefore absent in the final extract.

Production host plant. The well-known food crops **beet**, **spinach** and **lettuce** are used for production of recombinant colicins. The choice of food plant host used in the manufacturing process does not impact the safety of the colicin product. For production of colicins, Notifier has preferentially selected three food crops on the basis of safety and productivity criteria:

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 2005). Beet belongs to the Chenopodiaceae family (now classified in Amaranthaceae) and is a close relative of spinach, chard and quinoa (USDA NRCS BEVU2 2012; Purdue University 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 2014).

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 (*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 2014). 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 (*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 2014). There were no reports found of allergenic components present in lettuce.

Process impurities and their impact on safety. As summarized above, the production host used in manufacturing recombinant colicins is an edible plant, such as **red beet, spinach** or **lettuce**, which, by its inclusion as a "food," is 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 formulated as **COLICIN Isolate** may be >80-90 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 used to express the colicin genes and/or any residual reagents added in the manufacturing process.

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.

Agrobacteria are natural soil inhabitants and ubiquitous plant colonizers, present at levels as high as 30 million CFU/g of soil (Vicedo 1993; Bouzar 1993; Stockwell 1993; Penyalver 1999; Mougel 2001; Krimi 2002). *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. Furthermore, 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.

With respect to abiotic safety risks from process reagents, all processing aids, aqueous buffers 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.

Conclusion

The totality of evidence suggests that the health risks associated with materials used and process-derived impurities from the manufacture of recombinant plant-produced colicins would be very low.

A.2.3 Use and exposure, digestibility, safety in animal and cellular studies

Anticipated exposure from use of the COLICIN product

The maximum anticipated application rate for COLICIN is 10 mg/kg food (1 mg colicin/100 g food). That maximum rate applies regardless of whether COLICIN is comprised of a single colicin or a mixture of colicins with complementary activities. Assuming that all colicin reaches the stomach, a person consuming 100 g of colicin-treated food would be ingesting 1 mg colicin. In an average full stomach (1 to 1.5 L in food volume), the concentration of colicin in the upper digestive tract would be about 1 ppm (0.7 to 1 mg/kg) depending on the size of the meal and its density. Were the colicin not digested at all by gastric acid, pepsin or duodenal proteases and reach the colon, its concentration in the lower digestive tract would be about 33 ppm (1 mg/30 g fecal content dwb).

Compared to the estimated colicin exposure of 100 ppm from biosynthesis by human commensal gut microflora (see Section A.2.1), the potential “worst case” exposure level from ingesting COLICIN in the scenario cited would be 1/3 as much, on average. This scenario is not likely to materialize because, importantly, we and others have shown conclusively that colicins are denatured and inactivated by the gastric environment, are degraded to low MW peptides in a simulated gastric environment, and that either the native protein or its peptic breakdown products are further degraded in a simulated duodenal environment during the time that food would take to transit the gastrointestinal tract (see discussion immediately following, below). Therefore, exposure to colicins from ingestion of the product is projected to be much lower than from natural endogenous exposure in the gut.

Digestibility of colicin proteins under natural and simulated conditions

General recognition of colicin instability. Although the long history of natural human exposure to colicins without adverse consequences provides support for the safe use of colicins as food

biopreservatives, there are other attributes of colicins that further support their safety for use in food. Colicins have been shown to be very **susceptible to digestion by proteolytic enzymes**. By definition, colicins were identified as antimicrobial compounds that lost their efficacy after protease treatment.

For example, Schaller (Schaller 1981) showed that colicin M was not stable and that it degraded upon exposure to proteolytic enzymes, detergents and other environmental conditions, with a rapid ($t_{1/2}$ 10-40 min) breakdown of the protein upon incubation with such agents *in vitro*. Similarly, Slatin (Slatin 1986) demonstrated that colicin E1 is sensitive to pepsin digestion. Since then, other studies have shown that colicins are also susceptible to trypsin digestion (Elkins 1994; Murinda 1996; Zhang 1992).

In a study examining the efficacy of 24 different colicins, Murinda (Murinda 1996) noted that all of the colicins were highly susceptible to protease treatment and that treatment with either papain, pronase E, or trypsin eliminated the biological activity of the colicins. Since colicins are relatively large proteins (MW range approximately 30-60 kDa), when ingested they cannot be absorbed intact. Due to the presence of acid (stomach) and proteolytic enzymes in the human gastrointestinal tract, ingested colicins will be rapidly denatured and digested as any other protein in the diet.

Justification of digestibility assay conditions. To confirm that plant-made colicins were digested by stomach and intestinal enzymes as are their bacterial counterparts, degradation studies were carried out using two complementary paradigms:

1. High enzyme-to-substrate ratios. European guidance documents (e.g. EFSA 2011) for safety assessment of new proteins expressed in genetically modified crops intended for food or feed use suggest using high initial enzyme:protein ratios for assessing degradation. If poor degradation is observed after 60 min incubation, then additional digestibility assays are suggested (Thomas 2004). Colicin degradation was first evaluated using as high as ~3:1 wt:wt ratio of pepsin to colicin in SGF, and similarly 38:1 pancreatin (trypsin/chymotrypsin mix) to colicin (wt:wt) in SIF;

2. Low enzyme-to-substrate ratios. In order to assess colicin digestibility under a variety of physiologically relevant conditions, ratios of approximately 1:20 to 1:50 pepsin to colicin (wt:wt) in SGF were used to simulate various stomach food loads and peptic activity first, followed by subsequent exposure to 1:4:400 trypsin:chymotrypsin:colicin protein (wt:wt:wt) in SIF, to simulate duodenal degradation.

There is merit to assessing digestibility of proteins under paradigm 1, namely high enzyme to substrate ratios, as reported in the literature. For example, the concentration of pepsin in the fasting human stomach has been reported to be up to 1 mg/ml (range measured in human samples is 0.5 to 1.0 mg/ml; Zhu 2006). The volume of stomach fluid in a fasting person was estimated at 50 ml (Schiller 2005). Hence, the amount of pepsin in the stomach is 50 ml x 1 mg/ml = 50 mg pepsin. Using the maximum application rate for colicin of 10 mg/kg food, and assuming that all colicin reached the stomach, a person consuming 100 g of treated produce would be ingesting 1 mg colicin ($1/10^{th}$ of the 10 mg applied per kg). Thus, the 1 mg colicin would encounter 50 mg pepsin, or an enzyme:colicin ratio of 50:1 wt:wt.

Nevertheless, to assess digestibility under physiologic conditions in which low enzyme to colicin ratios may predominate, digestibility studies with plant-made colicins were also performed under low enzyme to substrate ratios (paradigm 2), as described below. Regardless of the digestibility paradigm used, and as shown below, plant-made colicins were degraded under all tested conditions.

Confirmation of colicin digestibility. Because the amino acid sequences of plant-produced colicins are homologous to those of bacterial colicins that have been shown to be susceptible to digestion by proteolytic enzymes (see above), the plant-made colicins are expected to be equally degraded upon exposure to said enzymes. To confirm, Notifier exposed representative plant-made colicins comprising various structures and modes of action, including **colicins M, E7, K and U**, to simulated gastric and duodenal digestion conditions. Exposures of the proteins to **Simulated Gastric Fluid** (**SGF**, commercial acidic pepsin extract) and **Simulated Intestinal Fluid** (**SIF**, commercial neutral pancreatic extract of trypsin and α-chymotrypsin) were done using established methods and standardized conditions (Thomas 2004; Dupont 2010), including regulatory guidance for safety assessment of new proteins expressed in genetically modified crops intended for food or feed use (EFSA 2011). The results generated from this study are summarized in Table A-2 for high enzyme:colicin ratios and in Table A-3 for low enzyme:colicin ratios. The methods applied are presented in more detail in APPENDIX C.

1. Degradation under high enzyme:colicin ratios. Plant-made colicin M was used as a model to assess digestibility under paradigm 1 (high enzyme:colicin ratios). Pepsin and colicin were incubated in acidic SGF at various ratios for up to 60 min with frequent sampling. Similarly, plant-made colicin M was incubated with pancreatin (trypsin/chymotrypsin mix) at a ratio of 38:1 in SIF (Thomas 2004). Colicin integrity was assessed by SDS-PAGE. Protein degradation times under these conditions are indicated in Table A-2.

Table A-2. Degradation of plant-produced colicin M in simulated gastrointestinal environments

Condition	Ratio of pepsin (in SGF) or trypsin/chymotrypsin (in SIF) to colicin protein (wt:wt)	Measured degradation time
Simulated gastric fluid (SGF)	2.82:1	0.5 min
Simulated gastric fluid (SGF)	1:10.8	2 min
Simulated gastric fluid (SGF)	1:20	10 min
Simulated intestinal fluid (SIF)	38:1	<0.5 min

Plant-produced colicin M was used as a model to assess digestibility under high enzyme:substrate ratios. Colicin protein was exposed to pepsin in acidic simulated gastric fluid (SGF) at various ratios, or to trypsin/chymotrypsin (pancreatin) in simulated intestinal fluid (SIF) at a ratio of 38:1 pancreatin:colicin wt:wt. Incubation was for up to 60 min with frequent sampling; protein integrity determination was by SDS-PAGE; colicin activity was assessed by degradation of substrate.

2. Degradation under low enzyme:colicin ratios. To assess digestibility of plant-made colicins in a variety of physiologically relevant scenarios, several plant-made colicins were exposed to simulated gastric and duodenal conditions and the time of degradation of the proteins was measured. Table A-3 summarizes the results of these studies. The results showed that plant-produced representative colicins M, E7, K and U are digested in 1-10 min upon exposure to SGF with low pepsin content, simulating breakdown of the protein in the human stomach containing food. Colicin degradation was also tested using sequential exposure of the proteins to pepsin (in gastric simulant) first, followed by exposure of pepsin breakdown products to trypsin/chymotrypsin in simulated duodenal fluid. The plant-produced colicins were not stable to exposure to intestinal proteases or to

sequential exposure to SGF/SIF, with results indicating digestion of the colicins at various rates, ranging from a few minutes to several hours (method details presented below Table A-3 and in APPENDIX C).

In this series of studies a fixed ratio of pepsin to colicin was used (1:20 wt:wt) in SGF, and the colicin or resultant pepsin-digestion products were subsequently exposed to SIF containing duodenal enzymes at a ratio of trypsin:chymotrypsin:colicin protein of 1:4:400 (wt:wt:wt; per Moreno 2005; Mandalari 2009; Eiwegger 2006).

Table A-3. Degradation of plant-produced colicins in simulated gastrointestinal environments

Plant-made colicin	Measured colicin protein degradation times under indicated simulated gastrointestinal exposure conditions using low enzyme:colicin ratios	
	A. Degradation of intact colicin by exposure to simulated gastric fluid (SGF) with pepsin[1]	**B. Degradation of SGF hydrolysate from "A" by exposure to simulated intestinal fluid (SIF) with trypsin + chymotrypsin[2]**
M	10 min	5 min
E7	1 min	180 min
K	1 min	120-180 min
U	1 min	180 min

[1]Plant-produced colicins were concentrated from total soluble protein (TSP) extracts to 0.2-1.0 mg/ml and exposed to SGF without or with pepsin supplementation, the latter at a ratio of 1:20 (wt:wt pepsin to colicin). Degradation/denaturation of intact colicins was significant in SGF alone, with 2-10-fold loss of colicin activity measured from 5-60 min (data not shown). **Column A** shows that degradation of intact colicins was significant upon exposure to pepsin in SGF, with complete disappearance of the intact proteins in 1-10 min. Concomitantly, colicin activities were destroyed by exposure to SGF/pepsin.

[2]Colicins or their peptidic breakdown products generated by exposure to SGF or SGF+pepsin in "A" were subsequently exposed to SIF alone (data not shown) or to SIF supplemented with trypsin + chymotrypsin, the latter at a ratio of 1:4:400 (wt:wt:wt trypsin to chymotrypsin to colicin protein). **Column B** shows that degradation of the proteins or their SGF breakdown products under simulated duodenal conditions took place albeit at a slower rate than digestion by pepsin.

Plant-made colicins were degraded under all tested conditions. Exposure of all plant-made colicins to pepsin in SGF quickly destroyed enzymatic activity and digested the proteins to low MW peptides, mostly in the 2 – 5 kDa range. Such small peptides are known not to interact with the immune system and are considered **non-immunogenic/non-allergenic** (Dupont 2010). Further support for the lack of immunogenicity/allergenicity risks is provided by comparing the colicin peptide amino acid sequences to the sequences of known allergens in public databases (see Section A.2.5). With the possible exception of one potent allergen that can survive full proteolysis (peanut protein Ara h 1; Eiwegger 2006), allergenic epitopes on proteins can be degraded by proteolysis.

Importantly, although the partially purified plant-made colicins were tested in the presence of other plant soluble proteins and were therefore not 100% pure, nothing in the remaining plant material appeared to inhibit enzymatic degradation of the colicins either in SGF or SIF.

Conclusion. The conclusion that can be drawn from these studies is that plant-produced colicins are not stable to exposure to gastric acid or intestinal proteases, in the same way that the homologous bacterial colicins are not stable to exposure to simulated gastrointestinal digestion.

For brevity, data for 4 representative plant-made colicins (colicins M, E7, K and U) are included as an example in this notification. Adding digestibility results for all plant-made colicins was not seen as necessary given the preponderance of evidence on their instability from the public literature. The literature is replete with examples of colicin instability upon simulated ingestion, and hence colicins made via alternative hosts, but comprising the amino acid sequences of their corresponding bacterial proteins, are expected to be digested by proteases in the same manner (Schaller 1981; Slatin 1986; Elkins 1994; Murinda 1996; Zhang 1992; Cascales 2007).

Safety of colicins in high-dose oral exposure in monogastric animals

The safety of colicins was demonstrated in two oral exposure studies evaluating the dietary inclusion of colicin in the prevention of post-weaning diarrhea in pigs that were orally challenged with porcine pathogenic *E. coli* strain F18 (Cutler 2007a; Cutler 2007b). Using colicin E1 as a model, piglets were fed E1 in doses ranging from 11 to 20 mg/kg of feed. Pigs fed 20 mg/kg of feed for 4 weeks, to evaluate the effect of dietary inclusion of E1 alone, showed no significant differences in body weight compared to control pigs at any point in the study. The study with 24 pigs orally challenged with *E. coli* strain F18 and fed E1-supplemented or a control diet (0, 11 or 16.5 mg colicin E1/kg feed for four days) demonstrated typical body weight gain and feed consumption for E1-treated animals, whereas control animals succumbed to the effects of diarrhea and had significantly lower feed consumption and weight gain.

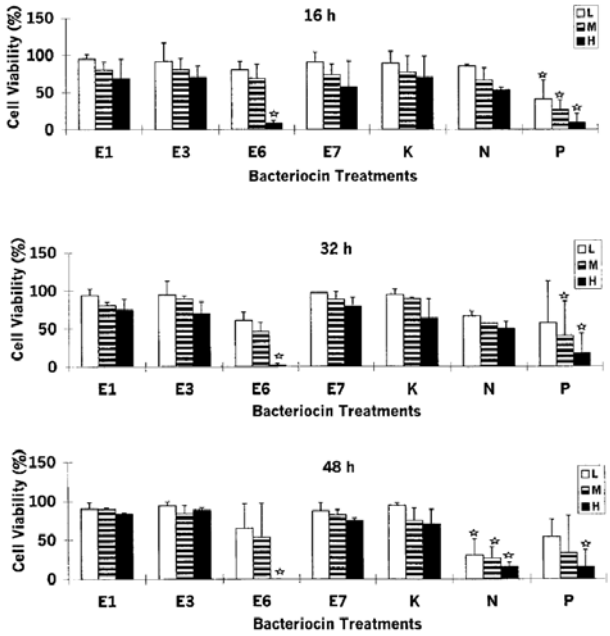
The study did not show growth promotion by colicin. Colicin E1 is as labile as other colicins but its ingestion at high levels (11-20 ppm) when mixed in feed enabled a protective effect. The main conclusion from these studies was that feeding high levels of colicin E1 to a monogastric species has no negative effects on health with respect to weight gain and feed intake criteria.

Safety of colicins in human and primate cells exposed *in vitro*

Murinda (2003) showed that various colicins had little if any toxicity on immortalized human colon cells (Figure A-1) and monkey kidney cells (Figure A-2). This study compared the toxicity of colicins and nisin against both of these cell lines *in vitro*. Colicins were generally less toxic than nisin to both cell lines, with nisin showing significant reductions in cell viability in both models, especially under prolonged exposure. Kordel 1986 evaluated the toxicity of nisin *in vitro* against normal (non-immortalized) mammalian cells, utilizing a nisin dose that was over 200-fold greater than the dose utilized by Murinda (2003). This study found no cytotoxic effect for nisin against either human or sheep erythrocytes.

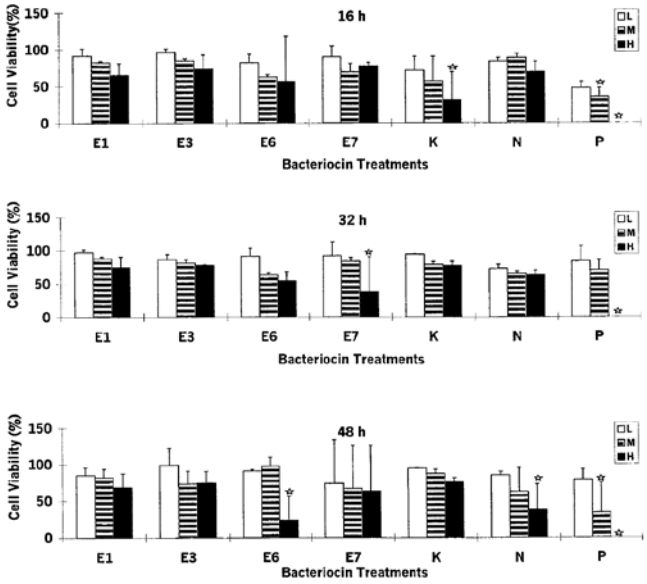
Similarly, colicins have been shown to have selected toxicity to malignant cells and have been examined as potential antineoplastic agents (Farkas-Himsley 1976, 1995), although the levels of exposure and route of administration were different than those contemplated here. Colicins therefore appear to have little if any toxic effects on normal mammalian cells.

Figure A-1. Inhibition of SV40-HC cells by bacteriocins.



Cells were treated with Col E1, Col E3, Col E6, Col E7, Col K, nisin (N), and pediocin (P), individually added at low (L 5 170 AU/ml), medium (M 5 350 AU/ml), and high (H 5 700 AU/ml) doses. Viable cell levels were expressed as percentages of those for the respective untreated controls. The viability values are means for two separate experiments. The vertical bars represent standard deviations of means. Stars indicate means that were significantly different from viability values for SV40-HC cells treated with 175 AU/ml of Col E6 and incubated for 16 h (Murinda 2003).

Figure A-2. Inhibition of Vero cells by bacteriocins.



Cells were treated with Col E1, Col E3, Col E6, Col E7, Col K, nisin (N), and pediocin (P), individually added at low (L 5 170 AU/ml), medium (M 5 350 AU/ml), and high (H 5 700 AU/ml) doses. Viable cell levels were expressed as percentages of those for the respective untreated controls. The viability values are means for two separate experiments. The vertical bars represent standard deviations of means. Stars indicate means that were significantly different from viability values for SV40-HC cells treated with 175 AU/ml of Col E6 and incubated for 16 h (Murinda 2003).

A.2.4 Low potential for development of resistance

We have also considered the potential for development of resistance to colicins by the native gut microflora and its implications on safety. Since colicins are endogenously produced in the gut, the issue is whether ingestion of incremental amounts of colicins in foods treated with the product could lead to resistance by the natural commensal population of coliforms. We conclude that the use and consumption of colicins through ingestion, regardless of level, is unlikely to allow for selection of colicin-resistant bacteria in the intestinal tract of humans, for three main reasons:

- Cooking colicin-containing foods prior to ingestion will lead to inactivation of colicin proteins by heat;
- Even without cooking/heating, ingested colicins will be denatured by the low pH of the stomach; and
- Colicin-class proteins will be rapidly digested by proteases in the upper and mid gastrointestinal tract before the reach the colon.

Therefore, we anticipate that exposure of the resident *E. coli* population in the colon to intact colicins derived from ingestion of foods treated with our product will be highly unlikely.

To confirm these observations derived from literature reports, Notifier conducted a series of tests wherein a tester strain of *E. coli* (pathogenic strain O157:H7) was applied to apples, subsequently treated with COLICIN product or a control plant extract containing no colicins, and any surviving colonies were re-grown and re-exposed to COLICIN product, repeating the experiment in series and testing susceptibility to the active ingredients.

Under realistic application conditions in produce, no development of resistance to COLICIN components (including a two-component mixture of colicins M + E7, and a five-component mixture of colicins M + E7 + K + B + 5) was observed in these studies. The methodology used in these assays and the results obtained are presented in more detail in APPENDIX C.

A.2.5 Low potential for development of allergenicity or immunogenicity

Because the recombinant colicins have the same amino acid content as the native, *E. coli*-produced proteins resident in the human colon, allergenic or immunogenic reactions to the recombinant colicins added as food antimicrobials are not expected because, as with other endogenous proteins, humans would have become tolerized to colicins through continued exposure. Also, it is highly unlikely that ingestion of significant amounts of added colicins will be experienced due to the instability of these proteins (Section A.2.3 above).

Nevertheless, for verification, the potential for induction of allergic responses was investigated at the molecular level by analyzing the sequence of plant-produced colicins for known immunogenic/allergenic domains against databases of known allergenic epitopes (e.g. AllergenOnline 2015). Proteins with greater than 50% identity to known allergens are considered potentially allergenic (Aalberse 2000). CODEX Alimertarius (CODEX Alimentarius 2003), lists 35% as the minimum homology required for categorizing a protein as potentially allergenic. More detailed sequences searches, at the 80-mer and even at the 8-mer level, were also conducted because searching by different peptide sequence lengths reduces the probability of both false-positive and false-negative results.

Results of allergenicity/hypersensitivity bioinformatic searches

The complete amino acid sequence of each plant-produced colicin was scanned for potentially allergenic/hypersensitive sequences in the AllergenOnline database. The results are summarized in Table A-4 and described in the accompanying text for each colicin entry.

Note that none of the plant-produced colicins examined had similarities to known food allergens of greater than 50% (Aalberse 2000) and therefore did not meet the threshold for potential allergenicity. Table A-4 shows similarity at the more stringent threshold of 35% identity (CODEX Alimentarius 2003). In addition, intact colicins are predicted (and shown by Notifier and from the literature) to fragment enzymatically (pepsin, trypsin, chymotrypsin) into peptides that are not known food allergens, thus further lowering the allergenicity concern from colicin ingestion.

Table A-4. Bioinformatic amino acid scan for potentially allergenic sequences in plant-made colicins

Colicin	≥35% allergen similarity at indicated search granularity? If yes, highest % similarity			Allergenicity Potential
	Full seq	80-mer	8-mer	
E1	0	0	0	very low
E7	46.7	45.2	1 exact match	low
Ia	0	0	0	very low
M	0	0	0	very low
N	0	0	0	very low
K	35.4	0	0	very low
U	43.2	0	0	very low
5	0	0	2 exact matches	low
B	0	0	0	very low

Colicin E1. A bioinformatic FASTA search of the full 522 amino acids of plant-produced colicin E1 revealed only a distant relationship to tropomyosin from the insect *Lepisma saccharina* (30.9% identity). A more specific bioinformatic comparison at the 80-mer level (sliding window of 443 80-mers in 522 aa) revealed 38 hits (mostly tropomyosin from various sources) but no (zero) matches with greater than 32% identity. An even more precise search at the 8-aa level revealed no (zero) exact matches. In sum, no amino acid sequences in colicin E1 are found to match known proteinaceous food allergens and hence the allergenic potential of colicin E1 should be considered very low.

Colicin E7. A bioinformatic FASTA search of the full 576 amino acids of colicin E7 revealed only a distant relationship to a known allergenic protein from ragweed (*Ambrosia artemisifolia*; 46.7%

identity). A more specific bioinformatic comparison at the 80-mer level (sliding window of 497 80-mers in 576 aa) revealed 6 sequences with similarity, the highest being 45.2%. An even more precise search at the 8-aa level revealed one match to a subtilisin precursor peptide (AKAKLDKE); not a food allergen. In sum, one 8-amino acid sequence in 576 amino acids in colicin E7 matched that of a non-food allergen, with no matches to known food allergens. It is not known whether the sequence was an allergenic epitope or simply a sequence in the protein. No sequences were homologous by 50% or more. Because colicin E7 is susceptible to heat, acid and enzymatic hydrolysis upon ingestion, the allergenic potential of colicin E7 should be considered low.

Colicin Ia. A bioinformatic FASTA search of the full 626 amino acids of Colicin Ia revealed only a distant relationship to a known allergenic protein (tropomyosin) from the German cockroach *Blattella germanica* (30.1% identity). A more specific bioinformatic comparison at the 80-mer level (sliding window of 547 80-mers in 626 aa) revealed no (zero) matches with greater than 35% identity. An even more precise search at the 8-aa level revealed no (zero) exact matches. In sum, no amino acid sequences in colicin Ia are found to match known proteinaceous food allergens and hence the allergenic potential of colicin Ia should be considered very low.

Colicin M. A bioinformatic FASTA search of the full 271 amino acids of colicin M revealed only a distant relationship to a known allergenic protein from the fungus *Fusarium* (27.9% identity). A more specific bioinformatic comparison at the 80-mer level (sliding window of 191 80-mers in 271 aa) revealed no (zero) matches with greater than 35% identity. An even more precise search at the 8-aa level revealed no (zero) exact matches. In sum, no amino acid sequences in Colicin M are found to match known proteinaceous food allergens and hence the allergenic potential of Colicin M should be considered very low.

Colicin N. A bioinformatic FASTA search of the full 387 amino acids of colicin N revealed only a distant relationship to a known allergenic protein from the common olive tree pollen (*Olea europaea*; 30.6% identity). A more specific bioinformatic comparison at the 80-mer level (sliding window of 308 80-mers in 387 aa) revealed no (zero) matches with greater than 35% identity. An even more precise search at the 8-aa level revealed no (zero) exact matches. In sum, no amino acid sequences in colicin N are found to match known proteinaceous food allergens and hence the allergenic potential of colicin N should be considered very low.

Colicin K. A bioinformatic FASTA search of the full 548 amino acids of colicin K revealed only a distant relationship to a known allergenic protein, pollen from common ragweed (*Ambrosia artemisifolia*; 35.4% identity). A more specific bioinformatic comparison at the 80-mer level (sliding window of 469 80-mers in 548 aa) revealed 18 similar sequences, but all at less than 31.1% identity. An even more precise search at the 8-aa level revealed no (zero) exact matches. In sum, no amino acid sequences in colicin K are found to match known proteinaceous food allergens and hence the allergenic potential of colicin K should be considered very low.

Colicin U. A bioinformatic FASTA search of the full 619 amino acids of colicin U revealed only a distant relationship to a known allergenic protein, pollen from common ragweed (*Ambrosia artemisifolia*; 43.2% identity). A more specific bioinformatic comparison at the 80-mer level (sliding window of 540 80-mers in 619 aa) revealed no (zero) matches with greater than 35% identity. An even more precise search at the 8-aa level revealed no (zero) exact matches. In sum, no amino acid sequences in colicin U are found to match known proteinaceous food allergens and hence the allergenic potential of colicin U should be considered very low.

Colicin 5. A bioinformatic FASTA search of the full 490 amino acids of colicin 5 revealed only a distant relationship to tropomyosin from the insect *Lepisma saccharina* (28.0% identity). A more specific bioinformatic comparison at the 80-mer level (sliding window of 411 80-mers in 490 aa) revealed 25 hits, but no (zero) matches with greater than 35% identity. An even more precise search at the 8-aa level revealed only two exact peptide matches (AALAKAKS; ALAKAKSQ) to domains in the 100 kDa paramyosin protein from *Haliotis discus* (abalone). Thus, two 8-aa sequences in 490 amino acids in colicin 5 match sequences in a known proteinaceous food allergen. However, the immunodominant domains in paramyosin have not been reported and hence it is not known whether the sequences are allergenic epitopes or simply constituents in common between the two proteins. Because colicin 5, like other colicins, is heat, acid and protease labile and is not expected to survive digestion, the allergenic potential of colicin 5 should be considered low.

Colicin B. A bioinformatic FASTA search of the full 570 amino acids of colicin B revealed no (zero) similarities to known allergenic proteins. A more specific bioinformatic comparison at the 80-mer level (sliding window of 432 80-mers in 570 aa) revealed no (zero) matches with greater than 35% identity. An even more precise search at the 8-aa level revealed no (zero) exact matches. In sum, no amino acid sequences in colicin B are found to match known proteinaceous food allergens and hence the allergenic potential of colicin B should be considered very low.

Conclusion. In the nine colicin proteins analyzed for sequence identity, comprising a total of 4,550 amino acids, only three 8-aa peptide sequences matched sequences in known proteinaceous allergens. Of those, one sequence in colicin E7 was related to a subtilisin precursor (not a food allergen), and two sequences in colicin 5 matched those of an abalone protein. In neither case was there a confirmation of similarity to allergenic epitopes in the known allergens, only a peptide sequence identity in the whole protein.

Hence, the potential for allergenicity/hypersensitivity of colicins is considered low to very low. This low risk is corroborated by the lack of reports in the literature linking colicins with allergenicity or hypersensitivity development.

Furthermore, intact colicin proteins are not expected to survive stomach acid or digestive enzymes once ingested and the low MW peptides generated during digestion are not known to interact with the immune system in deleterious ways, further lowering the concern over allergenicity.

A.3 Suitability of Use

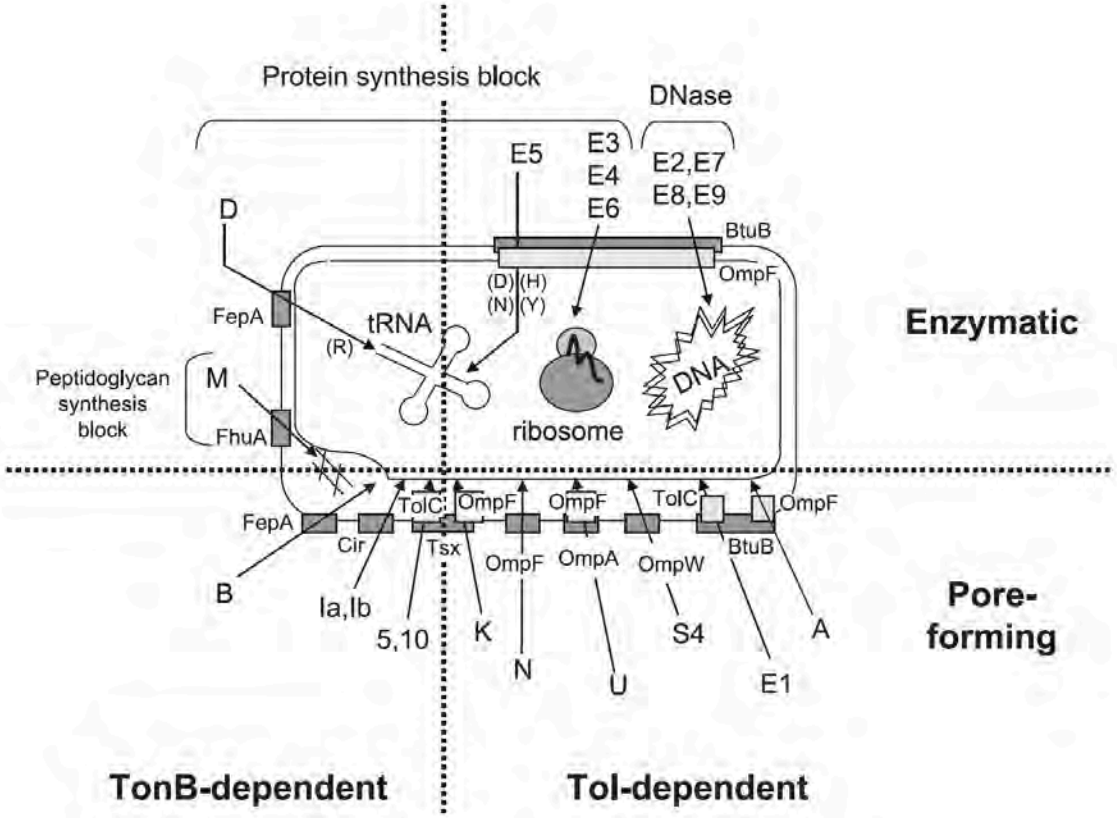
A.3.1 Biological activity of COLICIN on target pathogenic *E. coli* strains

Structure and mechanism of action underlying potency

Colicins are a group of bacteriocin-class antimicrobial proteins produced by and effective against *Escherichia coli* and closely related bacteria. Colicins are classified based on their mode of bactericidal activity, their membrane receptor, and by the mechanism they utilize for translocation through the outer membrane and across the periplasmic space of Gram-negative bacteria (see Table A-1 for full listing).

Cascales (Cascales 2007) provided a comprehensive review on colicins and their biological and ecological functions. A graphic depiction of colicins' mode of antibacterial action is shown in Figure A-3.

Figure A-3. Reception, translocation and mode of action of colicins on target bacterial cells



Schematic summary of reception, translocation, and mode of action of most studied colicins (from Cascales 2007). Colicins are distinguished by their general modes of action (upper section, enzymatic; lower section, pore forming) and transit machineries (right section, TonB; left section, Tol) separated by dotted lines. For each colicin (the name is indicated at the arrow base), the outer membrane protein used for the reception step (and sometimes for outer membrane translocation) (ColB, D, Ia, Ib, M, and N) and the OM protein involved in the translocation step (OmpF, colicins A, E2 to E9, K, and U; TolC, colicins 5, 10, and E1) are indicated. For enzymatic colicins, the mode of action (peptidoglycan synthesis block [colicin M], protein synthesis block by cleavage of tRNA [colicins D and E5] or 16S rRNA [colicins E3, E4, and E6], and DNA degradation [colicins E2 and E7 to E9]) is also indicated. For tRNA colicins (colicins D and E5), the specific tRNA targeted is indicated by the one-letter code in parentheses (D, aspartate; H, histidine; N, asparagine; R, arginine; Y, tyrosine).

Range of activity of plant-produced colicins against pathogenic strains of *E. coli*

Colicin proteins were evaluated for bactericidal activity at the levels of purity expected for the commercial COLICIN product. Examples of biological activity in intended applications ("suitability") of plant-produced colicins in controlling the growth of target pathogens are provided. Plant-produced colicins were evaluated for activity against indicator strains as well as pathogenic *E. coli* strains relevant to food contamination, using laboratory settings as well as treatment of intended foods such as fruits and vegetables.

Colicins produced in plants that may be formulated singly or in combination into Notifier's COLICIN product, and their modes of antibacterial action, are summarized in Table A-5.

Table A-5. Plant-produced colicins comprising components of COLICIN product

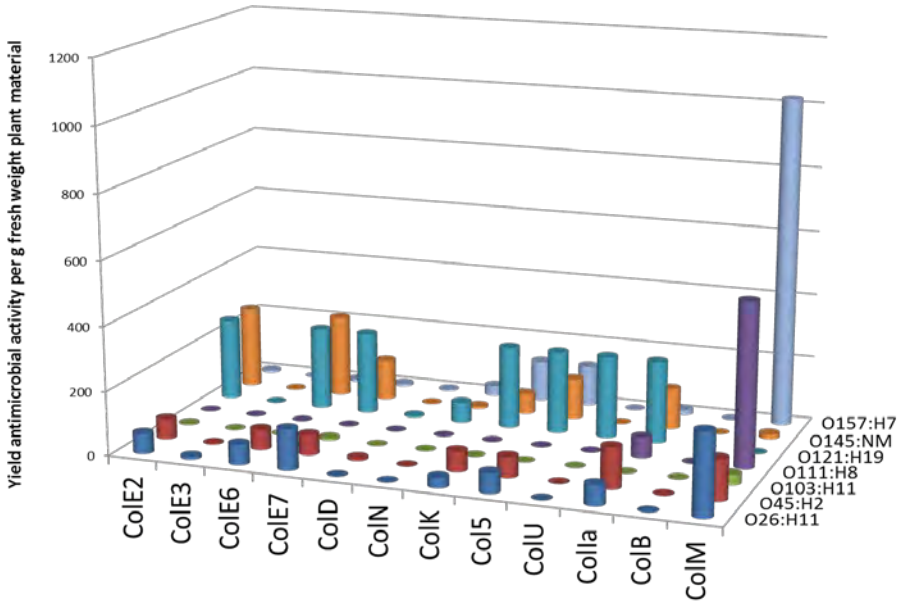
Colicin	Mode of Action	Receptor / Translocator	Targets
E1	Pore-forming	BtuB / TolC, TolAQ	ETEC/EHEC/STEC
E7	DNase	BtuB / OmpF, TolABQR	ETEC/EHEC/STEC
Ia	Pore-forming	Cir / TonB-ExbBD	ETEC/EHEC/STEC
M	Peptidoglycanase	FhuA / TonB-ExbBD	ETEC/EHEC/STEC
N	Pore-forming	OmpF / TolAQR	ETEC/EHEC/STEC
K	Pore-forming	Tsx / OmpAF, TolABQR	ETEC/EHEC/STEC
U	Pore-forming	OmpA / OmpF, TolABQR	ETEC/EHEC/STEC
5	Pore-forming	Tsx / TolC, TonB-ExbBD	ETEC/EHEC/STEC
B	Pore-forming	FepA / TonB-ExbBD	ETEC/EHEC/STEC

Figure A-4 shows the relative antibacterial activity per gram fresh weight of plant material against *E. coli* "Big Seven" pathogenic serotypes. These results clearly show that colicins exhibit target strain specificity.

Notably, Colicin M shows activity against 6 of the strains tested, with very high activity against O157:H7. Colicin E7 shows high potency against 4 of the targets, most notably against O121:H19, O45:H2 and O26:H11.

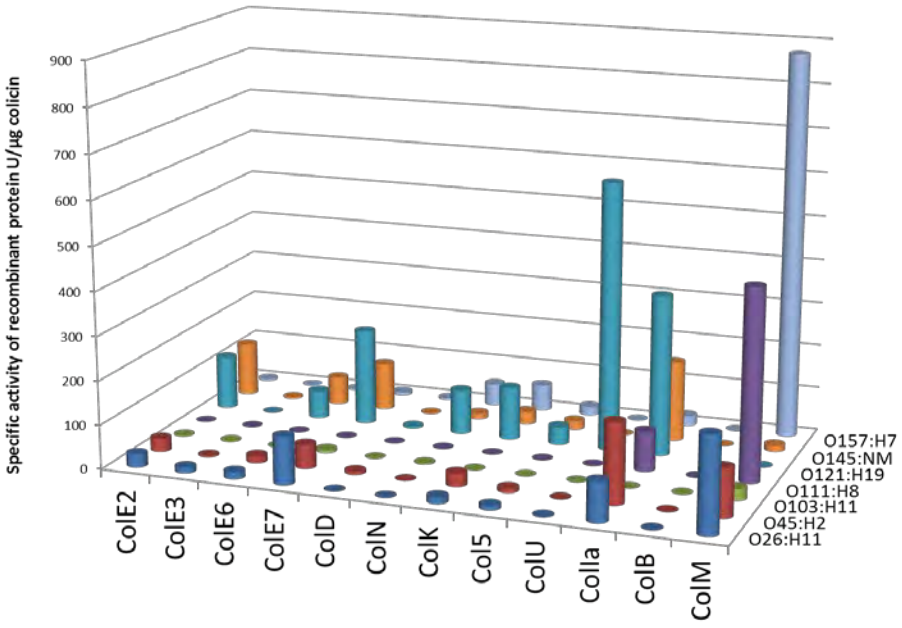
Other colicins provide complementary activity against other strains. Specificities and potencies are further illustrated when the data are plotted as specific activities (units normalized per mg protein) against target strains. Results are shown in Figure A-5.

Figure A-4. Yield of antibacterial activities in extracts of plant-produced colicins against pathogenic ("Big Seven") strains of *E. coli*.



Colicin activity is represented by bar height; tested recombinant colicins and *E. coli* strains are indicated. Values are relative activity units based on soft-agar clearing zone assays.

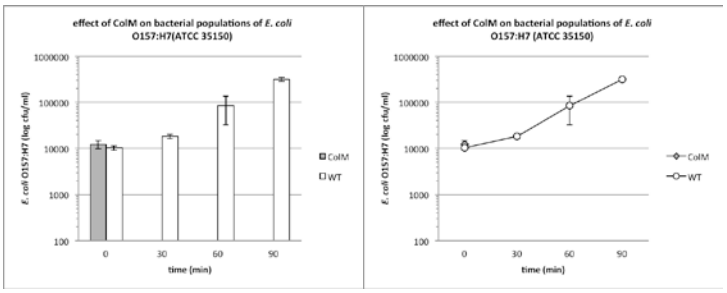
Figure A-5. Specific activities (U/mg protein) of plant-produced colicins against pathogenic ("Big Seven) strains of E. coli.



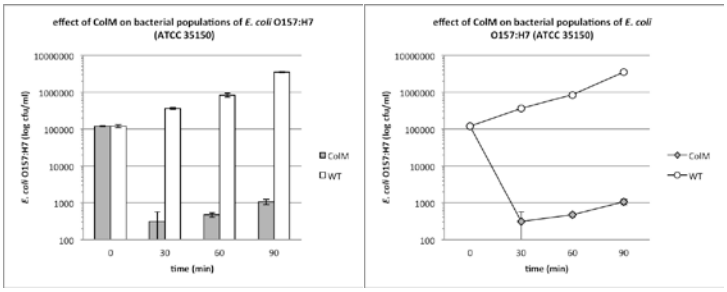
Colicins show differential potencies against target strains of *E. coli.* For example, colicin M is highly active against O157:H7; its activity against various STEC strains was further characterized, with results summarized in Figure A-6.

Figure A-6. Representative antibacterial activities of colicin M against various strains of *E. coli*.

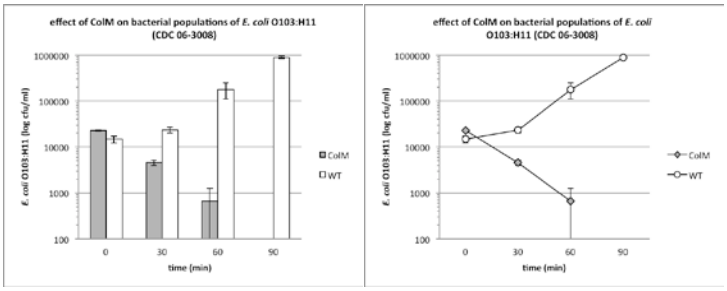
E. coli O157:H7 exposed to Colicin M at 20 μg/mL



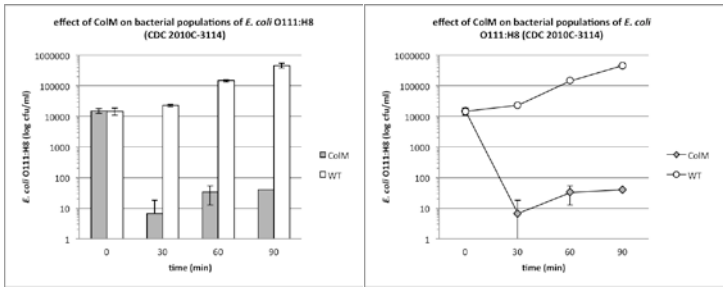
E. coli O157:H7 exposed to Colicin M at 1 μg/mL



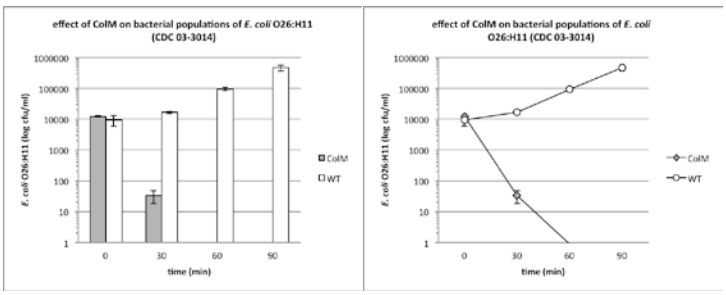
E. coli O103:H11 exposed to Colicin M at 20 μg/mL



E. coli O111:H8 exposed to Colicin M at 5 μg/mL



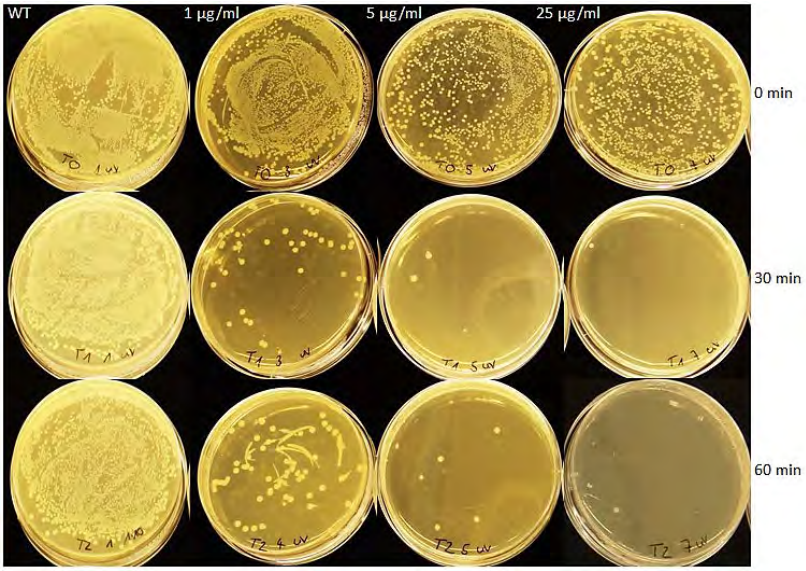
E. coli O26:H11 exposed to Colicin M at 5 μg/mL



Bacterial growth in liquid LB medium supplemented with plant extracts containing colicin M are shown as grey bars (left panels) or closed circles (right panels); growth in LB medium with extracts from wild-type control plants not expressing colicin (WT) are shown as white bars (left panels) or open circles (right panels).

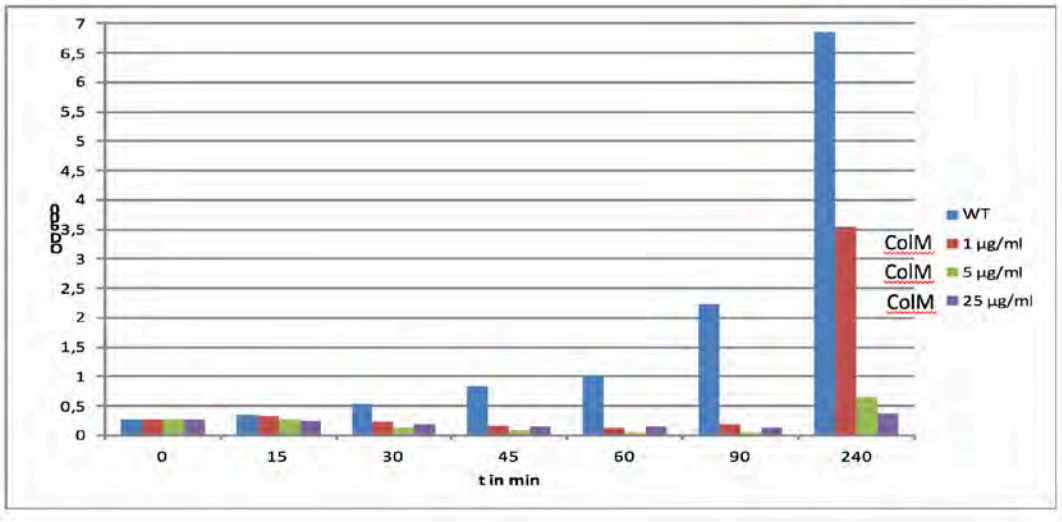
Dose titration experiments were conducted to further characterize colicin M's activity against O157:H7. Figure A-7 shows images of the effects of various concentrations of plant-produced colicin M on growth inhibition of O157:H7 (0, 30 and 60 min shown). Inhibition ranged from 3-5 log CFU relative to control (WT, without colicin treatment, left column). Likewise, Figure A-8 shows that colicin M causes a drastic reduction in the growth of O157:H7 in broth culture at all concentrations of colicin evaluated and at all analyzed time points.

Figure A-7. Antibacterial activity of colicin M against *E. coli* O157:H7.



Exposure of the pathogen in suspension culture to 0, 1, 5 and 25 µg/ml (0-25 ppm) of colicin M-containing plant extracts results in rapid multi-log reductions in CFU; 0, 30 and 60 min exposures shown (WT, wild-type plant extract controls without colicin).

Figure A-8. Colicin M inhibition of *E. coli* O157:H7.



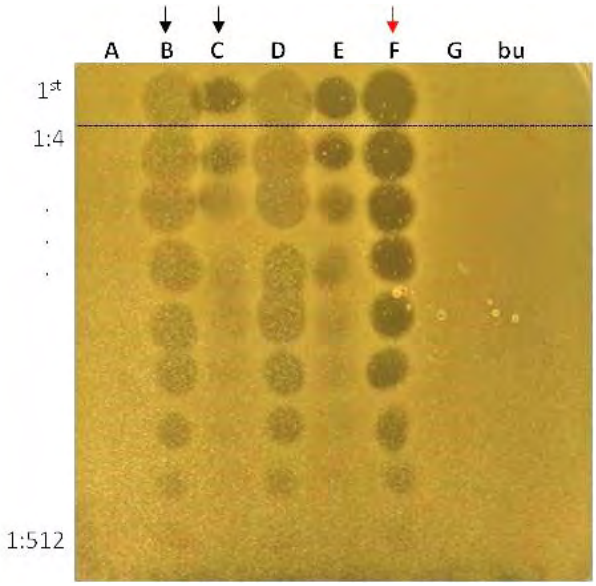
Exposure of the pathogen in suspension culture to plant extracts containing 0, 1, 5 and 25 µg/ml of colicin M (0-25 ppm; 1st-4th bar in each series) results in rapid and significant growth inhibition at all time points tested; 0-240 min shown (WT, exposure to extracts from wild-type plants without colicin as controls).

Synergy among colicins

Because colicins exert their antibacterial action through a variety of mechanisms, mixing or blending different colicins can potentially increase the potency of the mix by introducing multiple simultaneous modes of attack on target cell components. The additive, and sometimes synergistic, effects of mixing colicins with complementary modes of action have been verified. These results support the use of multicomponent COLICIN formulations.

Colicin M and colicin E7. Colicin M exhibits synergy when used in combination with other colicins. This is particularly notable with combinations of colicin M and colicin E7 against *E. coli* "Big Seven" strains. Figure A-9 shows clearing zones in a lawn of mixtures of pathogenic *E. coli* strains after exposure to serial dilutions of colicins. Enhanced killing activity is evident when mixtures of equal amounts of colicin M and colicin E7 are applied relative to equal amounts of each antibacterial protein applied individually.

Figure A-9. Synergy among colicins against pathogenic strains of *E. coli*.



Equal numbers (approximately 10^4) of STEC *E. coli* strains were mixed and plated. Serial dilutions of individual colicins or colicin mixtures in plant extracts were applied. Clearing zone diameter and color indicate degree of antibacterial potency. Lane B, colicin M applied singly. Lane C, colicin E7 applied singly. Lane F, colicin M + colicin E7 applied as a mixture in equal amounts of each protein. Lane G, bacteria treated with control extract from wild-type (WT) plants without colicins. Enhanced killing by colicin M + colicin E7 (Red arrow, Lane F, clearing zone diameter and color) is observed.

Similarly, Table A-6 and Table A-7 show the individual antibacterial activity of colicin M and colicin E7 (Table A-6) and mixtures of these two colicins at various ratios (Table A-7) against STEC strains (*E. coli*). Approximately 10^4 CFU/ml of each bacterial strain were exposed in liquid culture to the indicated concentrations of colicins for 90 min. Serial dilutions of the exposed cultures were then plated and counted after incubation at 37 °C.

Table A-6. Antibacterial activity of colicin M and colicin E7 applied individually to STEC strains

STEC Strain	Individual colicin and dose (μg/ml; ppm)	Ratio of viability reduction (log CFU control : colicin treated)
O121:H19	Col E7 (10)	2.7
O145:NM	Col E7 (10)	0.8
O103:H11	Col M (7.5)	2.6
O45:H2	Col M (7.5)	2.7
O111:H8	Col M (3.75)	4.1
O26:H11	Col M (3.75)	5.0
O157:H7	Col M (1.0)	3.5

Table A-7. Antibacterial activity of colicin M and colicin E7 applied as mixtures to STEC strains

STEC Strain	Colicin : Colicin Mixture (μg/ml of each colicin; ppm)	Ratio of viability reduction (log CFU control : colicin treated)
O121:H19	Col M : E7 (1:1)	2.8
O145:NM	Col M : E7 (1:1)	1.1
O103:H11	Col M : E7 (7.5:0)	2.6
O45:H2	Col M : E7 (7.5:0)	2.7
O111:H8	Col M : E7 (0.5:0.5)	5.2
O26:H11	Col M : E7 (0.5:0.5)	4.4
O157:H7	Col M : E7 (0.25:025)	3.6

As clearly shown in this series (i.e. Table A-6 and Table A-7), colicin M and colicin E7 can individually reduce the viability of target pathogenic *E. coli* strains, as well as show synergy of antibacterial potency when used as mixtures. Reductions in cell viability ranged from one to multiple log CFU relative to control bacteria exposed to plant extracts not containing colicins.

Colicin M, colicin E7, colicin B, colicin K and colicin 5. Based on the results obtained with colicins M and E7, and capitalizing on the differential antibacterial activity of various individual colicins, mixtures of colicins M, E7, 5 and K were also evaluated for enhanced activity against pathogenic *E. coli*. Synergistic activity was confirmed using samples of produce (apple slices) and tester strain *E. coli* O157:H7 (see next section).

Conclusion. From Notifier's own scientific studies on colicins M, E7, E1, B, K, U, 5 and others, and from the published literature on colicins, we conclude that colicins can effectively reduce the viability of enteropathogenic strains of *E. coli* at concentrations that are relevant in food contamination and intervention scenarios. Further, we have confirmed that the different modes of antibacterial action of individual colicins enable formulations of mixtures of colicins with enhanced potency and host range.

A.3.2 Suitability of COLICIN for use in vegetables and fruit

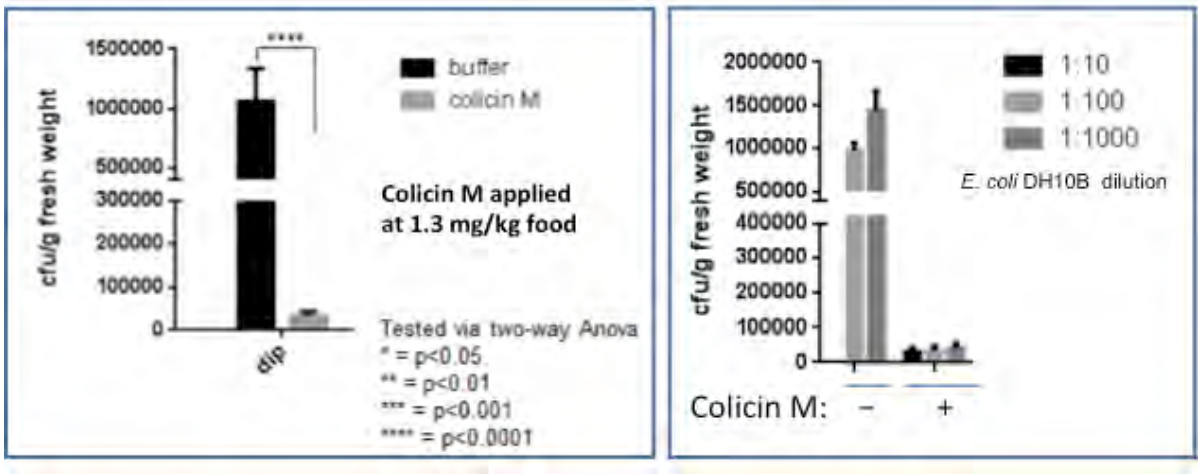
COLICIN on leafy vegetables applied as produce dip wash or spray

Plant-produced colicins were also tested for antibacterial activity on samples of produce to determine the product's suitability. For example, **fresh lettuce** and **arugula** were contaminated with *E. coli* tester strain DH10B as the indicator organism. The bacterial population in a suspension culture (CFU/ml) was determined by plating on agar medium.

With **lettuce**, the bacterial uptake contaminating the produce was determined to be 1.25 x 10^6 CFU per g food. The produce was then sprayed with a solution of colicin M as a representative colicin at an exposure level of 1.3 mg colicin M per kg of food; exposure to buffer served as the control. Samples of the exposed produce were collected and incubated to determine bacterial growth.

The results of **lettuce** treatment are summarized in Figure A-10. Bars show growth of bacteria in the controls but significant reductions in viability (>3 log CFU) in the colicin M-treated groups.

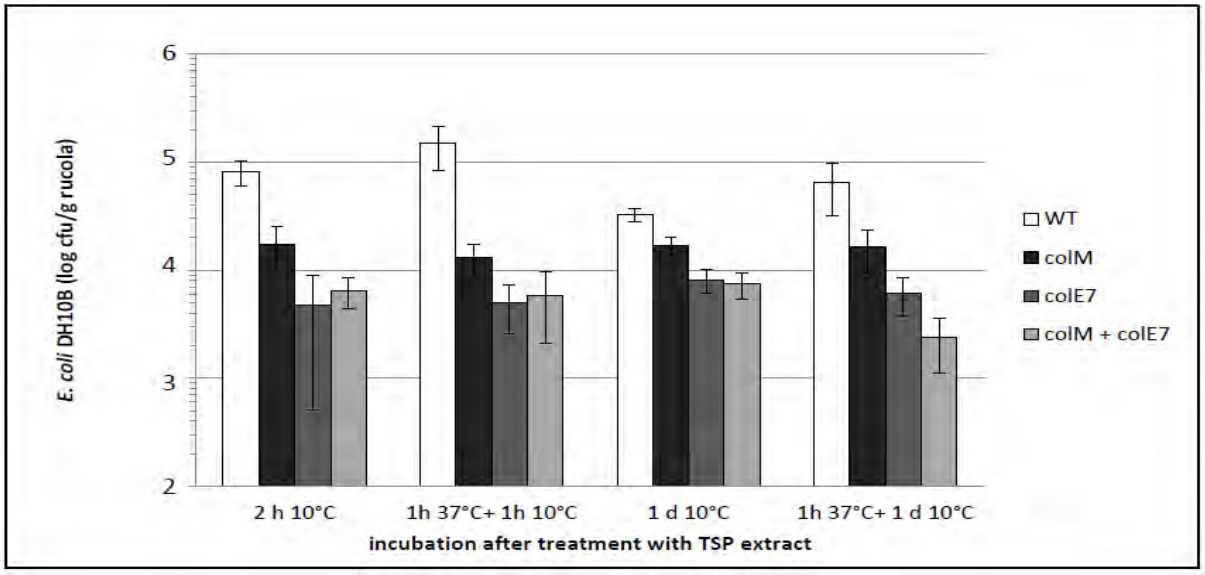
Figure A-10. Antibacterial activity of COLICIN on samples of lettuce contaminated with *E. coli* DH10B as the indicator organism



Lettuce samples (200 g) were dipped in a bacterial suspension that resulted in a contamination level of 1.25 x 10^6 CFU/g food. The samples were then sprayed with buffer control or with a solution containing colicin M; exposure was at a rate of 1.3 mg colicin M/kg produce. Differential counts on serial dilutions were performed. Results indicate significant reductions in viable cells (>3 log CFU) in the colicin-treated samples relative to buffer-exposed controls.

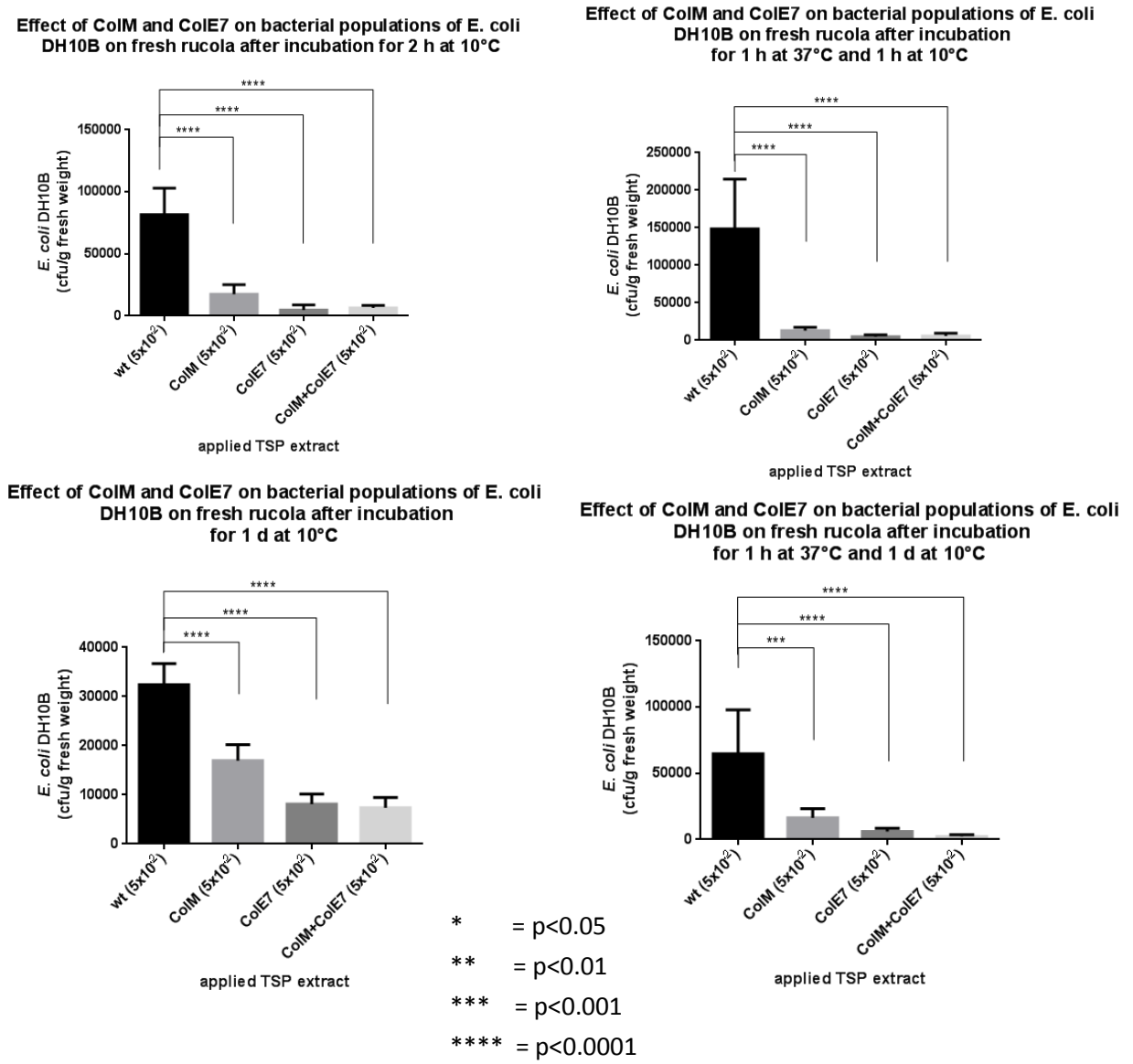
With **arugula** (a.k.a. rucola; rocket), weighed leaf samples were contaminated with 1.0 x 10^6 CFU/g of strain DH10B and exposed to 1 mg/kg semipurified colicin M extracts by dip wash. Dip washing is a preferred procedure for vegetables with corrugated surfaces, to ensure more effective disinfection. Exposures were conducted for various times and at different holding temperatures. Results are summarized in Figure A-11 and Figure A-12.

Figure A-11. Antibacterial activity of COLICIN on samples of arugula contaminated with *E. coli* DH10B as the indicator organism.



Arugula samples were dipped in a bacterial suspension that resulted in a contamination level of 1 x 10^6 CFU/g food. The samples were then washed by dipping in buffer containing colicins. The buffer was drained, and the samples were incubated for various times at different temperature regimes as shown in the panels. Exposure to colicin M was at 1 mg/kg produce. Differential counts on diluted samples were performed. Results indicate that growth was delayed in cold-stored samples (10 °C), but ensued at high-low temperature shifts in the buffer-treated control samples (white bars, first in each series). Washing alone reduced the contaminant by 1 log (10^5 CFU/g; white bars). Colicin M-treatment reduced the number of viable cells by one additional log (10^4 CFU/g; black bars, second in each series) across all temperature regimes tested. Colicin E7 was also tested for comparison, singly (third bar in series) and in combination with colicin M (fourth bar in series). Additive effects can be observed.

Figure A-12. Statistical analysis of results from COLICIN antibacterial treatment of contaminated arugula (rucola)



In every case a significant reduction in *E. coli* DH10B viability was observed after treatment with COLICIN solution. For comparison, colicins M and E7 were included singly or in combination to observe the additive potency of the two colicins. Statistical significance was determined by one-way Anova followed by post-hoc Dunnets test.

COLICIN on RTE fruit segments applied as a spray

Plant-produced colicins were also tested on fruits when applied as a spray. In these demonstrations of suitability, ready to eat (RTE) cut segments of **apples** and **cantaloupe** were infected with *E. coli* O157:H7 as an indicator pathogen and then sprayed with either a two-component (colicins M + E7) or a five-component (colicin M + E7 + K + B + 5) COLICIN formulation and the results compared to no treatment and to spraying a control vehicle solution containing plant extract without colicins.

The methods employed to assess suitability in fruit are described in APPENDIX C. Briefly, whole fruits were surface sterilized with 200 ppm sodium hypochlorite solution, rinsed in sterile water and cut into pieces using aseptic techniques. To develop analytical methods, *E. coli* tester strain DH10B was used first and the experiments were repeated with O157:H7. Fruit segments were dipped into a suspension of DH10B at ~$5x10^5$/ml density, resulting in a calculated inoculum of ~$1x10^4$/g fruit, or with O157:H7 as indicated below.

Fruit segments were then treated with control solution or sprayed with colicin-containing solutions comprised of total soluble protein (TSP) from the plant host. Samples of fruit were then pooled and homogenized, and aliquots were extracted in peptone water, diluted and plated to assess bacterial growth. No background bacteria were present in the fruit after sterilization; thus, all bacterial growth was due to inoculation with the tester strains.

Reduction of *E. coli* O157:H7 on fresh-cut RTE pieces of melon. The preparation of fruit pieces was done as described above. Serotype O157:H7 (strain DSM19206) inoculum culture used was ~1×10^5 cfu/ml, which after dipping, resulted in a measured actual load of ~2×10^3 cfu inoculated per g fruit. Treatments consisted of either carrier-only treatment control (colicin-free WT plant extract); two-component COLICIN treatment of 3+1 mg/kg fruit (TSP colicin M + colicin E7 mixture); or five-component COLICIN treatment 3+1+1.3+0.3+0.3 mg/kg fruit (TSP colicins M + E7 + K + B + 5). Samples were generated in 4 replicates for each timepoint of analysis and treatment by pooling 3 fruit pieces, which were analyzed for microbial populations upon 1h, 1d and 3d storage at 4°C (no effect of treatments on appearance of fruit pieces was observed) by dilution plating upon homogenization with 5 volumes of peptone water.

Statistically significant reduction of O157:H7 CFU of up to ~1 log were observed for all timepoints comparing carrier-treated cantaloupe melon pieces and colicin-treated melon pieces.

Results of COLICIN spray treatment of RTE cantaloupe melon segments are summarized in Figure A-13.

Reduction of *E. coli* O157:H7 on fresh-cut RTE pieces of apple. A similar exposure study to the one described above with RTE melon was performed on Golden Delicious apples. For this series, the density of the O157:H7 (strain DSM19206) inoculum was set to OD_{600}=0.005 (~5×10^5 cfu/ml) resulting in a measured actual load of ~1×10^4 CFU/g fruit, and the pooling of 4 apple pieces per sample. A statistically significant difference between carrier-only control treatment and colicin treatment was observed upon 1h, 1d and 3d storage at 4°C.

Results of COLICIN spray treatment of RTE apple segments are summarized in Figure A-14.

Figure A-13. Reduction of *E. coli* O157:H7 (DSM19206) in contaminated fresh-cut RTE melon pieces by spray treatment with two-component and five-component COLICIN product

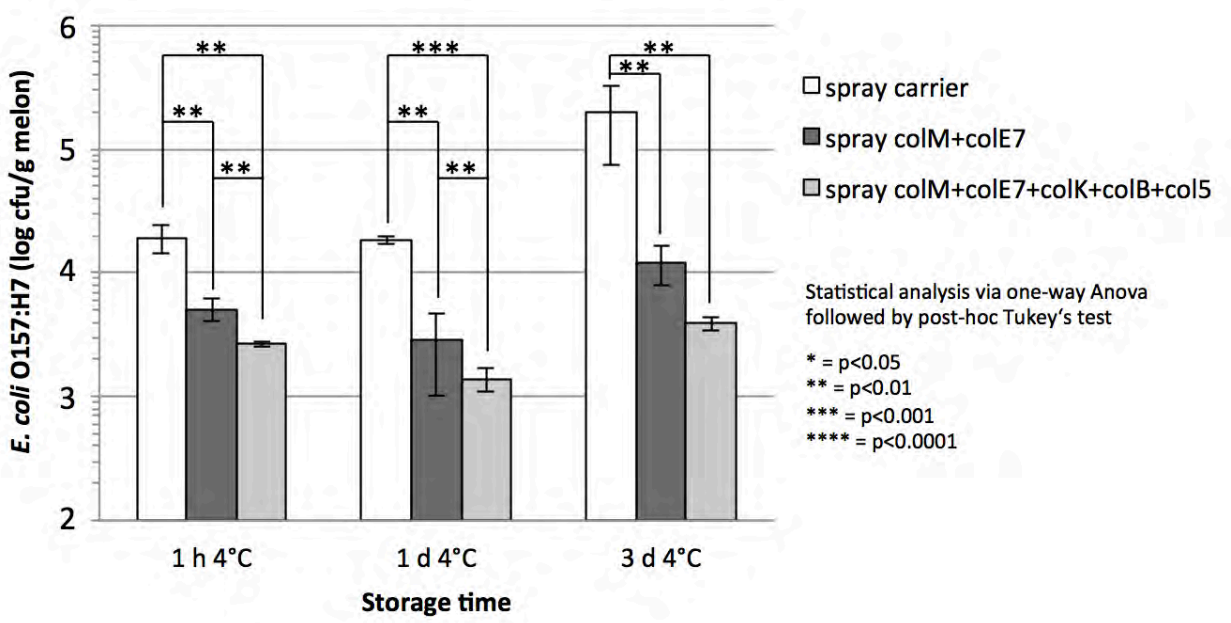
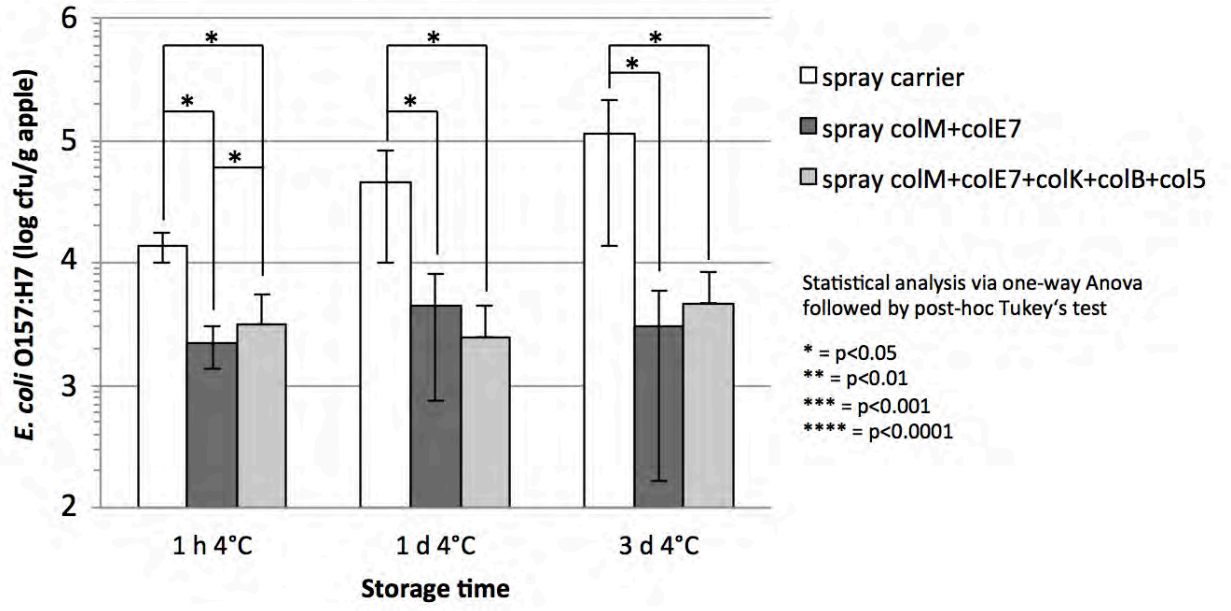


Figure A-14. Reduction of *E. coli* O157:H7 (DSM19206) in contaminated fresh-cut RTE apple pieces by spray treatment with two-component and five-component COLICIN product



A.4 Overall Conclusion

Extensive reviews of the published literature on colicins were conducted and pertinent safety, molecular characteristics and suitability results were complemented by Notifier's own findings. Colicins' selective mode of action against pathogenic bacteria, the presence of these proteins in the human intestine through synthesis by commensal strains of enteric bacteria, their instability in the upper gastrointestinal

environment upon ingestion, the safety of the plant-based manufacturing process, and the anticipated levels of use on food, support the safe use of plant-produced colicin proteins on produce (vegetables and fruits) with the goal of controlling *E. coli* contamination.

There is a long history of safe and natural gastrointestinal exposure to colicin proteins in healthy humans and animals. Colicins in general have been shown in cell culture models to be less toxic than a currently approved bacteriocin (nisin), and the intended use of our product would be at significantly lower levels than nisin. Cooking foods treated with colicins would ensure the proteins' inactivation. Even without cooking, ingested colicins are too large for intact absorption in the gastrointestinal tract. Ingestion of colicins would lead to the proteins' denaturation and digestion in the acidic environment of the stomach and the action of pepsin, and, in the unlikely event that intact colicins were to find their way to the intestine, their degradation by intestinal proteases would assure their destruction. Hence, the antimicrobial treatment of food products would not result in the presence of extraneous colicins in the gastrointestinal tract and consequently there would be no subsequent effects on the gut's resident microflora. Evidence of the digestibility and breakdown of colicins in simulated gastrointestinal environments is provided from the literature and from Notifier's own studies.

Furthermore, a bioinformatics analysis revealed that the multiple colicins analyzed have only a few amino acid sequences in common with proteins that are known food allergens, but that the sequences in common are not necessarily the allergenic epitopes, only shared peptide sequences between the proteins. Hence the potential for development of allergenicity/hypersensitivity through ingestion of colicins is judged to be of very low risk. No reports appear in the literature linking colicins with allergenicity/hypersensitivity.

The plant-based manufacturing process used to produce colicins is adapted directly from processes used to produce injectable vaccines and monoclonal antibodies that have shown safety in clinical studies under FDA IND regulatory oversight, indicating that neither the plant-produced biopharmaceuticals nor any process-derived impurities have been a source of concern, even when using non-food host plants from the genus *Nicotiana* (relatives of tobacco). In the production of colicins for use on food, food host plants such as spinach, lettuce or beet are used (all plant hosts are themselves "GRAS" by virtue of being food crops). The totality of scientific evidence therefore suggests that colicins produced by the plant-based manufacturing process described are safe for their intended uses at the levels proposed.

Individual colicins can be used singly or as components in a COLICIN product comprising a mixture of colicin proteins, each exhibiting complementary bactericidal activity and collectively yielding a broader-spectrum, higher potency product. Data are presented for individual colicins and mixtures of colicins in two-component and five-component formulations. In each case, potent antibacterial activity was quantified against relevant strains of enteropathogenic *E. coli*, including the Big Seven serotypes that are known to contaminate various foods.

Suitability for use of the COLICIN product on various produce (vegetables such as lettuce and arugula, and fruits such as melons and apples) is supported by the data presented, and the product was shown to be effective at low use levels (e.g. 1-10 mg colicin/kg food product, or 1-10 ppm).

Notifier asserts that the totality of evidence presented in this notification supports the classification of plant-produced colicin proteins that comprise COLICIN, individually and as mixtures, as Generally Recognized as Safe, and furthermore, that the product meets suitability criteria as an antimicrobial for treatment of produce (vegetables and fruits) under the conditions of use specified herein.

APPENDIX B. COLICIN Manufacturing Process

B.1 Introduction and Rationale

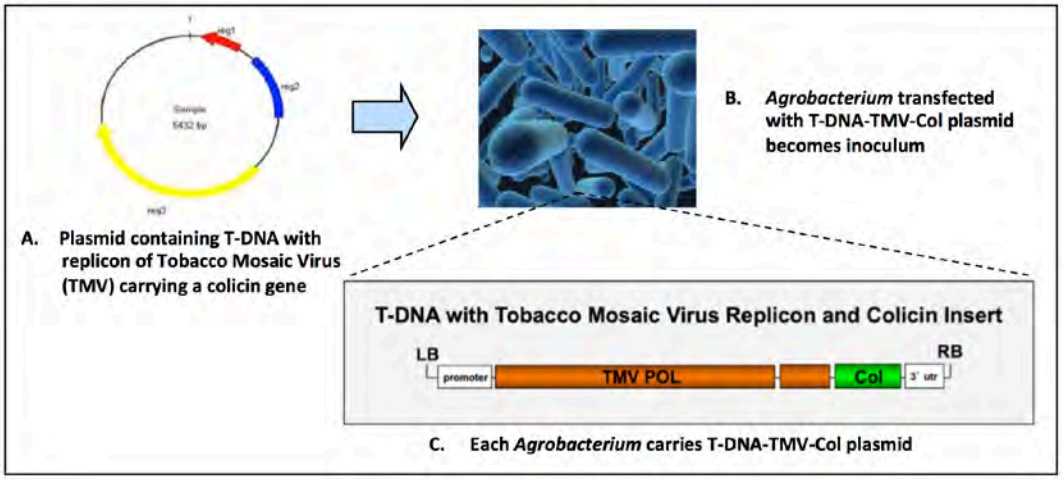
Notifier produces colicin proteins recombinantly using a plant-based manufacturing process. This approach minimizes concerns over toxicity of colicins to the producing host and offers a more scalable and cost-effective manufacturing option relative to fermentation. In Notifier's process, leaf tissue of the food plants *Beta vulgaris* (beet), *Spinacia oleracea* (spinach), or lettuce (*Lactuca sativa*) 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 food plants 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. A description of the process with respect to production of any colicin protein is summarized below.

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 (source: Nomad Bioscience)



B.3 Procedure

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

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

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 the ultra- and dia-filtration procedures during plant biomass extraction and processing. All raw materials and processing aids are food grade. A multi-vial Master Vector Bank of the vector is prepared and stored at -80°C, from which aliquots are removed as Working Vector Banks of the inoculum for each manufacturing batch.

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

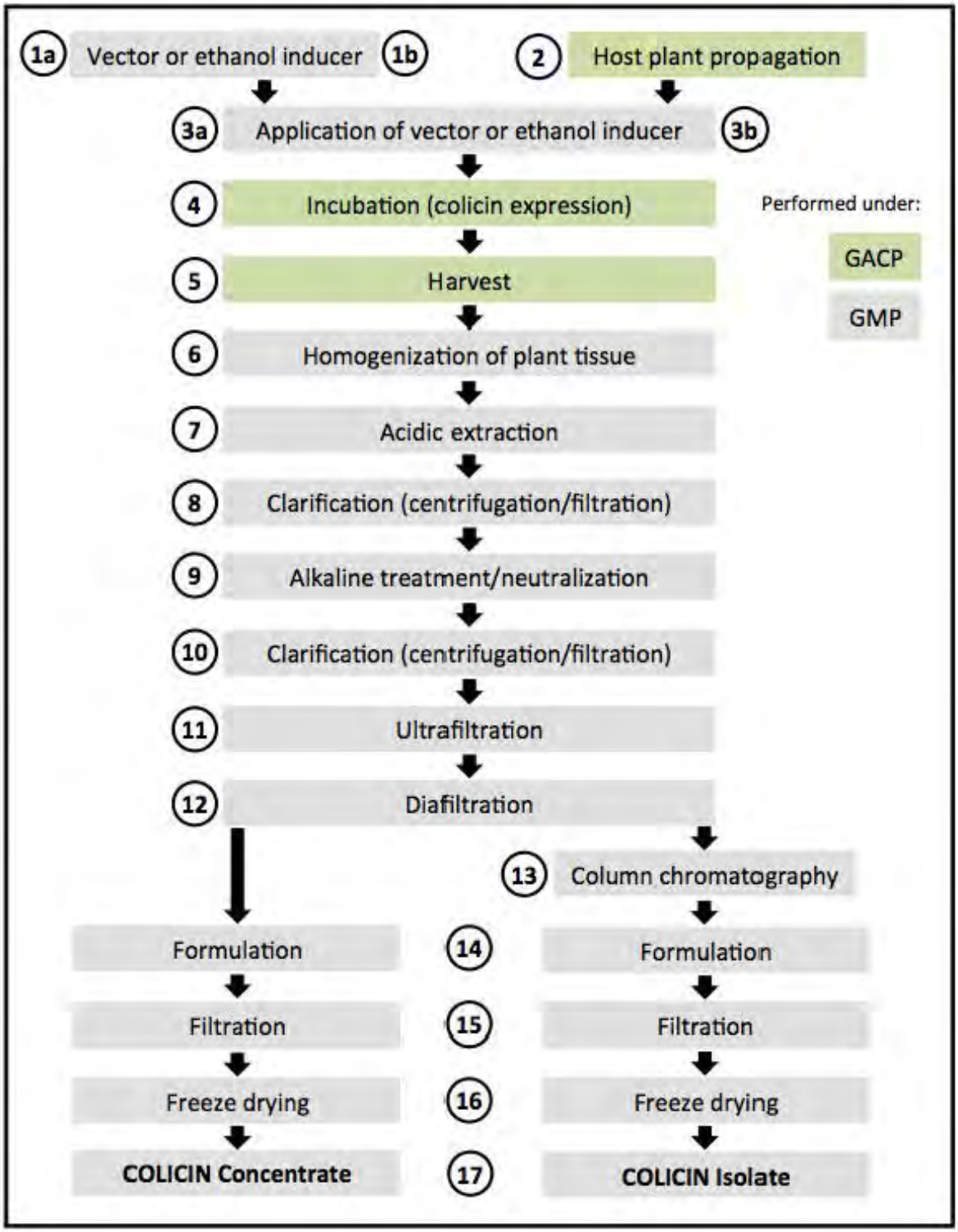
Step 1b. Ethanol induction of transgenic plants

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

Step 2. Host plant preparation

For agroinduction, normal seeds of *Spinacia oleracea* (spinach), *Beta vulgaris* (beet) or *Lactuca sativa* (lettuce) 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, 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; 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.

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 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, 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 and to meet this level of purity can be blended into a final solution that will be further processed into **COLICIN Isolate**. If a less purified bulk product will suffice for certain applications, the chromatography step is eliminated, and this solution (containing one or a blend of colicin proteins) is further processed into **COLICIN Concentrate**.

Steps 14 – 17. Formulation, fill and finish

The final **COLICIN Concentrate** or **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, or freeze dried to produce a dry, off-white to light tan powdered product. Prior to release, the bulk products are tested to ensure compliance with the respective final product specification for COLICIN Concentrate or 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 Specifications

Specifications for each grade of COLICIN produced by this process are shown in Table B-1 (**COLICIN Concentrate**) and Table B-2 (**COLICIN Isolate**).

Table B-1. Specification for COLICIN Concentrate Product

COLICIN Concentrate		
Parameter	**Specification limit**	**Method**
Appearance	Powder, beige to brownish	Visual
Specific Activity	>10,000 AU/g	Serial-dilution based assay
pH of a 1% solution	6.5-8.5	Potentiometric
Heavy metals (sum of Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, Sn)	≤30 ppm	USP38<233>
Lead	≤5 ppm	USP38<233>
Bioburden	≤5,000 CFU total per g	USP32<61>
Agrobacterium per 10 g sample	0 (absent)	Selective plate-based assay
Undesirable microorganisms, including *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp.* or coagulase-positive *Staphylococcus spp.*, per 25 g	0 (absent)	USP32<1111>
Stability (dry concentrate; 0-10°C)	>6 months	Specific activity by serial dilution-based assay

Table B-2. Specification for COLICIN Isolate Product

COLICIN Isolate		
Parameter	**Specification limit**	**Method**
Appearance	Powder, white to beige	Visual
Specific Activity	>25,000 AU/g	Serial-dilution based assay
pH of a 1% solution	6.5-8.5	Potentiometric
Heavy metals (sum of Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, Sn)	≤30 ppm	USP38<233>
Lead	≤5 ppm	USP38<233>
Bioburden	≤10 CFU total per 25 g sample	USP32<61>
Agrobacterium per 10 g sample	0 (absent)	Selective plate-based assay
Undesirable microorganisms, including *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp.* or coagulase-positive *Staphylococcus spp.*, per 25 g	0 (absent)	USP32<1111>
Stability (dry concentrate; 0-10°C)	>6 months	Specific activity by serial dilution-based assay

B.5 Manufacturing Facilities

Notifier can manufacture COLICIN 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 pilot – 68 mt/shift commercial
- 75 L of Green Juice (post-grind/pre-clarification extract) per minute
- Continuous processing prior to UF
- 35,000 L of tank storage capacity
- Heating, cooling of in-process material
- Manufacturing clean rooms with controlled environments
- Computer-controlled processing and data collection
- Clarification options (UF/DF/Microfiltration/Nanofiltration/Reverse Osmosis)

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

B.6 Waste Handling and Disposal

Waste streams containing plant-derived residuals are treated per local regulations and discarded. No by-products or residuals of the process are used in food or feed products, supplements, additives or treatment aids.

APPENDIX C. Methodology

The methods employed to assess the properties and characteristics of plant-made colicin proteins that are candidate components of the COLICIN product are described herein.

C.1 Methods for assessing digestibility of colicins in simulated gastric and intestinal fluids

Due to the amino acid sequence homology shared by plant-made colicins and the natural proteins produced by intestinal bacteria, plant-produced colicins are expected to show equal susceptibility as their bacterial counterparts to degradation by proteolytic enzymes.

To confirm, Notifier exposed several plant-made colicins that comprise the COLICIN product, including colicins M, E7, K and U, to simulated gastric and duodenal digestion conditions. These colicins were selected as representative examples of colicins based on their different and complementary modes of action.

Exposures of the proteins to Simulated Gastric Fluid (SGF, commercial acidic pepsin extract) and Simulated Intestinal Fluid (SIF, commercial neutral pancreatic extract of trypsin and α-chymotrypsin) were done using established methods and standardized conditions (Thomas 2004; Dupont 2010), including regulatory guidance for safety assessment of new proteins expressed in genetically modified crops intended for food or feed use (EFSA 2011).

In addition, sequential exposure studies using low enzyme-to-substrate ratios were conducted to increase the stringency and relevance of the digestibility assays. For these studies, the colicins were first exposed to either SGF without pepsin or SGF with pepsin, followed by exposure of the resultant hydrolysate products to SIF supplemented with trypsin and chymotrypsin (Moreno 2005; Mandalari 2009; Eiwegger 2006).

The rationale and justification for the exposure conditions and the ratios of enzymes:colicin used in these studies were provided in Section A.2.3.

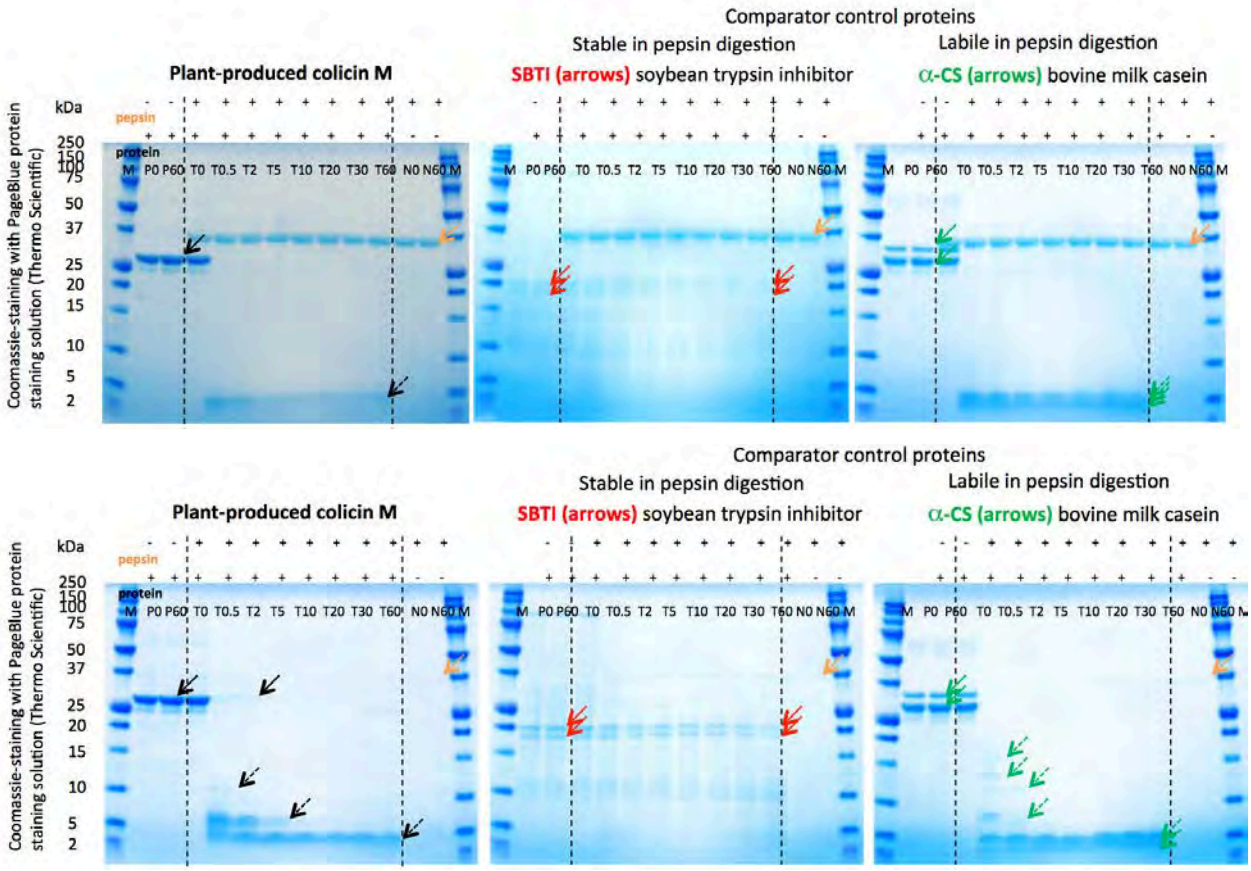
Digestion by Simulated Gastric Fluid

Methods were derived from the literature cited. Briefly, plant-produced individual colicins were mixed with SGF at the recommended concentration and incubated for up to 60 min, sampling every few minutes and assessing the digestion of the protein into fragments by SDS-PAGE. Coomassie and silver staining on gels were used to visualize protein decomposition and estimate the MW of peptide products.

Figure C-1 presents the results of SGF (gastric) digestibility using two concentrations of SGF and plant-produced colicin M as a model to illustrate the acceptability of the method. These results show that plant-produced colicin M is digested in 1-10 min upon exposure to different concentrations of SGF (with pepsin:substrate ratios ranging from ~3:1 to 1:11), simulating breakdown of the protein in the human stomach at various enzyme/protein ratios.

In addition to plant-made colicin M, the same methods were applied to assess digestibility of colicins E7, K and U. The results of these studies are shown in Section A.2.3.

Figure C-1. Digestion of plant-produced colicin M in Simulated Gastric Fluid



This study conclusively shows the digestion of plant-made colicin M as a representative colicin in **Simulated Gastric Fluid (SGF)** following established procedures for assessing protein stability. Assay conditions described for each series.

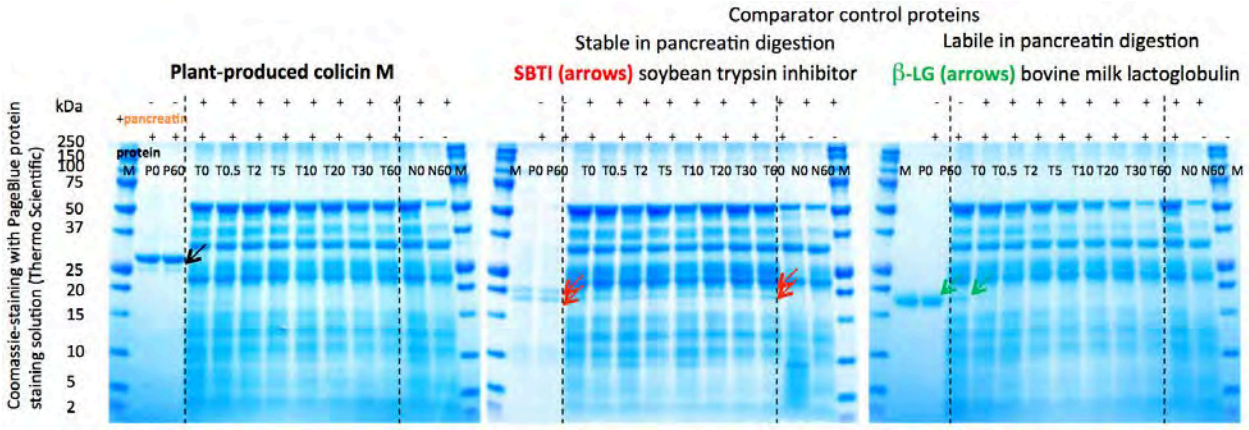
Top series, 10 U pepsin/µg protein; pepsin:protein 2.82:1 (wt:wt); 10-20% Mini-PROTEAN® Tris-Tricine gels (Biorad); M = Precision Plus Protein Dual Xtra Standards (Biorad) at 10 µl; 15 µl of each sample loaded per lane. Pepsin appears as the stable band between 25 and 37 kDa markers (orange arrow). **Left panel**, Colicin M band (black arrows) appears just above the 25 kDa marker at Time P0 and P60 (pretreatment in vehicle) and is shown to quickly degrade in approximately 0.5 minutes of exposure to SGF, with degradation fragments shown as faint bands accumulating during the 60-min incubation and stabilizing at 2 kDa (arrow). Comparator proteins (SGF-stable soybean trypsin inhibitor, **middle panel**, red arrows, and SGF-unstable bovine milk α-chymotrypsin, **right panel**, green arrows), added as stable and labile controls, respectively, show stability and instability upon exposure.

Bottom series, repeat of above experiments but using 0.33 U pepsin/µg protein, pepsin:protein ratio 1:10.8 (wt:wt).

Digestion by Simulated Intestinal Fluid

Similar experiments were performed using SIF to mimic digestion of colicin in the duodenum by pancreatic proteases. Two types of studies were conducted. In one series, plant-produced individual colicins were mixed with SIF at the recommended concentration and incubated from 60 to 180 min, sampling every few minutes and assessing the digestion of the protein into fragments by SDS-PAGE. Coomassie and silver staining on gels were used to visualize protein decomposition and to estimate the MW of peptide fragments. Figure C-2 presents the results of SIF (intestinal/pancreatic) digestibility using plant-produced colicin M as a model to illustrate the method. The results show that plant-produced colicin M is almost instantaneously digested by intestinal proteases.

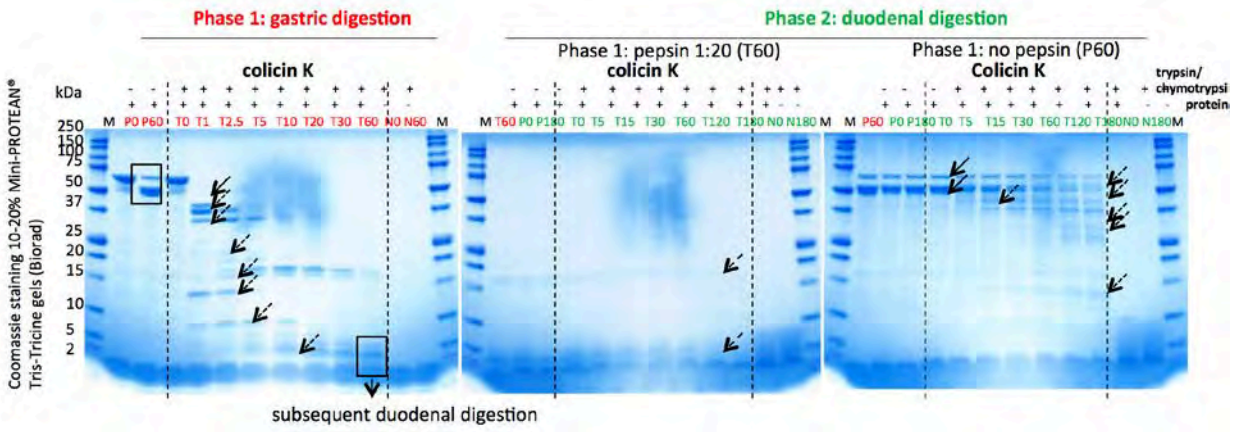
Figure C-2. Digestion of plant-produced colicin M in Simulated Intestinal Fluid



This study conclusively shows the digestion of plant-produced colicin M as a representative colicin in **Simulated Intestinal Fluid (SIF)** following established procedures for assessing protein stability upon simulated ingestion. Assay conditions included: 1% pancreatin mixture; pancreatin:protein 38:1 (wt:wt); 10-20% Mini-PROTEAN® Tris-Tricine gels (Biorad); M = Precision Plus Protein Dual Xtra Standards (Biorad) at 10 µl; 15 µl of each sample loaded per lane. **Left panel**, Colicin M band (black arrow) appears just above the 25 kDa marker at Time P0 and P60 (pretreatment in vehicle solution) and is shown to quickly degrade (almost instantaneously) just after T0 treatment upon exposure to SIF. No pancreatin-resistant peptides were detected for up to 60 min incubation. Comparator proteins (SIF-stable soybean trypsin inhibitor, **middle panel**, red arrows, and SIF-unstable bovine milk β-lactoglobulin, **right panel**, green arrows), added as stable and labile controls, respectively, show stability and instability upon exposure.

In a second series of studies, colicin breakdown products generated in SGF by pepsin digestion were subsequently exposed to SIF (with trypsin+chymotrypsin) to examine further breakdown of the products. Figure C-3 presents the results of SIF digestibility of peptidic degradation products of plant-produced colicin K as a model to illustrate the method.

Figure C-3. Sequential digestion of plant-produced colicin K in SGF followed by SIF



This study conclusively shows the sequential digestion of plant-produced colicin K as a representative colicin in **Simulated Gastric Fluid (SGF; Phase 1)** followed by **Simulated Intestinal Fluid (SIF; Phase 2)** using referenced procedures.

As shown in Figure C-3, exposure of colicin K to pepsin (1:20 pepsin:colicin wt:wt) in acidic SGF (Phase 1) results in the rapid breakdown of the protein to lower MW degradation products (indicated by arrows in the left panel). Sampling times are shown in minutes.

Resistant peptides from SGF hydrolysis (shown in **box at bottom of the left panel in Figure C-3**) were collected and subsequently incubated with SIF containing trypsin and chymotrypsin at a ratio of 1:4:400 (wt:wt:wt trypsin:chymotrypsin:colicin peptides). Two assay conditions were used: (a) exposure of colicin in Phase 1 to SGF without pepsin prior to treatment in Phase 2, or (b) exposure of colicin in Phase 1 to SGF+pepsin (1:20 wt:wt pepsin:protein ratio), followed by treatment in Phase 2. As shown in the figure, prior exposure to stomach pepsin facilitates further breakdown of peptidic products in simulated duodenum conditions (**center panel**), while exposure to SGF alone (no pepsin) prior to SIF leads to further breakdown of the peptides albeit at a slower rate (**right panel**).

These methods were applied to assess digestibility in SGF and SIF of colicins M, E7, K and U as representative components of the COLICIN product. The results are summarized in Section A.2.3.

Conclusion

Methods to demonstrate the digestion of colicins under simulated gastrointestinal conditions exist in the public literature and these methods were applied to determine the digestibility of plant-made colicins. Our results show that plant-produced colicins are not stable to exposure to gastric or intestinal proteases, in the same way that their homologous bacterial colicins are not stable to exposure to simulated gastrointestinal digestion.

C.2 Methods for confirming colicin amino acid sequences by MALDI-MS

Methods

Host plants were inoculated with *Agrobacterium* vectors carrying inserts for individual colicins (see APPENDIX B for colicin expression methods). The gene sequences for the colicin inserts (Section 2.3) in the expression vectors are verified by DNA sequencing. Plants were extracted to yield total soluble protein (TSP) extracts containing colicins; each TSP was run on SDS-PAGE gels and stained with Coomassie for visualization or maintained in appropriate buffers for further processing.

The composition of the plant-expressed proteins is verified by 2 complementary mass spectrometry methods, including peptide mass (PMF) and peptide fragment fingerprinting (PFF):

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 (500-3500 Da range) for detection (Methods: in-gel and/or in-solution proteolytic digestion; 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 "2" above for PMF are fragmented and the mass of each amino acid is measured. Predicted and actual masses of the sequences are analysed are then compared.

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. 100% coverage for colicin M, 99% for colicin E7, 95% for colicin K, 89% for colicin U, and 91% for colicin B). All plant-made colicin proteins conform to their predicted composition and match the consensus amino acid sequences of the bacterially produced native colicin proteins.

Table C-1 summarizes the results of amino acid sequence analyses by MALDI MS for 6 representative colicins. 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 processing, if present, is identical regardless of source host.

Table C-1. Identity confirmation of plant-made colicins by peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF) MALDI TOF mass spectrometry

Colicin	Plant-made (determined by Notifier)			Bacterial (literature values)
	N-terminus	C-terminus	Full-length coverage	N-terminus (method). Reference
E7	Confirmed. N-terminal met is cleaved off; mature protein starts with N-terminal ser.	Confirmed	99%	No data available
M	Confirmed. N-terminal met is present.	Confirmed	100%	N-terminal met is present (Edman degradation). Dreher 1985
N	Confirmed. N-terminal met is cleaved off; mature protein starts with N-terminal gly.	Confirmed	76%	No data available
K	Confirmed. N-terminal met is present.	Confirmed	95%	No data available
U	Confirmed. N-terminal met is cleaved off; mature protein starts with N-terminal pro.	Confirmed	89%	No data available
B	Confirmed. N-terminal met is cleaved off; mature protein starts with N-terminal ser.	Confirmed	91%	N-terminal met is cleaved off; mature protein starts with N-terminal ser (Edman degradation). Pressler 1986
Ia	Confirmed. N-terminal met is cleaved off; mature protein starts with N-terminal ser.	Confirmed	93%	N-terminal met is cleaved off; mature protein starts with N-terminal ser (DNSC method; Edman degradation). Konisky 1972; Mankovich 1986

Sample processing and output of analyses

Figure C-4 shows typical Coomassie-stained gels of samples of various plant-produced colicins (colicin band MW indicated by arrow plus plant-derived protein impurities) prior to MS/MS confirmation of colicin identity.

Figure C-5 presents a summary of peptide mass fingerprint (PMF) analysis of colicin M as a representative colicin, for which combined proteolytic cleavage by three enzymes (pepsin, trypsin and chymotrypsin) generated peptide fragments covering 100% of the protein. All colicins are analyzed by the same methods.

Figure C-4. Colicins extracted from plant material prior to identify confirmation by mass spectrometry

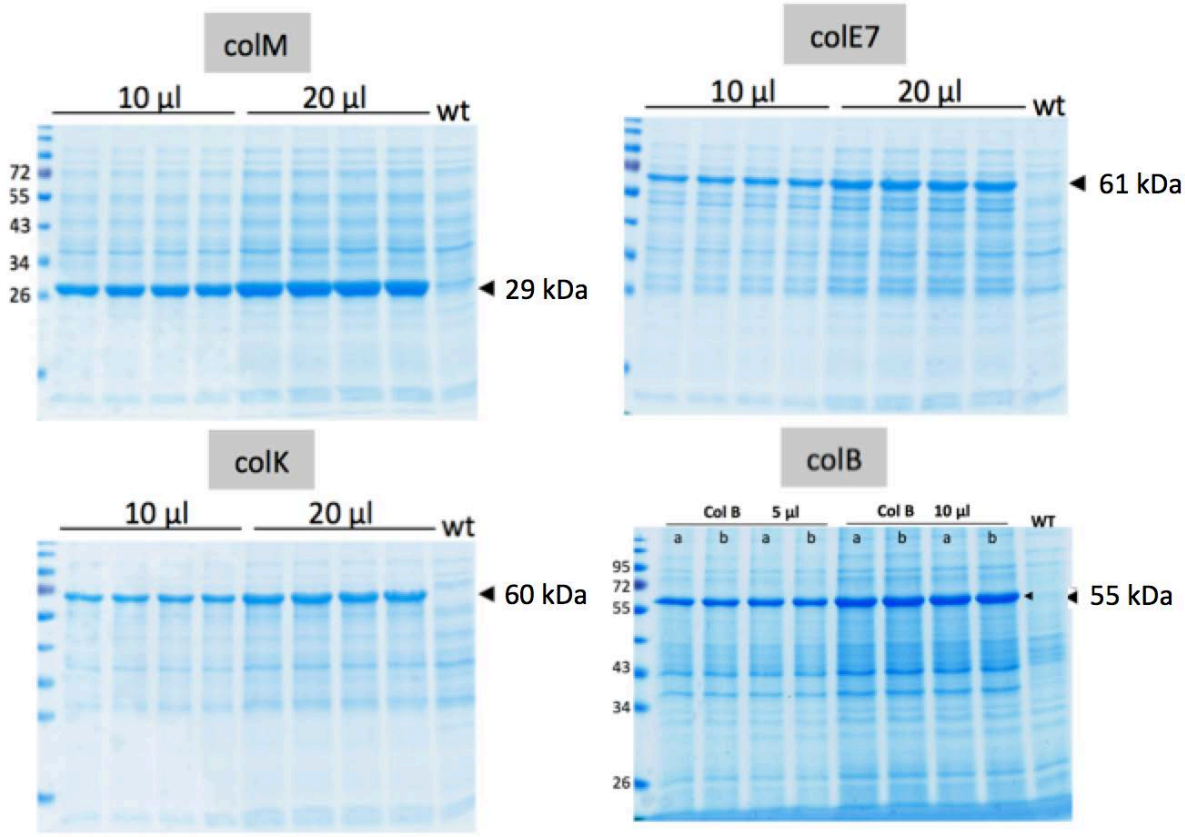


Figure C-5. Proteolytic coverage of colicin M for identity confirmation

1) Sequence annotation of colicin M (GeneBank AAA23589.1; 271 aa)

METLTVHAPS PSTNLPSYGN GAFSLSAPHV PGAGPLLVQV VYSFFQSPNM CLQALTQLED

YIKKHGASNP LTLQIISTNI GYFCNADRNL VLHPGISVYD AYHFAKPAPS QYDYRSMNMK

QMSGNVTTPI VALAHYLWGN GAERSVNIAN IGLKISPMKI NQIKDIIKSG VVGTFPVSTK

FTHATGDYNV ITGAYLGNIT LKTEGTLTIS ANGSWTYNGV VRSYDDKYDF NASTHRGIIG

ESLTRLGAMF SGKEYQILLP GEIHIKESGK R

2) Protease cleavage of colicin M

Tryptic peptides
Chymotryptic peptides
Pepsin peptides

Protein coverage as function of protease used (peptide mass range 500-5000 Da):

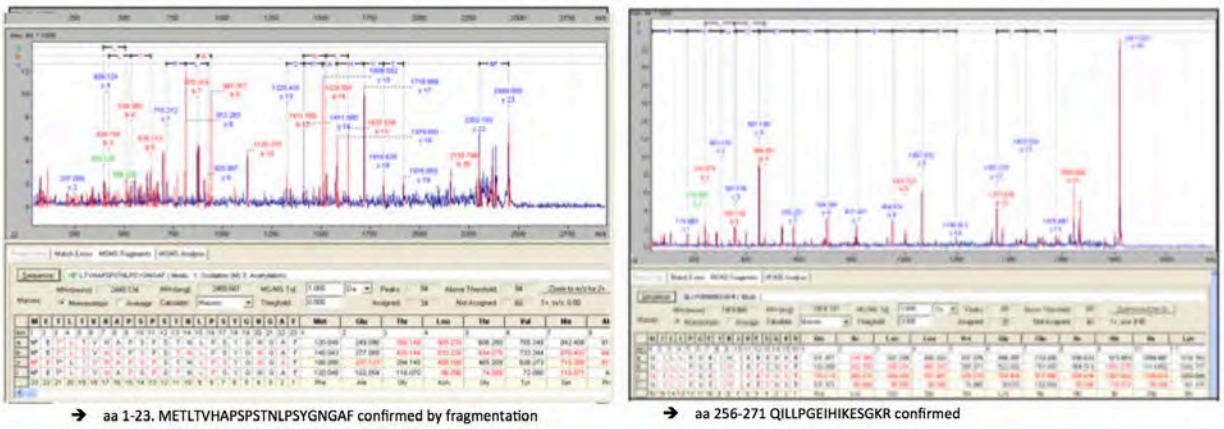
- Trypsin: 76%
- Chymotrypsin: 96%
- Pepsin: 83%
- **Combined sequence coverage: 100%**

3) Confirmation of colicin M identity by MS/MS

- N-terminal Colicin M sequence confirmed by fragmentation of chymotrypsin peptide 1-23 (m/z 2449.019), METLTVHAPSPSTNLPSYGNGAF
- C-terminal Colicin M sequence confirmed by fragmentation of chymotrypsin peptide 256-271 (m/z 1818), QILLPGEIHIKESGKR

Figure C-6 shows typical results from analysis of peptide fragments using the BioTools software. Fragmentation of N- and C-terminal peptides from colicin M digestion is shown (left panel, N-terminus; right panel, C-terminus). By adding all fragments *in silico*, each peptide's molecular weight and amino acid sequence can be confirmed.

Figure C-6. Example of verification of N- and C-terminal amino acid sequences by fragmentation of peptides (peptide fragment fingerprinting; PFF) for colicin M



Notifier confirms all plant-produced colicins using these methods in order to ensure that the amino acid sequence composition of recombinant colicins match those reported for the naturally produced bacterial proteins.

C.3 Methods for assessing development of resistance

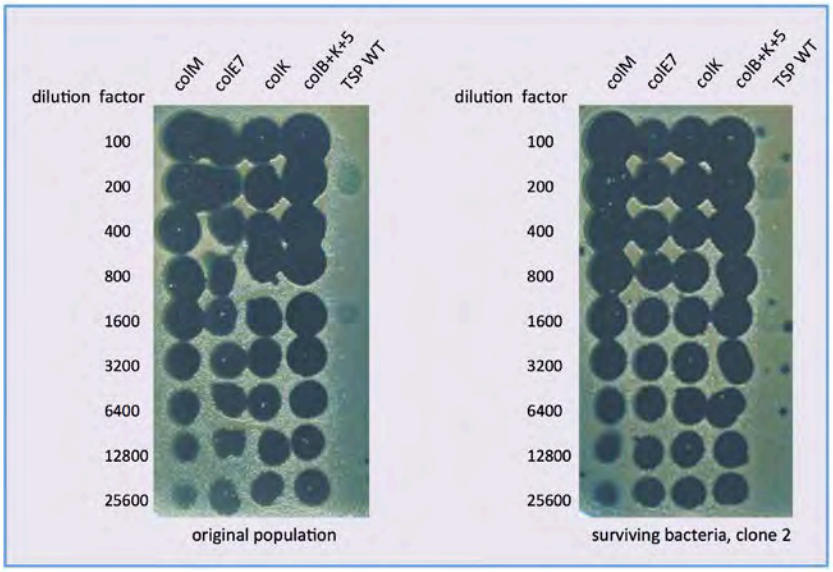
Methodology to assess resistance potential

As discussed in Section A.2.4, we find the development of resistance to colicins by commensal *E. coli* through exposure of food treated with the product to be extremely unlikely. All colicins are unstable to heat (cooking), acidic environments (stomach acid) and proteolytic degradation (endogenous proteases in the gastrointestinal tract).

The development of resistance by pathogens present in food treated with the product is also unlikely. To demonstrate, *E. coli* DH10B that survived bactericidal action at first exposure under laboratory conditions were re-exposed sequentially to the same colicin to determine their susceptibility or resistance. Figure C-7 shows plate-clearing zones upon re-exposure of DH10B to various colicins at the indicated dilutions. Surviving bacteria were found to be equally sensitive to colicin treatment upon re-exposure.

The complex mechanism of action of colicins suggests that highly improbable multiple simultaneous mutations would need to occur in the target cell, either in the peptidoglycan composition, internal macromolecular structures, the colicin receptor or transporter, or in all these systems, for significant resistance to develop.

Figure C-7. Susceptibility of *E. coli* DH10B to repeated exposure to COLICIN components



After exposure of the pathogen to various plant-made colicins, surviving cells were re-exposed to colicins to assess susceptibility or resistance. As shown by clearing zones (dark circles on opaque background) at multiple serial dilutions, surviving bacteria were as sensitive as the original population to the bactericidal effects of the antibacterials (WT, exposure to extracts from wild-type plants without colicin, as controls).

To assess the potential for development of resistance under realistic use conditions, sections of **apples** were contaminated with *E. coli* O157:H7 and then treated with a two-component COLICIN formulation consisting of colicins M + E7. After a period of incubation and recovery, 10 bacterial clones surviving the initial treatment were serially re-exposed to individual colicins and their mixtures to assess their susceptibility and/or resistance. As summarized in Figure C-8, no resistance was documented when applying the two-component product in an actual use scenario.

A parallel study was conducted using a five-component COLICIN product, consisting of colicins M, E7, K, B and 5. Tester strain *E. coli* O157:H7 on apples was again used for comparison, exposed to the colicin mixture and subsequently to individual colicins at the dilutions indicated. The result of this series is shown in Figure C-9. No resistance to any colicin was documented when applying the five-component COLICIN product in an actual use scenario.

In both studies, a suspension of *E. coli* O157:H7 (5 x 10^5 CFU/ml; 1.48 x 10^4 cfu/g food) was applied to the surface of apple pieces. After incubation, bacteria were sprayed with a first round of COLICIN (3 mg/ml colicin M and 1 mg/ml colicin E7 in a two-component mixture, or the same amount of M and E7 plus 1.3 mg/ml colicin K and 0.3 mg/ml each colicin B and colicin 5 in a five-component mixture) followed by an incubation period of 3 days at 4°C. Surviving bacteria were plated for single colonies and re-exposed to the same colicins (singly or as mixtures) via soft agar overlays to assess growth after re-exposure to the product. Plating results for six of ten recovered clones are shown in the figures. WT indicates plant extract without colicin, followed by growth inhibition upon re-exposure to colicins M, E7, K or B + K + 5.

Conclusion

No resistance to the two-component or the five-component COLICIN product was evident.

Figure C-8. Lack of resistance development upon repeated exposure of *E. coli* O157:H7 to COLICIN (component colicins M and E7) on apples

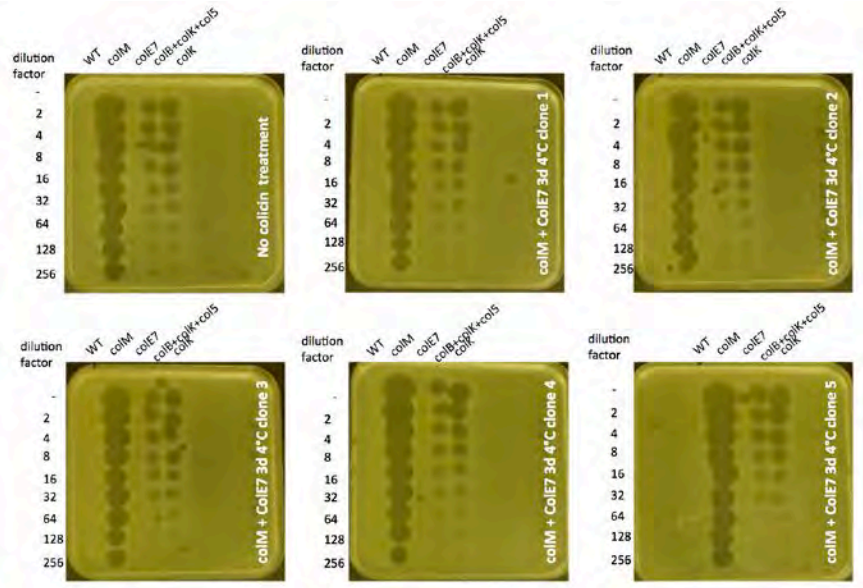
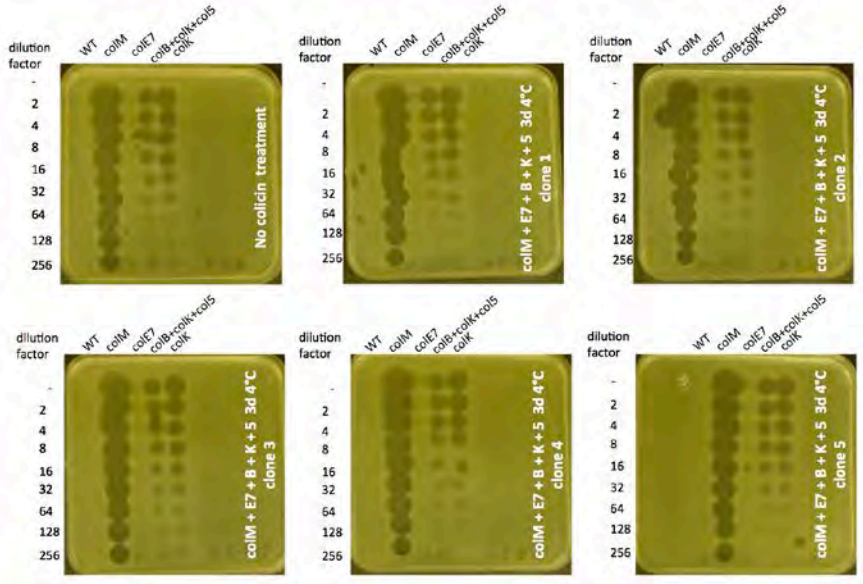


Figure C-9. Lack of resistance development upon repeated exposure of *E. coli* O157:H7 to COLICIN (component colicins M, E7, B, K and 5) on apples



C.4 Methods for determining suitability using fruit segments and spray application

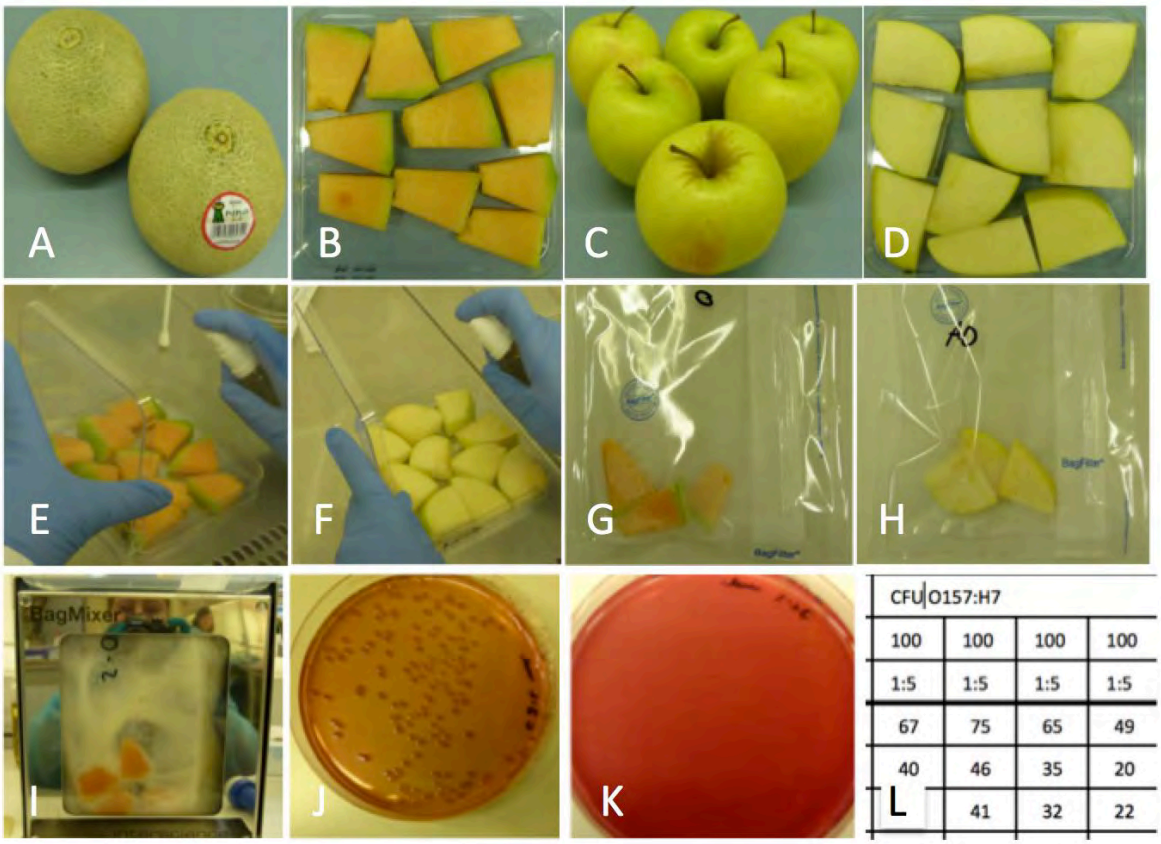
Method development

Method development is illustrated in Figure C-10, with references to main steps indicated by the capital letters of each panel in the figure. In order to test handling of as well as recovery of *E. coli* from fresh-cut fruit pieces, one pre-experiment using *E. coli* strain DH10B for contamination of RTE pieces of Golden Delicious **apple** and **cantaloupe melon** was performed. This methodology was subsequently

applied to quantify activity of COLICIN (two- and five-component mixtures) on the same fruit using pathogenic *E. coli* O157:H7 as the indicator. Whole fruits were surface-sterilized with 200 ppm sodium hypochlorite solution and cut into pieces of ~0.8 mm thickness (10 pieces corresponding to about 65 g fresh weight) using knifes sterilized with Bacillol (panels **A-D**). A suspension of *E. coli* strain O157:H7 (DSM19206) of OD_{600}=0.005 (~5 x 10^5 cfu/ml) or OD_{600}=0.001 (~1 x 10^5 cfu/ml) was used as inoculum and fruit pieces were inoculated by dipping into this bacterial solution with both sides. Consumption of bacterial solution was measured and the theoretical inoculum was calculated to be ~1 x 10^4 cfu/g fruit.

Subsequently, fruit pieces were sprayed with carrier solution (typically 8 puffs/application; panels **E-F**) and incubated at various conditions in sterile bags (**G-H**). For microbial analysis, 3-4 fruit pieces (corresponding to ~20 g) were pooled and homogenized with 5 volumes of peptone water (panel **I**, showing melon), followed by serial dilution and plating of control-treated and colicin-treated samples (panels **J-K**) and enumeration (**L**). The evaluation of CFU showed good correlation between bacterial numbers recovered from and theoretically inoculated on fruit pieces, although there was more growth on melons compared to apples and hence the inoculum used was lower for the melon RTE experiments. No background CFU were detected on recovery medium supplemented with 50 µg/ml cefixime and 100 µg/ml X-Gluc for non-treated fruit pieces.

Figure C-10. Overview of methods used to assess suitability of COLICIN treatment on samples of RTE melon and apples.



Reduction of *E. coli* O157:H7 on fresh-cut RTE pieces of melon

The preparation of fruit pieces was done as described above. The density of the O157:H7 (strain DSM19206) inoculum culture was set to OD_{600}=0.001 (~1 x 10^5 cfu/ml) with a measured actual ~2 x 10^4 cfu inoculated per g fruit. For colicin treatment, TSP extracts were prepared from plant leaf material and antimicrobial treatment was performed by spraying with 3 different treatments: A) carrier-only control treatment (plant WT TSP extract containing no colicin); B) two-component COLICIN treatment, 3+1 mg/kg fruit (TSP colicin M + colicin E7); or C) five-component COLICIN treatment, 3+1+1.3+0.3+0.3 mg/kg fruit (TSP colicins M + E7 + K + B + 5). Samples were generated in 4 replicates for each timepoint of analysis and treatment by pooling 3 fruit pieces and were analyzed for microbial populations upon 1h, 1d and 3d storage at 4°C (no effect of treatments on appearance of fruit pieces was observed) by dilution plating upon homogenization with 5 volumes of peptone water. The results of this study are shown in Figure A-13.

Reduction of *E. coli* O157:H7 on fresh-cut RTE pieces of apple

This experiment was performed as the experiment on melons described above with 2 exceptions: The density of the O157:H7 (strain DSM19206) inoculum culture was set to OD_{600}=0.005 (~5 x 10^5 CFU/ml) with a measured actual ~1 x 10^4 CFU inoculated per gram of fruit and the pooling of 4 apple pieces per sample (different weight of apple and melon pieces). The results of this study are shown in Figure A-14.

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