ORIGINAL SUBMISSION

consultants to the regulated industry

Center for Regulatory Services, Inc.

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

#676

October 31, 2016

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

Dear Dr. Mattia:

SUBJECT: Transmittal of the NOMAD BIOSCIENCE GmbH –
GRAS Notice for Colicin Antimicrobial Product
For use on MEAT Products

Enclosed you will find the GRAS notice for colicin antimicrobial product as submitted by NOMAD BIOSCIENCE GmbH antimicrobial treatment to reduce the levels of E. coli bacteria (bactericidal) on meat food products.

This filing is a paper copy of the GRAS notice, as well as a CD of the GRAS notice and as all the cited references.

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

Sincerely,

(b) (6)

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

Attachments

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

cc: Yuri Gleba, Nomad

consultants to the regulated industry

Center for Regulatory Services, Inc.

5200 Wolf Run Shoals Road
Woodbridge, VA 22192-5755
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cc: Yuri Gleba, Nomad

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

RECEIVED NOV 1 2016 OFFICE OF FOOD ADDITIVE SAFETY

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
000676	
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 na	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.			
ty 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		

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

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NAME FOR INTERNET	
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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: na	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.			
ty: 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 593
☐ b) GRAS Affirmation Petition No. GRP _____
☐ c) Food Additive Petition No. FAP _____
☐ d) Food Master File No. FMF _____
☐ e) Other or Additional *(describe or enter information as above)* _____

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, EAEC and STEC. COLICIN is intended to be used as a solution and applied as a spray, although other forms of application can be contemplated, to control *E. coli* on meats (e.g. beef, pork, lamb, mutton and veal), at an application rate of 1-10 mg COLICIN (total colicin protein) per kg of meat 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

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, ...n, 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, efficacy and suitability of the notified substances was provided in GRN 593 and is further incorporated in the documents provided in this Notice.

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

[illegible] ☐ 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)
	Kristi O. Smedley, Ph.D.	10/31/2016

PART VIII – LIST OF ATTACHMENTS

: 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 Aid in Processing Meat Products, Including Protocol Used to Determine Efficacy and Duration of Technical Effect (APPENDIX A) (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

Date: 31 October 2016

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

Re: GRAS Notice for COLICIN as an Antimicrobial Food Processing Aid for Meat Products

Dear Dr. Mattia,

Nomad Bioscience GmbH ("Nomad"; "Notifier") is submitting printed copies and a CD containing duplicate electronic files of this GRAS Notice for its **COLICIN antimicrobial for meat** products. NOMAD has concluded, under FDA's Final Rule pertaining to 21 CFR 170 (August 17, 2016), 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) on meat food products. Consequently, this Notice is submitted for **dual review** by FDA and USDA's Food Safety and Inspection Service personnel.

Nomad previously submitted a GRAS notice to the Center for Food Safety and Applied Nutrition (CFSAN) on August 7, 2015, supporting COLICIN's general recognition of safety on the basis of scientific procedures, for the use of COLICIN on fresh or processed fruits and vegetables at a level of 1-10 milligrams (mg) of COLICIN preparation per kilogram (kg) of fruit or vegetable product. The Agency assigned our notice the identifier **GRN 593**. On December 18, 2015, we received from FDA a "No Questions" letter in response to GRN 593.

The current Notice expands the GRAS conclusion for the use of Nomad's COLICIN antimicrobial product to include meat. As such, we are referencing GRN 593 in its entirety. Nomad's COLICIN product is intended for use on raw and processed meats and can be delivered via spray, as described in this Notice, and potentially via other delivery methods.

COLICIN for application to meat is comprised of the same protein blends that were described in GRN 593. Further, the same rates of application of COLICIN used on produce will be used in the treatment of meats (i.e. 1-10 mg COLICIN per kg treated meat). Therefore, Nomad concludes that the product used on meats should be Generally Recognized as Safe (GRAS) under 21 CFR 170.36, and exempt from pre-market approval requirements as specified in Section 201(s) of the Federal Food, Drug, and Cosmetic Act. Because the product is the same and is manufactured by the same process as that described in GRN 593, extensive references are made to sections of GRN 593 for brevity and to rely on FDA's original review memoranda.

In previous interactions, the staff of CFSAN recommended that we provide an updated safety assessment for colicins based on data that may have been published since submission of GRN 593, if available. Nomad is not aware of any new safety related information in the public domain. Results of biological activity (efficacy) of plant-produced colicins have been published by Notifier and collaborators since submission of GRN 593 (Schulz 2015); a copy of this manuscript is included in this package for Agency review.

In the current Notice, Nomad provides (1) new estimated COLICIN exposure information based on consumption (intake) of meats in a typical US diet, including a risk assessment, and (2) suitability and residual technical effect information for using COLICIN during meat processing; the latter to be reviewed with assistance from the US Department of Agriculture's Food Safety and Inspection Service (FSIS) and any other departments or agencies, at FDA's discretion.

Also included in this package as APPENDIX A is the specific protocol used by Nomad to determine efficacy/suitability and residual technical effect. A draft of the protocol was shared and discussed with USDA, with the Agency affirming the adequacy of the methods used in a letter to Notifier on May 16, 2016.

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

Bound copies

1. FDA Form 3667 Nomad Bioscience GRN for COLICIN antimicrobial for meats
2. GRN for COLICIN as an Antimicrobial Food Processing Aid for Meat Products (Parts 1-7)
3. APPENDIX A: Standard Operating Procedure NMD 901-01 ("Protocol") – Determination of Efficacy and Duration of Bactericidal Effect of COLICIN (Colicin Mixtures) on Pathogenic Strains of *Escherichia coli* Applied to Meat Matrices

Electronic files provided in enclosed CD

1. FDA Form 3667 Nomad Bioscience GRN for COLICIN antimicrobial for meats (PDF)
2. GRN for COLICIN as an Antimicrobial Food Processing Aid for Meat Products (Parts 1-7), including APPENDIX A: Standard Operating Procedure NMD 901-01 ("Protocol") – Determination of Efficacy and Duration of Bactericidal Effect of COLICIN (Colicin Mixtures) on Pathogenic Strains of *Escherichia coli* Applied to Meat Matrices (PDF)
3. Copies of references cited (PDF)

If the Agencies have any questions or require additional information to aid their review of NOMAD's findings and conclusions, please contact us at the address listed above. For convenience, you may also contact our regulatory 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

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1 General Introduction and Claim of Exemption from Premarket Approval Requirements for COLICIN as an Antimicrobial Processing Aid for Meat Products

Nomad Bioscience GmbH ("Nomad"; Notifier) COLICIN product is produced recombinantly using a plant-based manufacturing process to match the amino acid sequence of naturally occurring colicin-family antimicrobial proteins. On August 7, 2015, Notifier submitted a GRAS Notice to FDA/CFSAN citing its conclusion that COLICIN should be generally recognized as safe as determined through scientific procedures, and that the product should therefore be exempt from the requirement of premarket approval under proposed 21 CFR 170.36(a). FDA assigned the notice the identifier GRN 593, and subsequently reviewed the merits of Notifier's submission claims. On December 18, 2015, Notified received from FDA a "No Questions" letter for COLICIN antimicrobial as a processing aid for fresh and minimally processed produce (fruits and vegetables) under the conditions of intended use described in GRN 593.

This current Notice aims to expand the use of COLICIN as a GRAS processing aid on foods other than produce, specifically red animal meats (e.g. beef, pork, lamb, mutton and veal), with the goal of reducing the number of pathogenic *Escherichia coli* (*E. coli)* bacteria on such food products. Because the manufacturing method and the composition of the resultant COLICIN product for use on meats are identical to the methods and compositions described in GRN 593, for brevity, the current Notice refers to sections of GRN 593 whenever possible and as appropriate.

From a safety perspective, this Notice complements GRN 593 by providing exposure estimates from consumption of meat products treated with COLICIN, and includes a corresponding risk assessment. Notifier concludes that under the conditions of use described herein, COLICIN is also generally recognized as safe and therefore should be exempt from premarket approval procedures under 21 CFR 170.36(a)(I). Furthermore, the current Notice includes a detailed description of the use of the product on meats, the methods used to assess efficacy/suitability as well as residual technical effect, and the results obtained.

1.1 Submission of Notice

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

1.2 Name and Address of Notifier

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

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

1.3 Common or Usual Name of the Notified Substance

COLICIN

1.4 Conditions of Use

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

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. Full details on colicins, their safety and range of biological activities were provided in GRN 593. A summary list of colicins' safety attributes when used as intended, including the source of supporting information, is provided in Table 7-1.

COLICIN is intended to prevent or minimize contamination of meat products by pathogenic strains of *E. coli*. Specifically, the intended use of COLICIN is as a spray (other means of application not described in this Notice are contemplated) to control pathogenic strains of *E. coli* on fresh or processed mammalian meats such as beef, pork, lamb, mutton and veal (a.k.a. "red meats"), at an application rate of 1-10 mg COLICIN per kg of meat product (approximately 0.5-5 mg/lb). As in GRN 593, this current Notice encompasses 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): Spray, dip, wash or marinade for processed meats

COLICIN CONCENTRATE (lower purity): Spray, dip or wash for bulk meat processing

The **subpopulation** potentially exposed to COLICIN is comprised of individuals of all ages who consume mammalian meat products ("red meats") treated with Notifier's antimicrobial.

1.5 Statutory Basis for Notifier's GRAS Conclusion

The statutory basis of the GRAS status in through scientific procedures in accordance with 21 CFR 170.30(b): GRAS Conclusion.

Based on the information provided in the Notice, it is Nomad Bioscience's conclusion that COLICIN is generally recognized as safe when used to prevent or minimize contamination of meat products by pathogenic strains of *E. coli* at an application rate of 1-10 mg COLICIN per kg of meat product, and as such is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

1.6 Availability of Information for FDA and USDA Review

The data and all information that serve as a basis for the GRAS and suitability conclusions are part of this Notice, but are also available for FDA and USDA review during customary business hours 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

1.7 Public Disclosure

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

1.8 Certification

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

(b) (6)

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

2 Identity, Method of Manufacture, Specifications, Technical Effect

2.1 Identity, Structural and Functional Information

Identity

Detailed information about the components of COLICIN are presented in GRN 593. Table 2-1 lists the components of COLICIN product that may be used singly or in combination to achieve the desired antibacterial suitability on meat products.

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	*EHEC; *Salmonella*
E7	CAA45164.1	DNase	BtuB / OmpF, TolABQR	EHEC
Ia	ADW79574.1	Pore-forming	Cir / TonB-ExbBD, Cir	EHEC; *Salmonella*
M	AAA23589.1	Peptidoglycanase	FhuA / TonB-ExbBD	EHEC; *Salmonella*
N	CAA68592.1	Pore-forming	OmpF / TolAQR	EHEC
K	AAB41288.1	Pore-forming	Tsx / OmpAF, TolABQR	EHEC
U	CAA72509.1	Pore-forming	OmpA / OmpF, TolABQR	EHEC
5	CAA61102.1	Pore-forming	Tsx / TolC, TonB-ExbBD	EHEC; *Salmonella*
B	AAA98063	Pore-forming	FepA / TonB-ExbBD	EHEC

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

All colicins are produced naturally in the human gut by commensal strains of *E. coli* or related species and demonstrate complementary modes of action (See **Table A-1 in GRN 593, p 20**; from Yang 2014). Of the various colicins studied, **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.

Structural Information on COLICIN Components

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

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

Quantitative Composition

COLICIN is prepared in bulk as a concentrated solution or as a dry powder at two different purities as described in **GRN 593 APPENDIX B: COLICIN Manufacturing Process, Section B.4 Specifications (p 56).** 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 meats 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 meat products or infused as a marinade at a rate not to exceed 10 mg/kg (<4.6 mg/lb).

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

Modes of Action

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

2.2 Method of Manufacture

Notifier uses a **plant-based manufacturing process** for producing COLICIN proteins; the method is an adaptation of the process used to manufacture biopharmaceuticals, which have been administered in clinical trials under FDA IND. The colicins are produced using recombinant technology to yield concentrated extracts at two different purities. Current host plants include the food species **spinach** (*Spinacia oleracea*), **red beet** (*Beta vulgaris*) or **lettuce** (*Lactuca sativa*).

The colicin protein manufacturing process was described in **GRN 593 APPENDIX B: COLICIN Manufacturing Process (pp 51-57)**. The plant-derived biomass remaining after colicin protein extraction is treated and discarded (disposed) and is not used as a human food or animal feed product, additive or supplement.

2.3 Composition and Specification

Characteristic Properties

The characteristic molecular properties of all colicin proteins that can comprise the final COLICIN product were defined in **GRN 593, Section 2.3 Characteristic Properties (p 16).** Colicins, by definition, are unstable to proteolytic digestion (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 plant-produced colicins, Notifier has confirmed the rapid degradation of colicins in simulated gastric fluid and simulated intestinal fluid. Results of these digestion and degradation studies were presented in **GRN 593, Section A.2.3 (pp 28-32) and APPENDIX C: Methodology (pp 58-61).**

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

Formulation

COLICIN is provided as a 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, package fill or marinade depending on the intended use.

Content of Potential Human Toxicants in COLICIN

None.

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 was presented in **GRN 593, APPENDIX B: COLICIN Manufacturing Process,** specifically in **Section B.3: Procedure, and Section B.4: Specifications, pp 52-56).**

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

2.4 Technical Effect and Suitability of Use

In GRN 593 we described the properties, modes of action and antibacterial potency of plant-produced colicins. Results of these studies have now been published by Notifier and collaborators (Schulz 2015); a copy of this manuscript is included in this package for Agency review. Salient points first described in GRN 593 and in Schulz et al. (2015) about COLICIN's efficacy against *E. coli* strains are reproduced herein for convenience to reviewers. In this Notice we also describe the efficacy and suitability of COLICIN as an antimicrobial processing aid for red meat products, as well as the longevity of COLICIN's technical effect.

2.4.1 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 (Cascales 2007). Schematic depictions of colicins' mode of antibacterial action were presented in GRN 593.

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 red meat cuts. 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 2-4.

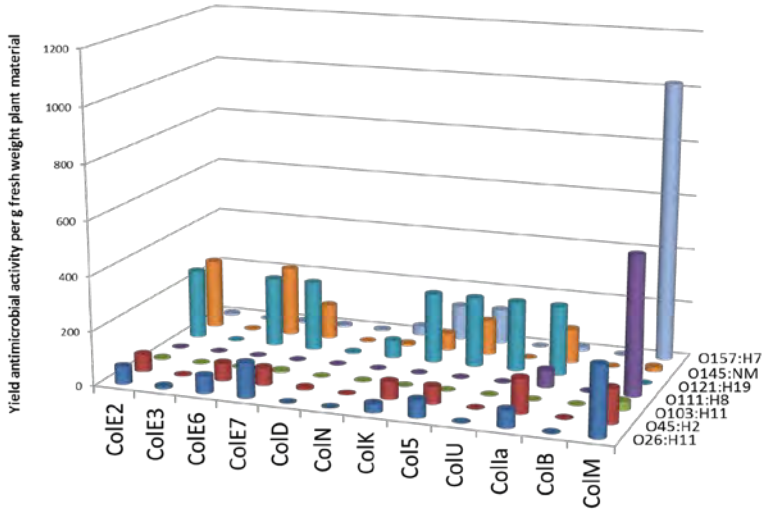
Table 2-4. Plant-produced colicin components of COLICIN product

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

Figure 2-1 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.

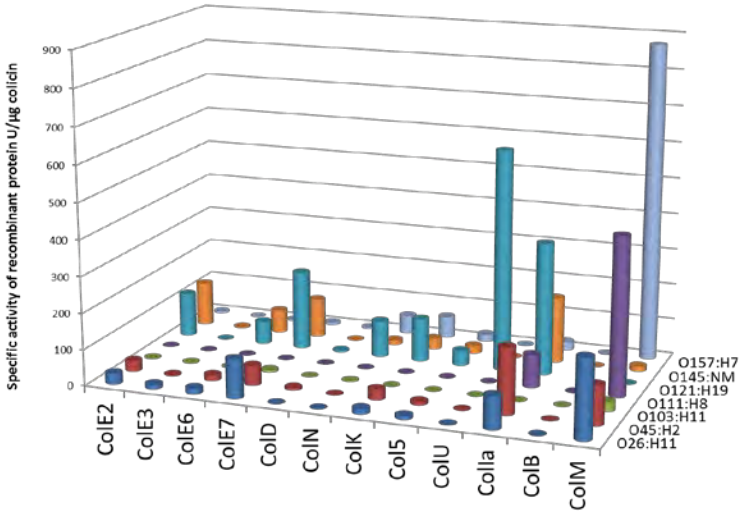
Figure 2-1. Yield of antibacterial activities in extracts of plant-produced colicins against pathogenic ("Big Seven") strains of *E. coli*



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.

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 2-2.

Figure 2-2. Specific activities of plant-produced colicins against pathogenic ("Big Seven) strains of *E. coli*

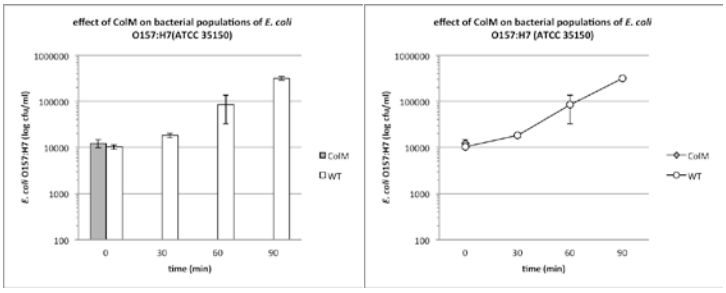


Colicins show differential potencies against target strains of *E. coli.* Activity is represented by bar height as U/µg colicin; tested recombinant colicins and *E. coli* strains are indicated.

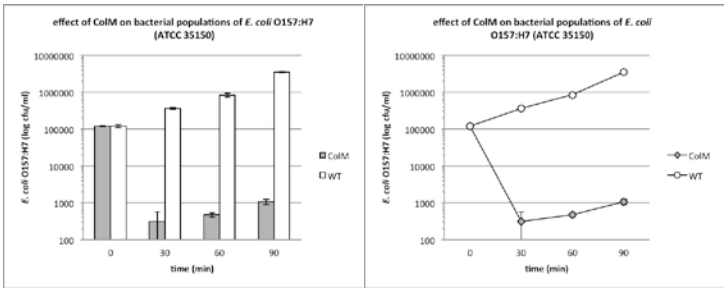
Colicin M is highly active against O157:H7; its activity against various EHEC strains was further characterized, with results summarized in Figure 2-3.

Figure 2-3. Representative antibacterial activities of colicin M against various strains of *E. coli*

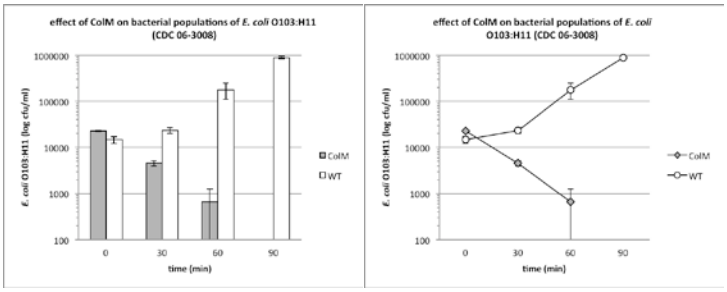
E. coli O157:H7 exposed to Colicin M at 20 μg/mL



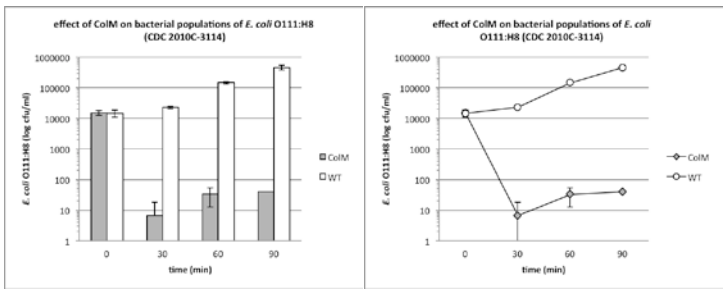
E. coli O157:H7 exposed to Colicin M at 1 μg/mL



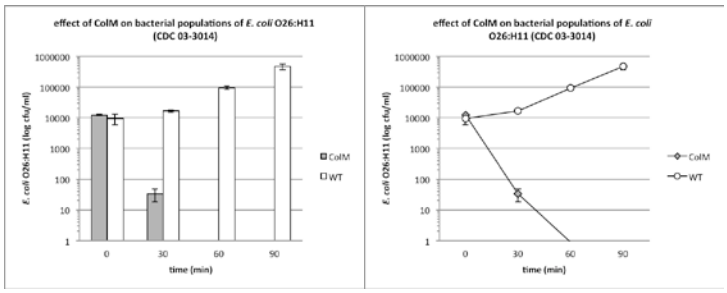
E. coli O103:H11 exposed to Colicin M at 20 μg/mL



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



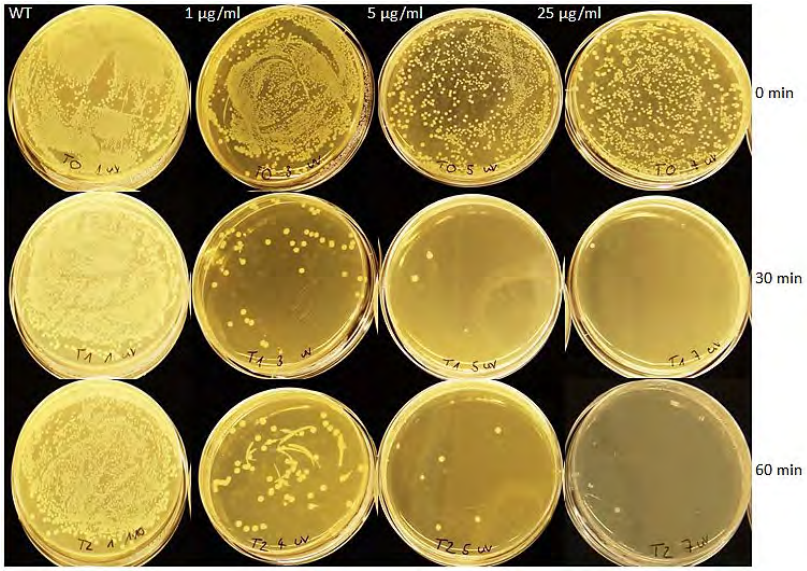
E. coli O26:H11 exposed to Colicin M at 5 μg/mL



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 2-4 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).

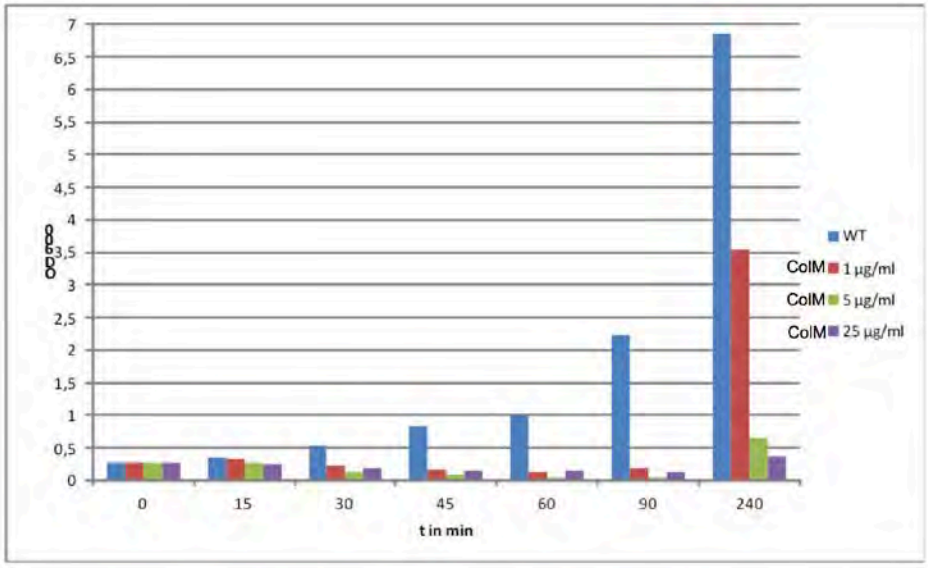
Figure 2-4. Antibacterial activity of colicin M against *E. coli* O157:H7



Exposure of the pathogen in suspension 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).

Likewise, Figure 2-5 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 2-5. Colicin M inhibition of *E. coli* O157:H7



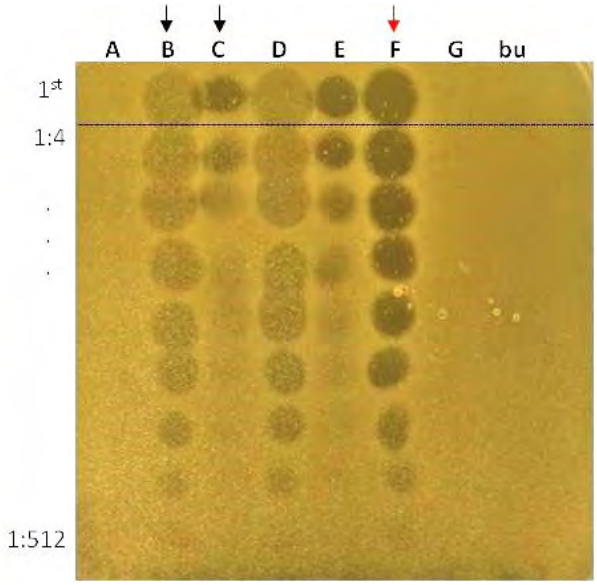
Exposure of the pathogen in suspension culture to plant extracts containing 0, 1, 5 and 25 µg/ml of colicin M (0-25 ppm; 1st-4th bar in each series) results in rapid and significant growth inhibition at all time points tested; 0-240 min shown (WT, exposure to extracts from wild-type plants without colicin 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 2-6 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 2-6. Synergy among colicins against pathogenic strains of *E. coli*.



Equal numbers (approximately 10^4) of pathogenic *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 2-5 and Table 2-6 show the individual antibacterial activity of colicin M and colicin E7 (Table 2-5) and mixtures of these two colicins at various ratios (Table 2-6) against pathogenic strains of *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 2-5. Antibacterial activity of colicin M and colicin E7 applied individually to EHEC 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 2-6. Antibacterial activity of colicin M and colicin E7 applied as mixtures to EHEC 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 2-5 and Table 2-6), 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 (**GRN 593 Section A.3.2; pp. 45-49**).

Conclusion. From Notifier's own scientific studies on colicins M, E7, E1, B, K, U, 5, including Schulz 2015, and from the published literature on colicins, we conclude that colicins can effectively reduce the viability of pathogenic 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.

2.4.2 Suitability of COLICIN for use in processing meat products

Application of COLICIN on meats reduces the viability of pathogenic strains of *E. coli*

Plant-produced colicins were also tested for antibacterial activity on samples of red meats to determine the product's suitability as an antimicrobial processing aid on different target matrices. To illustrate utility, **beef**, **pork** and **lamb** were contaminated with mixtures of *E. coli* Big Seven strains plus strain 0104:H4 at different levels as whole cuts, and also beef cuts prior to grinding into ground beef. The contaminated meats were incubated at RT for 30 min to allow for pathogen establishment, subsequently treated with control or COLICIN-containing solutions, held for 1-2 h at RT, followed by storage at temperatures ranging from 4 °C to 15 °C. After incubation, the treated meat samples were homogenized and extracted, and the remaining viable bacteria in the suspension (CFU/g meat) from each sample was determined by dilution plating on agar medium. Multiple studies were conducted, and specific methods and results obtained in each study are summarized herein. Detailed protocols for evaluation of COLICIN efficacy on meats and for determining residual technical effect are included in APPENDIX A to this Notice (**Nomad SOP NMD 901-01).**

Summary of methods

COLICIN's antibacterial effect was evaluated at two levels of pathogenic *E. coli* contamination, namely, Level I (low contamination, 1-2 log CFU/g meat) and Level II (high contamination, 4-5 log CFU/g meat). For low contamination (Level I), nalidixic acid resistant mutants of corresponding EHEC strains were used (Hane 1969; Sniegowski 1997) to enable detection of colonies of experimentally introduced EHEC in the presence of non-pathogens naturally present on meat, which could be an issue at low bacterial loads. For selection, the solid nutrient medium used for enumeration of viable cells was supplemented with 25 mg/L nalidixic acid. Nalidixic acid resistant mutants and original strains did not differ in their sensitivity to COLICIN (sample results presented in APPENDIX A Table 8-2). COLICIN's technical effect on various meats was determined in a series of exposure experiments using low and/or high contamination levels. Beef meat was analyzed at both contamination Levels I and II, pork at Level II, and lamb at Level I. The goal was not to duplicate only one set of conditions on different matrices, but rather to observe antibacterial trends as a function of levels of contamination, meat matrix, treatment, temperature and storage time.

The *E. coli* cultures were applied to whole cuts of meat by dipping meat samples in a bacterial suspension. For meat intended to be ground, bacterial solution at 10 ml/kg was equally applied. These methods ensured uniform exposure of the matrix to bacteria. The bacterial mixture consisted of the Big Seven strains (APPENDIX A Table 8-1), plus *E. coli* 0104:H4, which was associated with the major food-borne outbreak in Europe in 2011. Hence, the multi-strain contaminant used in these studies can be referred to as the "Big 7+1." Inoculated meat samples were held for 30 min at RT to allow adherence prior to COLICIN or control treatment. In studies with contaminated pork meat stored at low-temperature (4 °C), cocktails of from 2 to 8 colicins, including M, E7, Ia, K, E6, E2, 5 and U in the ratio 3 mg/kg meat Col M + 1 mg/kg of all other colicins in the mixture, were used to determine the contribution of each colicin in the mix and the additive safety value of colicins over refrigeration. In subsequent studies with beef and lamb, a mixture of only 6 colicins (M, E7, Ia, K, 5 and U at 3+1+1+1+1+1 mg colicin/kg meat) was determined to provide adequate antibacterial effects even at higher meat storage temperatures of 10 °C and 15 °C. Control treatment solution consisted of total soluble protein extract from the same production host plant not containing colicins ("carrier solution"). Statistical methods are described in APPENDIX A. Inscriptions in all figures refer to mean $\log_{10}$ (CFU/g) reduction of EHEC counts in meat samples treated with carrier vs. COLICIN (upper number), and mean percent (CFU/g) reduction of EHEC in COLICIN- vs. carrier-treated samples (number in brackets). Figures in this Notice show the level of statistical significance graphically as * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ and **** = $p<0.0001$.

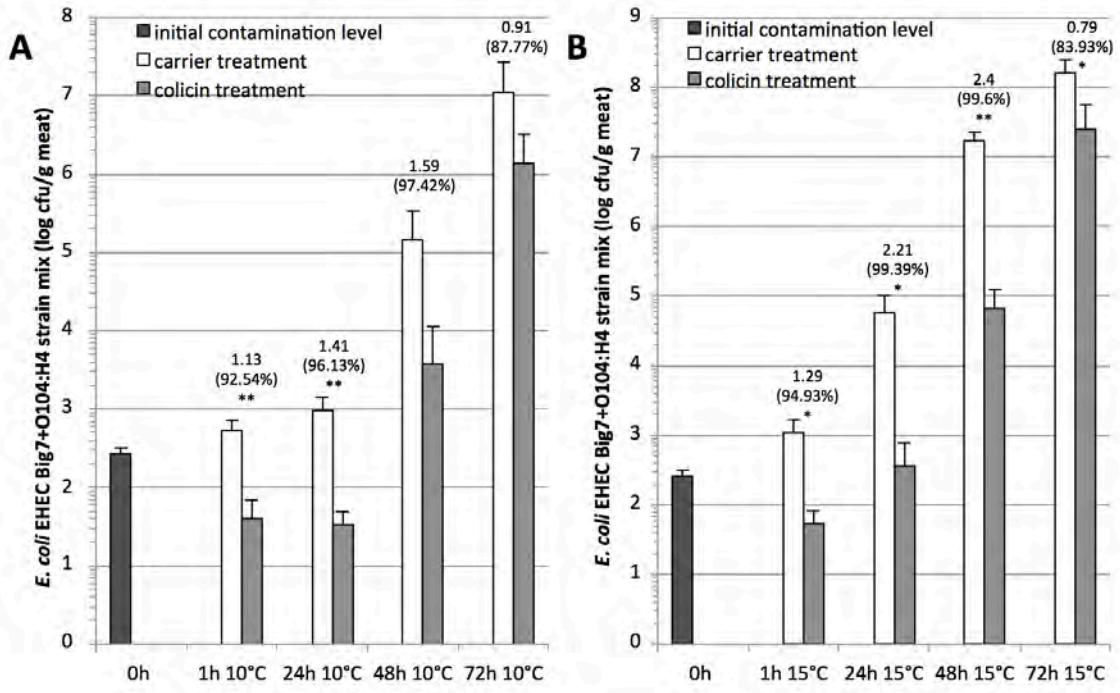
The COLICIN solutions were applied to meat samples using a hand-held spraying device (see Section 2.4.4). Bacterial populations on contaminated samples were typically analyzed at 1, 24, 48 and 72 h of storage at temperatures of 4 °C, 10 °C, or 15 °C; thus analyses began 2-3 h post COLICIN or carrier application. In some experiments bacterial populations were also enumerated just before COLICIN treatment (0 h time point) to compare applied vs recoverable inoculum density. Treated beef and lamb meat samples were stored at either 10 °C or 15 °C before enumeration. Pork meat samples were stored at 4°C only to assess the effect of COLICIN at a very low storage temperature. Results are summarized in the following sections.

Whole beef cuts, contamination Level I (low)

Two independent experiments were performed with beef steak samples at low contamination Level (1-1.5 log CFU/g meat). Each experiment included incubation at 10 °C and 15 °C. To discriminate between colonies of experimentally introduced EHEC and possible non-specific contamination, nalidixic acid resistant mutants of corresponding *E. coli* strains were used in these experiments. In both experiments at both temperatures, significant reductions of bacterial populations due to COLICIN treatment were found.

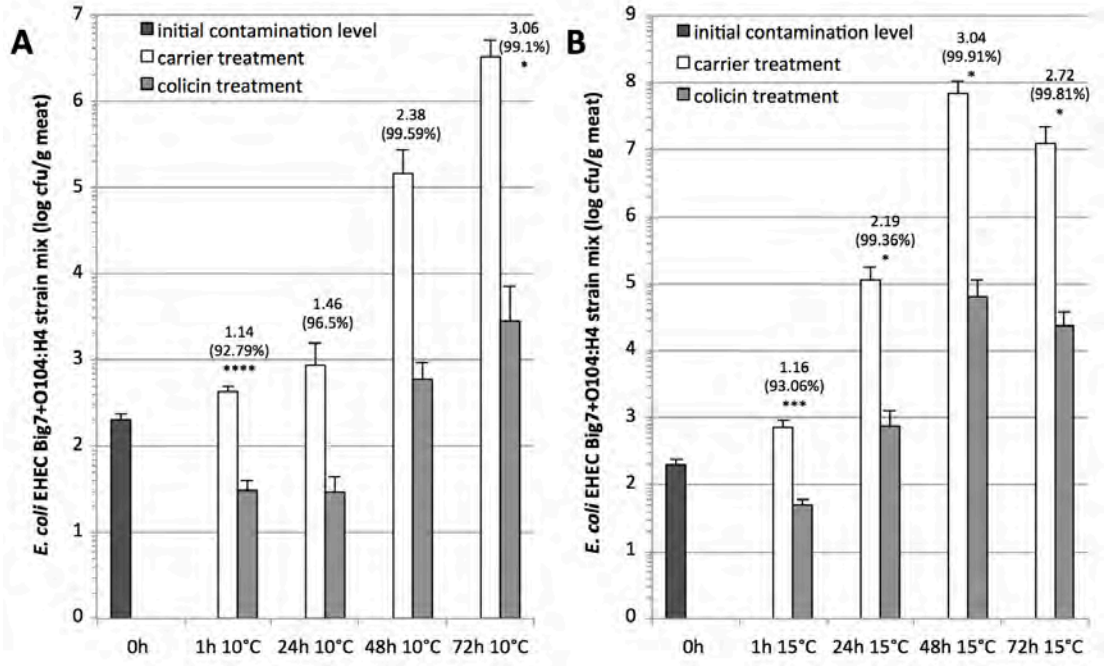
In Experiment I, at 10 °C (Figure 2-7 **A**), statistically significant initial reductions of bacterial populations (T = 0 h versus 1 h and 24 h, unpaired t-test) were followed by re-growth. In this experiment, up to 1.6 log CFU/g (97.4% reduction) difference in bacterial population in COLICIN-treated samples versus carrier treatment was observed. This reduction was statistically significant (unpaired t-test). Difference in mean values for bacterial populations observed at later time points was not significant (unpaired t-test, 48 h time point). At 10 °C bacteria that survived COLICIN treatment grew at normal rates compared to control beginning at 24 h. A similar trend was found upon storage at 15 °C (Figure 2-7 **B**), when up to 2.4 log CFU/g (99.6% reduction) difference between COLICIN and carrier treatment was observed. The bacterial re-growth was faster at 15 °C. Bacteria that survived COLICIN grew at normal rates relative to control beginning at 1 h post treatment, with significantly lower CFU/g at 72 h (unpaired t-test). These results indicate rapid antibacterial action by COLICIN, with a short duration of technical effect (0-24 h).

Figure 2-7. Effect of COLICIN treatment on low-dose EHEC contamination of beef steak meat (Exp I)



A similar trend was found in Experiment II. An initial reduction of bacterial population after COLICIN treatment (T = 0 h vs 1 h and 24 h at 10°C, Figure 2-8 **A**, and 0 h vs 1 h at 15°C Figure 2-8 **B**) was observed followed by re-growth. However, the bacterial population in COLICIN-treated samples was significantly lower than in carrier-treated samples. At either storage temperature, the bacteria that survived COLICIN treatment grew at similar growth rates beginning at 24 h at 10 °C and at 1 h at 15 °C, showing again that COLICIN induces rapid kill (significant reduction in CFU/g) but that the technical effect is short-lived (0-24 h).

Figure 2-8. Effect of COLICIN treatment on low-dose EHEC contamination of beef steak meat (Exp II)



Whole beef cuts, contamination Level II (high)

Five independent experiments were performed with beef steak at high EHEC contamination Level II (4-5 log CFU/g meat). Each experiment included incubation of treated samples at 10 °C and 15 °C. In Experiments I-IV, bacterial populations were analysed at 1, 24, 48 and 72 h post COLICIN or carrier treatment. In Experiment V, sampling included a 0 h time point to verify recoverable inoculum. In all 5 experiments, the net reduction of bacterial population due to the COLICIN treatment was observed at both incubation temperatures. The reduction in log CFU/g in COLICIN- versus carrier-treated samples was more pronounced at lower temperature (10 °C), as expected, because bacterial re-growth was more vigorous at 15 °C.

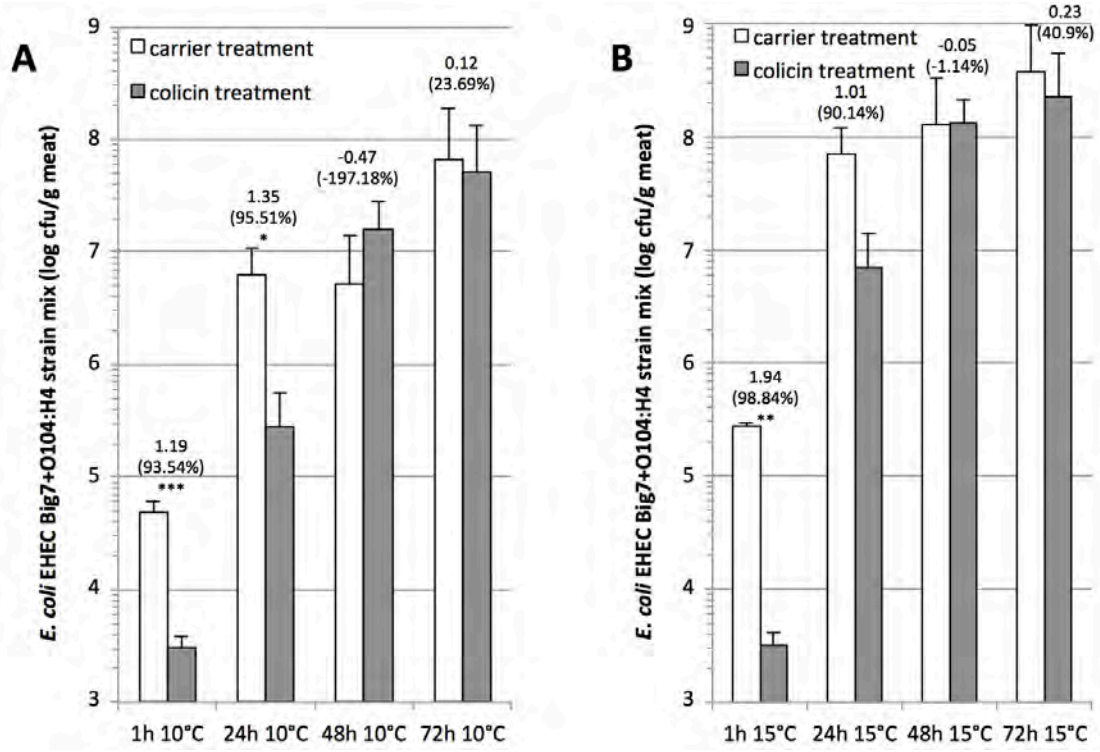
There was a range of antibacterial effects recorded among experiments, as can be expected given the heterogeneity of the meat matrices and the challenges in representative sampling. For example, in 4 of 5 experiments using 3 to 4 sampling/enumeration replicates each, bacterial population reductions in COLICIN-treated versus carrier-treated samples stored at 10 °C ranged from 1.28 to 2.58 log CFU/g (94.81%-99.74%). In only one study (Experiment III) the COLICIN effect was less pronounced (CFU/g reduction of 0.8 log; 83.6%); that experiment was not typical. As expected, differential CFU/g were less pronounced at the higher storage temperature (15°C), with a net range of 0.7 to 2 log CFU/g (79.7-99.0%) reduction in COLICIN-treated versus control, in 4 of 5 experiments using 3-4 sampling replicates each.

Results from Experiments IV and V may be the most representative because these studies were conducted after becoming more familiar with the methods used**.** Statistical evaluations of differences between

COLICIN-treated and control-treated samples were performed by pairwise comparison at the same time points using unpaired parametric t-test (GraphPad Prism v. 6.01 software). In Experiment IV, for 10 °C storage (Figure 2-9 **A**), statistically significant differences in CFU/g between COLICIN and carrier treatments were evident at earlier time points (1 h and 24 h post application) with no significant differences at later time points (48 h and 72 h). Bacteria surviving COLICIN treatment grew at normal rates at 10 °C after 24 h.

The same analyses performed for samples stored at 15 °C (Figure 2-9 **B**) showed significant differences in CFU/g between COLICIN and carrier treatments at 1 h, with no significant differences at later time points. Bacteria surviving COLICIN treatment grew at normal rates at 15 °C after 1 h post treatment. These data corroborate the rapid but temporal nature of COLICIN's technical effect (see Section 2.4.3).

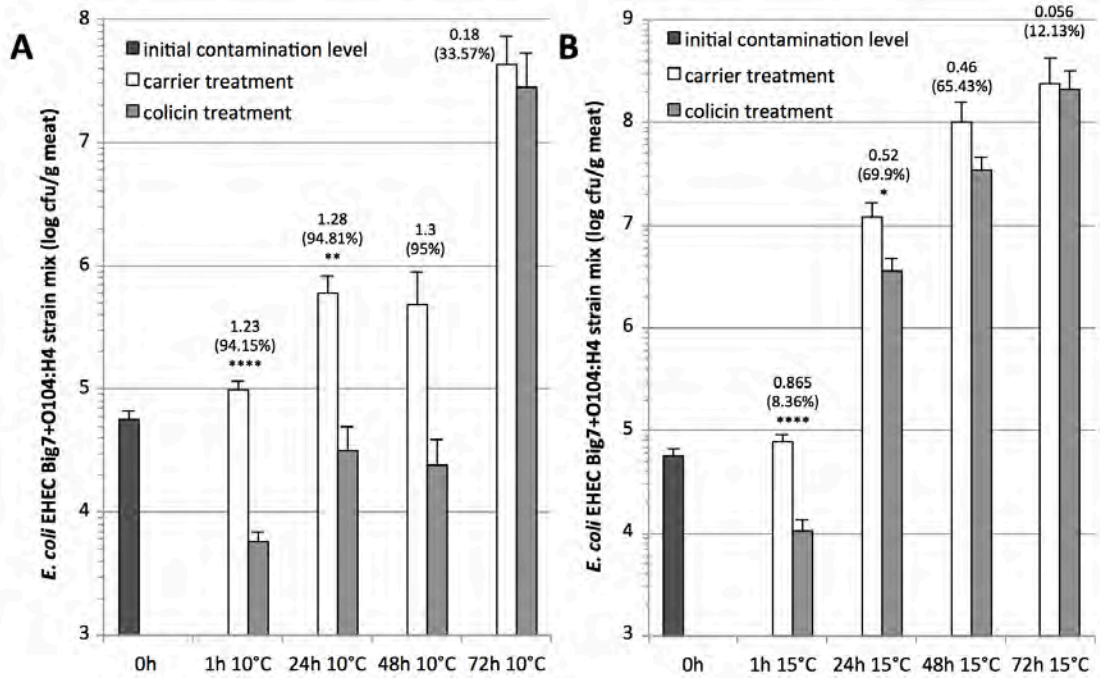
Figure 2-9. Effect of COLICIN treatment on high-dose EHEC contamination of beef steak meat (Exp IV)



A similar trend was observed in Experiment V. In this study, bacterial viability at T = 0 h was measured (i.e. samples taken immediately after the contamination with *E. coli*) to assess recovery of initial bacterial load. The values obtained from carrier-treated samples 1 h after storage (2 h post treatment; 9.85 x 10^4 CFU/g at 10 °C; 7.77 x 10^4 CFU/g at 15 °C) were very close to the initial contamination level measured before treatment of samples (5.68 x 10^4 CFU/g). As expected, growth in COLICIN-treated samples was less at 10 °C (Figure 2-10 **A**) than at 15 °C (Figure 2-10 **B**). With carrier (control) treatment and storage at 10 °C, significant increases of bacterial growth were found between 0 h and later time points (unpaired t-test). In contrast, meat samples treated with COLICIN showed statistically significant reductions (up to 1.3 log CFU/g at 48 h) in bacterial population relative to control (0 h versus 1 h, 24 h and 48 h). After treatment and 15 °C storage (Figure 2-10 **B**), the bacterial re-growth in COLICIN-treated samples was faster than at 10 °C (Figure 2-10 **A**); at 1 h the CFU/g was significantly lower than at 0 h, but at 24 h the bacterial count was already significantly higher than at 0 h. Statistically significant differences between COLICIN and carrier treatments were observed at 1 h and 24 h (unpaired parametric t-test). No statistically significant differences in CFU/g

between COLICIN and carrier treatment were observed at later time points. Bacteria surviving COLICIN grew at normal rates after 1 h storage, underscoring COLICIN's rapid but short-lived technical effect.

Figure 2-10. Effect of COLICIN treatment on high-dose EHEC contamination of beef steak meat (Exp V)



Beef prior to grinding; contamination Level I (low)

Two independent experiments were performed with beef steak at low EHEC contamination Level I (1-1.5 log CFU/g meat), with sampling extended to 144 h (Experiment II). In both studies, nalidixic acid resistant mutants of corresponding EHEC strains were used to discriminate between growth of experimentally introduced EHEC and microbes naturally present on meat. The nutrient medium used for enumeration of viable cells in these experiments was supplemented with 25 mg/L nalidixic acid. In both experiments, COLICIN's antibacterial effect was significant at either 10 °C or 15 °C storage temperature.

In Experiment I, the bacterial population in COLICIN-treated beef ground post treatment and incubated at 10 °C was practically constant and significantly lower than in carrier-treated samples until 72 h (Figure 2-11 **A**). Under these storage conditions, COLICIN treatment reduced viable *E. coli* to levels close to the limits of detection and re-growth of bacteria was not seen. Although a similar effect was also seen among the samples stored at 15 °C (Figure 2-11 **B**), clear re-growth was observed after 48 h of storage. Statistically significant differences in CFU/g between COLICIN- and control-treated samples were observed until 72 h. Statistical analyses at 144 h were not performed due to unspecified contamination in some plates. Once surviving bacteria re-grew, they appeared to have the same rate of growth as the control samples.

In Experiment II, the results obtained were definitive and confirmed the trend observed in Experiment I. In COLICIN-treated samples incubated at 10 °C, bacterial populations remained at very low levels until 72 h, with re-growth observed at 144 h (Figure 2-12 **A**). In contrast, bacteria in carrier-treated samples grew vigorously. At the 48 h sampling point, there was a difference of 4.92 log CFU/g or 99.9% reduction, between COLICIN-treated and control samples. Bacterial re-growth at 15 °C was faster than at 10 °C despite the obvious effect of COLICIN (3.59 log CFU or 99.79% reduction versus carrier treatment at 48 h). At 15 °C,

significant differences in CFU/g between COLICIN- and carrier-treated samples were found at all sampled time points (Figure 2-12 **B**). Surviving bacteria appeared to re-grow at normal rates relative to control.

Figure 2-11. Effect of COLICIN treatment on low-dose EHEC contamination of beef steak meat prior to grinding (Exp I)

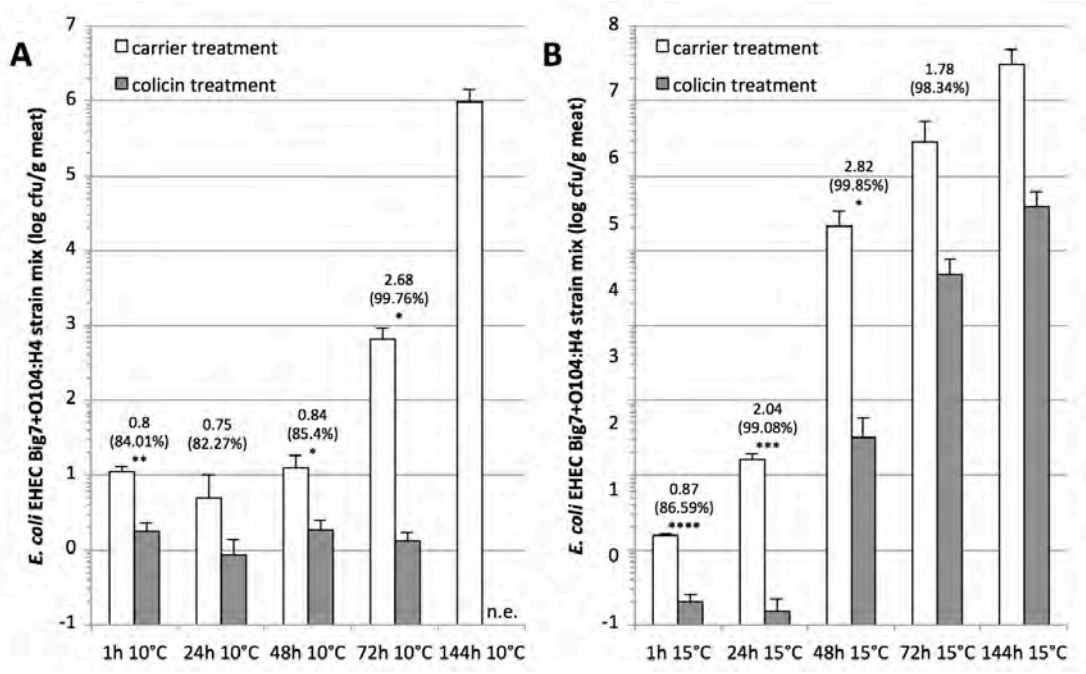
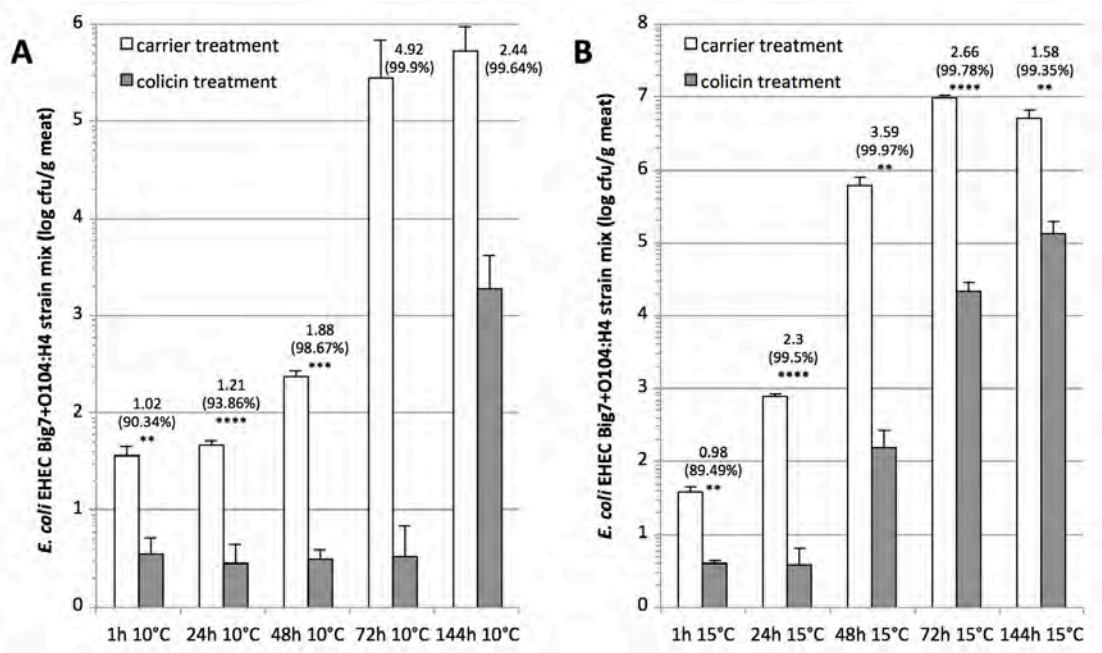


Figure 2-12. Effect of COLICIN treatment on low-dose EHEC contamination of beef steak meat prior to grinding (Exp II)



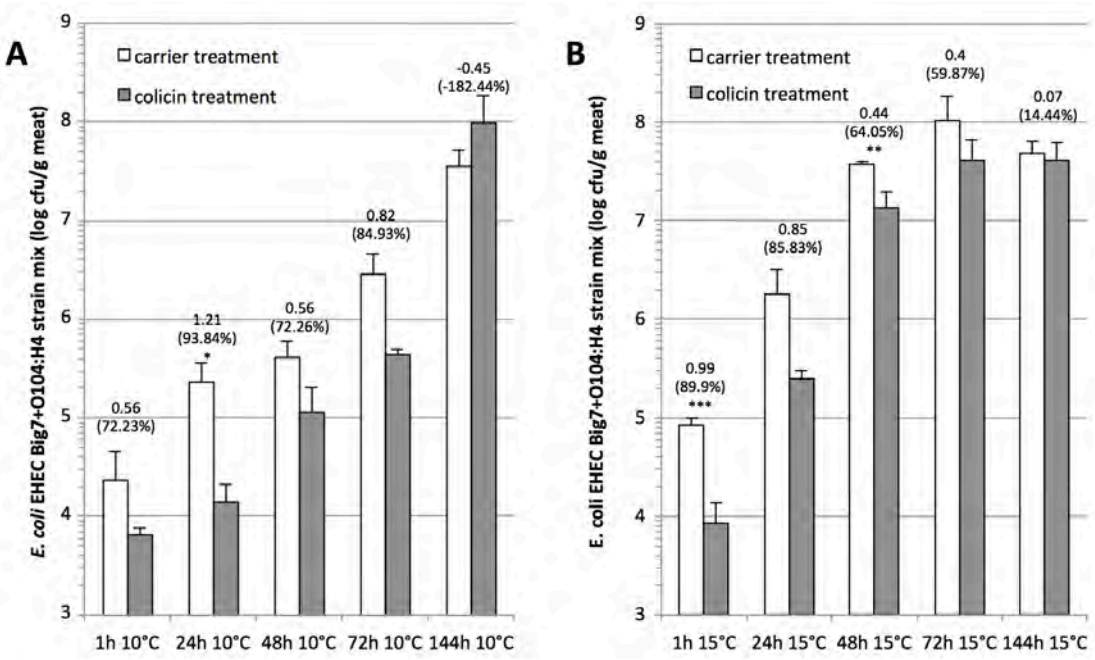
All three experiments displayed rapid yet temporary bactericidal effects in COLICIN-treated samples. The dramatic known-down of pathogens to nearly the limit of detection was not observed in other experiments

with whole cuts of beef. The difference could be due to the grinding process post treatment, which was only used in the comminution of the samples to produce ground beef in these studies.

Beef prior to grinding; contamination Level II (high)

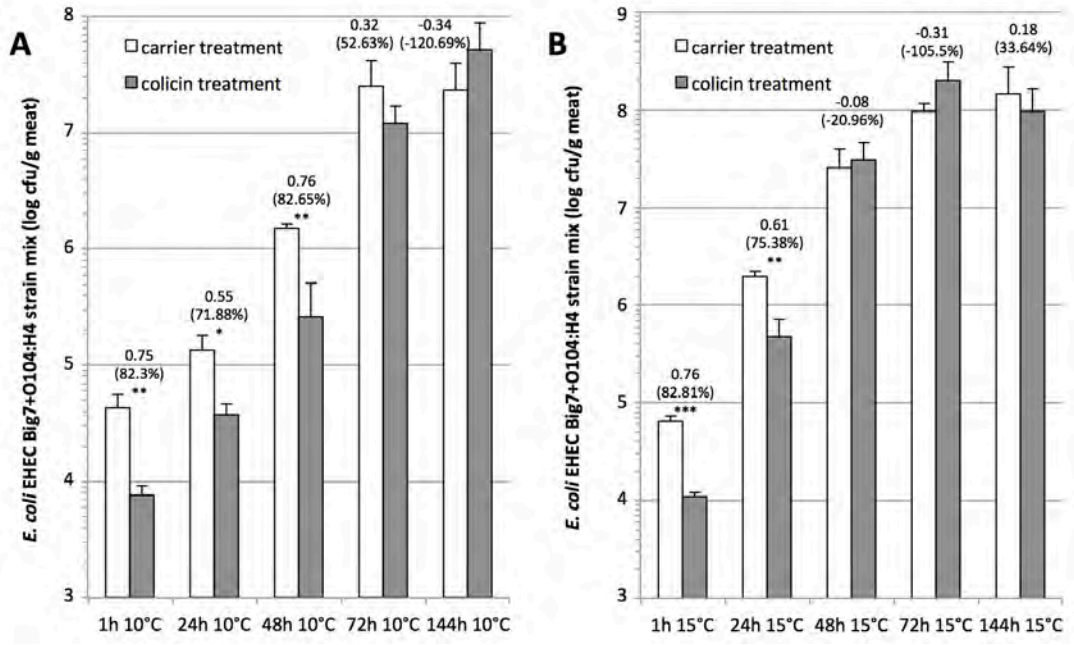
Three independent experiments were performed where beef prior to grinding was exposed to a high level (Level II) of EHEC contamination (4-5 log CFU/g meat). COLICIN or control carrier solutions were applied by spraying contaminated beef samples prior to grinding, samples were stored at 10 °C and 15 °C, and CFU enumeration was performed on triplicate samples at various times of incubation. Bacterial populations were analysed at 1, 24, 48, 72 and 144 hours post treatment. In all 3 studies, COLICIN reduced the number of viable bacteria. Differences in CFU/g between COLICIN- and carrier-treated samples were higher at earlier time points (up to 1.4 log CFU/g or 95.54% reduction) and lower at later time points. In Experiment I at 10 °C, CFU/g between COLICIN and control groups differed significantly up to 24 h (Figure 2-13 **A**). At 10 °C, a maximal difference of 1.21 log CFU/g or 93.8% reduction was seen at 24 h. Incubation of treated samples at 15 °C showed uniformly greater bacterial growth at the higher temperature, with COLICIN treatment reducing CFU/g significantly relative to controls for approximately 48 h. Maximal reduction of 0.99 log CFU/g or 89.9% was seen at 1 h; no statistically significant differences were found at the 72 h or the 144 h sampling points (Figure 2-13 **B**). In all samples at either incubation temperature, bacteria surviving COLICIN appeared to grow at normal rates.

Figure 2-13. Effect of COLICIN treatment on high-dose EHEC contamination of beef steak meat prior to grinding (Exp I)



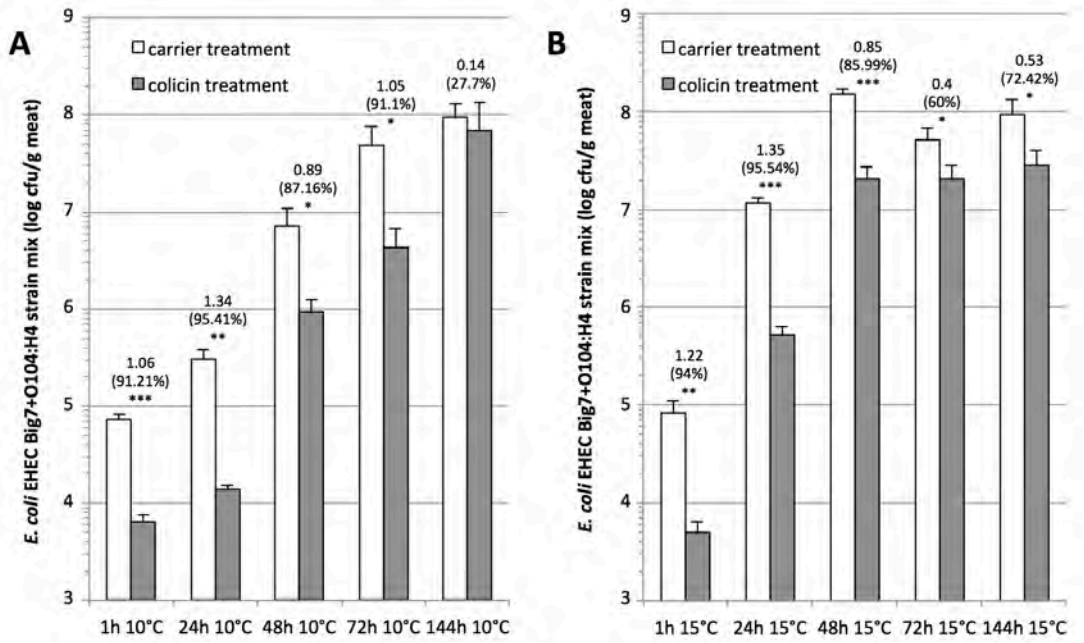
In Experiment II, antibacterial effects of COLICIN versus carrier were shown (maximal difference in 0.76 log CFU/g at both 10 °C and 15 °C, with bacterial re-growth being faster at 15°C). At 10 °C (Figure 2-14 **A**), the CFU/g in COLICIN-treated samples were significantly lower than in carrier-treated meat until 48 h (unpaired t-test). After initial kill, re-growth of surviving bacteria at 15 °C (Figure 2-14 **B**) was faster than at 10 °C, and 24 h was the last time point when a significant difference in CFU/g between the two treatments was observed. Bacteria surviving COLICIN treatment appeared to grow at normal rates relative to controls.

Figure 2-14. Effect of COLICIN treatment on high-dose EHEC contamination of beef steak meat prior to grinding (Exp II)



In Experiment III, the effect of COLICIN was stronger than in the prior studies. At 10 °C, significant reductions of CFU/g were observed up to 72 h (maximum of 1.34 log CFU/g or 95.41% reduction at 24 h; Figure 2-15 **A**). At 15 °C, maximal differences were also at 24 h (1.35 log CFU/g or 95.54% reduction; Figure 2-15 **B**). Population density differences remained significant until 144 h. Re-growth appeared normal.

Figure 2-15. Effect of COLICIN treatment on high-dose EHEC contamination of beef steak meat prior to grinding (Exp III)

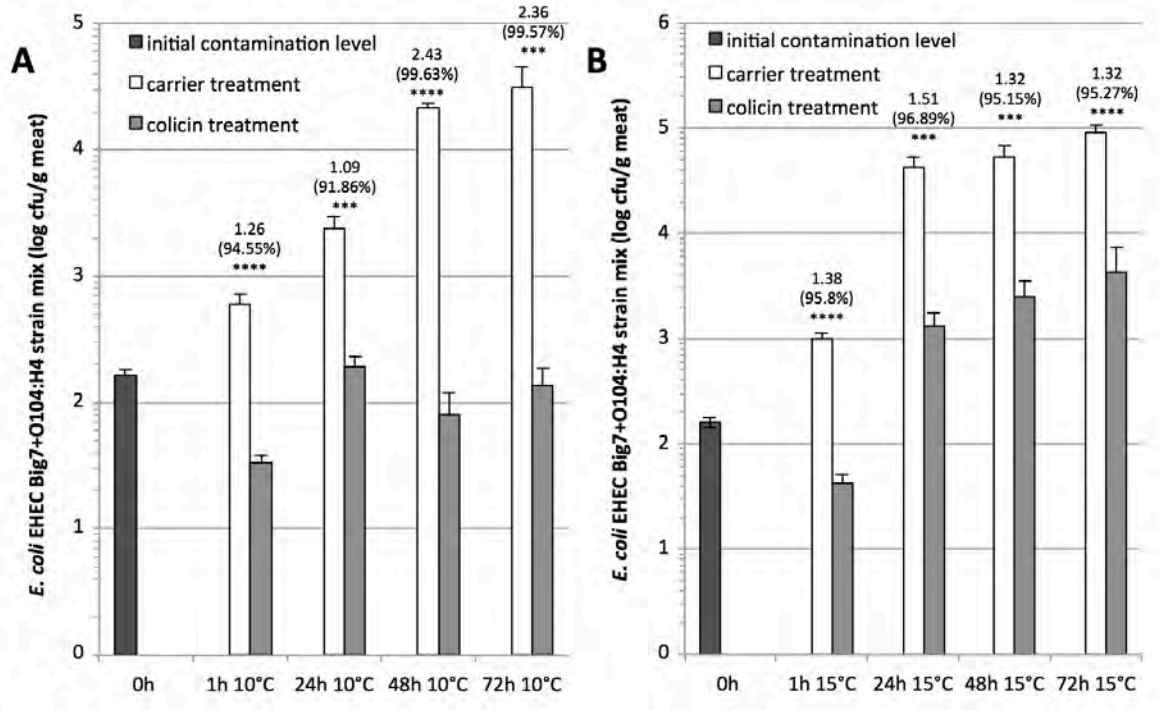


Lamb loin, contamination Level I (low)

Domestically, lamb and adult ovine meat (mutton) are consumed in relatively low quantities relative to other red meats such as beef or pork. Nevertheless, one experiment with lamb loin meat was performed to assess COLICIN's antibacterial effect on this matrix, using a Level I (low) bacterial contaminant load.

In Experiment I, lamb loin meat was inoculated with mixed EHEC suspension of nalidixic acid resistant mutants at ~2 log CFU/g meat. Bacterial populations remaining after COLICIN or control carrier treatment were measured in quadruplicate at 0, 1, 24, 48 and 72 h post treatment. Bacteria in carrier-treated samples steadily grew during storage at 10 °C (Figure 2-16 **A**). In contrast, after some initial reduction in CFU/g (1 h), bacteria in the COLICIN-treated samples did not grow well. This is the only study exhibiting poor re-growth of COLICIN survivors. COLICIN also induced significant reductions in CFU/g at 15 °C (Figure 2-16 **B**); re-growth of survivors was higher than at 10 °C, as expected, and the difference in CFU/g between COLICIN- and carrier-treated samples was narrower yet remained significant for the duration of the study.

Figure 2-16. Effect of COLICIN treatment on low-dose EHEC contamination of lamb loin meat (Exp I)



Pork steak, contamination Level II (high)

The goal of this series of experiments was to determine the effect of COLICIN followed by storage at a lower temperature (4 °C) than those evaluated in the beef and lamb experiments described above (i.e. 10 °C and 15 °C). Pork steak meat was used to evaluate yet another meat matrix. Various blends of colicins (from 2 to 8 components) were also evaluated to determine the relative antibacterial value of each mix.

Two independent experiments with pork cuts were conducted. In both experiments a high level of *E. coli* contamination (Level II; 4-5 log CFU/g meat) was used. The inoculum included the Big Seven EHEC strains and O104:H4 cultures mixed in equal proportions. Meat samples were contaminated with EHEC by dipping the samples in the bacterial suspension, to ensure an even application.

In these studies, up to 4 different colicin formulations were evaluated as shown below. In all formulations, Col M was applied at 3 mg/kg meat. All other colicins in the mixtures were applied at 1 mg/kg meat.

Experiment I

1) Two-component colicin mix containing Col M and Col E7;
2) Four-component colicin mix containing Col M, Col E7, Col K and Col Ia; and
3) Eight-component colicin mix containing Col M, Col E7, Col K, Col Ia, Col E2, Col E6, Col 5 and Col U.

Experiment II

1) Two-component colicin mix containing Col M and Col E7;
2) Four-component colicin mix containing Col M, Col E7, Col K and Col Ia;
3) Five-component colicin mix containing Col M, Col E7, Col K, Col Ia and Col E6; and
4) Eight-component colicin mix containing Col M, Col E7, Col K, Col Ia, Col E2, Col E6, Col 5 and Col U.

After 30 min incubation at room temperature to allow bacterial adhesion, meat samples were sprayed with a hand-held sprayer with either colicin solution or carrier (plant extract without colicin) control solution. Sprayed samples were incubated for 1 h at RT after treatment at then stored at 4 °C and held for various times for analysis. Bacterial populations were measured at 1, 24 and 72 h post treatment. COLICIN treated samples were compared with samples treated with carrier and with samples contaminated with bacteria without any treatment (no treatment control). In both experiments significant net reductions in bacterial populations after COLICIN treatment were observed. The antibacterial effect depended on the colicin cocktail composition of the COLICIN product. The overall potency of the eight-component cocktail was higher than that of the four-component mix, which in turn was higher than that of the two-component mix.

In Experiment I (Figure 2-17), significant reductions in CFU/g occurred between COLICIN-treated and carrier- or no-treatment groups. The maximal reduction occurred with the eight-component mix at 24 h incubation (1.95 log CFU/g or 98.87% reduction). The bactericidal effect did not improve over time for any colicin mix. For example, for the two-component colicin mix, there was no significant additional killing at 24 or 72 h relative to the 1 h sampling point. Similarly, for the four-component mix, the CFU/g value at 72 h was not significantly lower than at 24 h. And for the eight-component colicin mix, there was no significant CFU/g reduction in samples incubated for 24 h or 72 h over those incubated for only 1 h. These results again demonstrate COLICIN's rapid but transient technical effect.

In Experiment II (Figure 2-18), the effects of COLICIN were stronger (2 log CFU/g reduction for four- and eight-component cocktails at only 1 h post-treatment incubation; up to 2.6 log CFU/g maximal reduction for the eight-component mix at 24 h or 99.76% reduction in bacterial population). The carrier solution (plant extract without colicins) did not induce antibacterial or growth-promoting effects over the no treatment control. Also, no bacterial re-growth was found in either study for any kind of treatment. We surmise that this was due to the low post-treatment storage temperature, as 4 °C is too low a temperature for *E. coli* growth, considering that the optimum for enteric bacteria including *E. coli* is 37 °C although growth as low as 7.5 °C has been reported (Ingraham 1996).

Regardless, even at a low meat storage temperature of 4 °C the antibacterial effects of COLICIN were quite strong, suggesting that the product can complement low temperature refrigeration to help ensure safety. That is, COLICIN can effect rapid "knock down" in CFU of viable pathogens during the temporary handling or processing of meat at RT, complemented by proper low-temperature storage afterwards. These data corroborate the value of COLICIN when used in meat processing at higher temperatures such as 10 °C and 15 °C, which are more typical of processing conditions in industrial settings.

Another conclusion that can be drawn from low-temperature studies with pork cuts using various mixes of colicins is that the antibacterial effect is significant but short-lived. No improvement in technical effect was found at 72 h relative to 24 h or 1 h of post-treatment incubation. These results with multiple colicin mixtures corroborate the temporal nature of COLICIN's antibacterial effects seen also at 10 °C and 15 °C.

Figure 2-17. Effect of COLICIN treatment on high-dose EHEC contamination of pork cuts (Exp I)

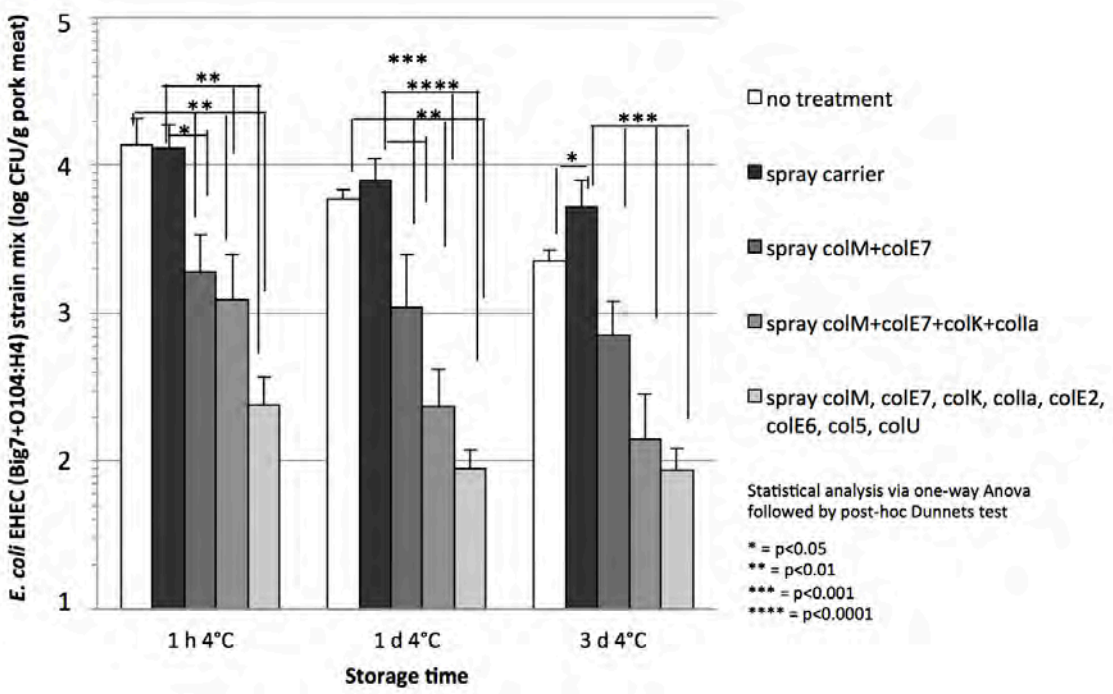
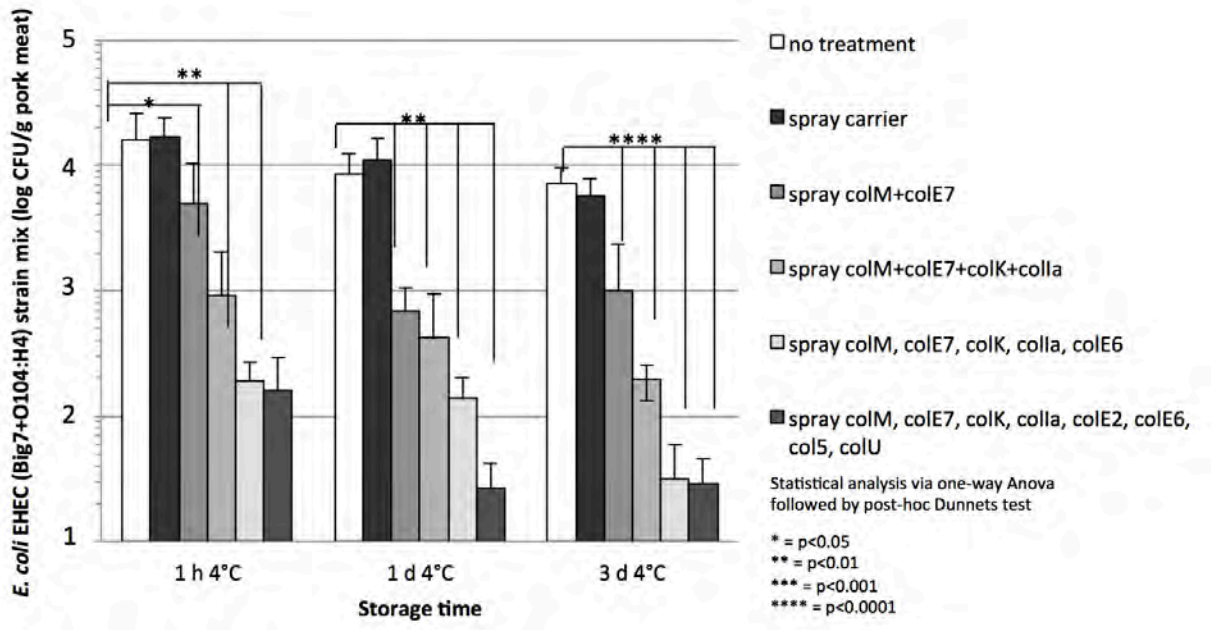


Figure 2-18. Effect of COLICIN treatment on high-dose EHEC contamination of pork cuts (Exp II)



2.4.3 Duration of COLICIN's technical effect

The duration of COLICIN's technical (antibacterial) effect after application to meat products was evaluated. Typical experiments are summarized here, and the methods applied are further detailed in APPENDIX A.

Definition of technical effect and duration of technical effect

In this Notice, the **technical effect** is defined as the bactericidal effect of COLICIN, and is quantified by determining the population of target pathogens remaining in an experimentally contaminated food matrix (CFU/g) after exposure to either COLICIN or a control solution (i.e. carrier solution containing no colicins). The **duration of technical effect** is the time after COLICIN treatment at which bacteria that have survived the effects of COLICIN are quantified to begin normal growth relative to the control treatment.

Summary of methods

Samples of red meat (e.g. beef) were contaminated with measured concentrations of pathogenic *E. coli* (EHEC) strains, incubated at RT for 30 min to allow bacterial colonization, treated with a metered volume of plant-produced COLICIN antimicrobial solution, and incubated at two controlled temperatures (10 °C and 15 °C). Control treatment solution consisted of total soluble protein extract from the same production host plant not containing colicins ("carrier solution"). At various times after treatment and storage, samples of treated meat were homogenized to recover bacteria for enumeration of viability. Bacteria-containing suspensions were serially diluted and plated on selective nutrient medium and incubated at 37 °C for 18-24 h, after which the number of colonies were enumerated. Viability was determined as a function of treatment, time of incubation and storage temperature.

Bacterial strains and contamination

Because contamination of meats with EHEC during food production and processing may include one or more strains, in these experiments we used a mixture of 8 EHEC strains that have been associated with food-borne outbreaks. The "Big7+1" mixture consists of the Big Seven strains shown in APPENDIX A Table 8-1, plus *E. coli* strain 0104:H4, which was associated with the major food-borne outbreak in Europe in 2011. The bacterial suspension contained approximately equal numbers of the Big 7+1 in buffer and was applied to meat samples at a rate of approximately 4-5 log CFU per gram of meat. The application procedure used is found in APPENDIX A.

COLICIN application

The contaminated meat samples were sprayed (47 mL/kg meat maximum; see methods in APPENDIX A) with a COLICIN solution comprising 6 different colicins at the following ratio: 3 mg/kg Col M, 1 mg/kg Col E7, 1 mg/kg Col Ia, 1 mg/kg Col K, 1 mg/kg Col 5, and 1 mg/kg Col U. Thus the application rate was 8 mg total colicins per kg treated meat. The carrier (control) solution was sprayed at an equivalent volume and total protein concentration but contained no colicins. After COLICIN or carrier solution application, the treated, contaminated samples were held at room temperature for 1 h to model the duration of meat processing, after which time they were moved to temperature-controlled incubators and held at either 10 °C or 15 °C.

Sampling

Samples of treated meat were collected at 0 h (before application of either COLICIN or carrier solution) and analyzed immediately or after storage at 1, 24, 48 and 72 h. For microbiological analysis, the samples were homogenized for uniform recovery of viable bacteria, concentrated by centrifugation to isolate pellets containing bacteria, which were then resuspended and serially diluted and plated on selective medium.

Exposure experiments were conducted in duplicate, and enumerations were done in triplicate. CFU were determined per gram of meat and results were analyzed for statistical significance. A full description of the methods applied can be found in APPENDIX A.

Results

The total CFU/g for samples treated with COLICIN or carrier solution as a function of storage temperature and storage time were calculated.

Bactericidal effect at 10 °C. Results of technical effect as a function of time for meat samples stored at 10 °C are shown in Figure 2-19. Under these conditions, inhibition of bacterial growth is a function of COLICIN bactericidal technical effect and low temperature.

Bactericidal effect at 15 °C. The same calculations were done for samples stored at 15 °C. At this higher temperature, more robust bacterial growth can be expected, so the conditions model bacterial growth on meat products that are poorly handled or improperly stored during and/or after processing.

Technical effect as a function of time on meat samples stored at 15 °C is shown in Figure 2-20. Under these conditions, low temperature has less influence on controlling bacterial growth.

Comments on level of contamination. For calculating residual technical effect over time, a high bacterial inoculum density of 4-5 log CFU/g of meat was used to model a worst-case scenario. Due to the organoleptic properties of meat contaminated to this extent, it is highly unlikely that the meat would be sold for consumption because it would be considered a spoiled food product.

Nevertheless, the results of these experiments clearly show COLICIN's technical effect is significant relative to control treatments and that COLICIN's duration or longevity of activity after application is brief.

Figure 2-19. Duration of COLICIN's technical effect on beef samples contaminated with pathogenic *E. coli* strains (Big Seven+0104:H4) and stored at 10 °C

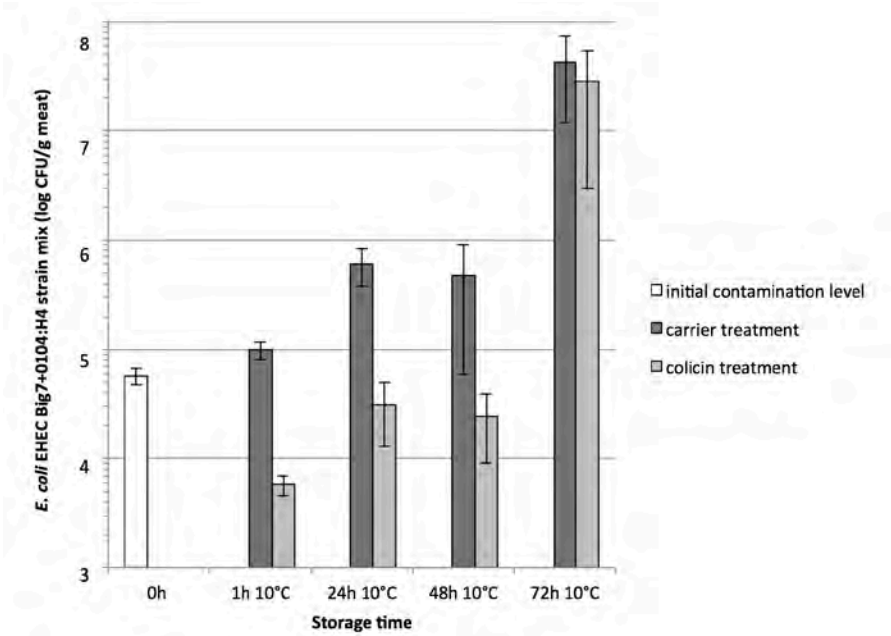
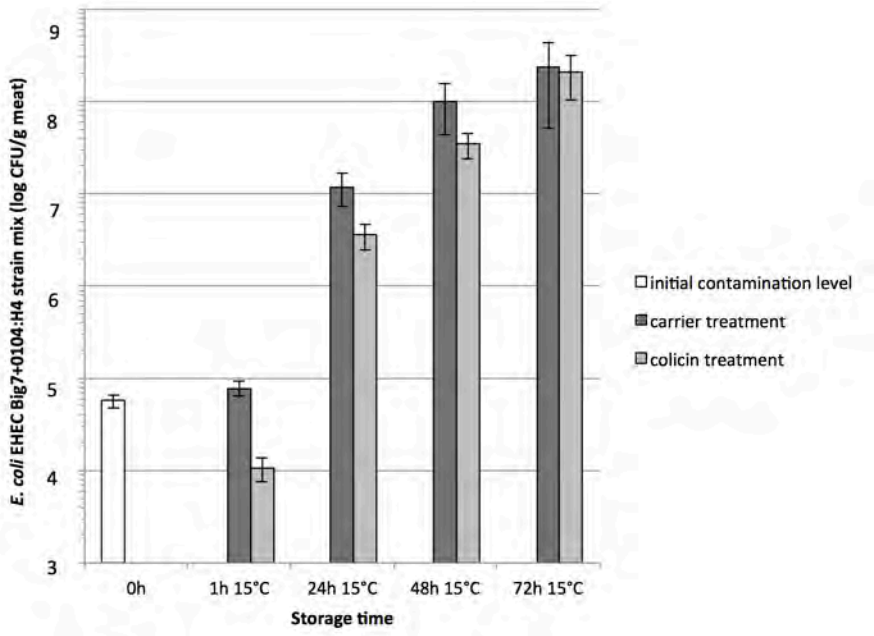


Figure 2-20. Duration of COLICIN's technical effect on beef samples contaminated with pathogenic *E. coli* strains (Big Seven+0104:H4) and stored at 15 °C



Summary and conclusion

Results of these experiments indicate that plant-produced COLICIN is antibacterial against a mix of pathogenic (including STEC/EHEC/EAEC) strains of *E. coli* that includes all Big Seven members plus O104:H4, at bacterial contamination levels (4.5-5 log CFU/g meat) that would typically render the meat not fit for consumption due to organoleptic effects even if the contamination was due to non-EHEC strains.

The technical effect provided by COLICIN treatment was seen when contaminated meat samples were stored at 10 °C and even when stored at an inappropriately high temperature of 15 °C. Due to the very high level of contamination, only about a 1-log reduction in CFU/g is observed between COLICIN- and control-treated groups. This is in sharp contrast to the >2 log CFU/g control afforded by COLICIN when more typical levels of contamination (i.e. Level I) of whole cuts and ground meat are used (see Section 2.4.2). However, more bacteria were killed by COLICIN at the high level of contamination than at the lower level tested.

Importantly, at the application rate of 8 mg/kg meat and post-treatment sample storage at 10 °C, COLICIN's technical effect was observable from T = 0 (application) until the 1 h sampling point, when the growth of COLICIN-surviving bacteria is clearly evident (Figure 2-19). In that study, the 48 h sample counts appear anomalous to the rest, but with or without that time point the rate of growth of bacteria in the COLICIN and control groups appear equivalent.

At the same rate of COLICIN application and 15 °C storage, the technical effect was observable for up to 1 h, after which time the bacteria surviving COLICIN treatment appear to grow normally (significant differences are evident only at the earlier time points), albeit more vigorously at the higher storage temperature (Figure 2-20).

These experiments demonstrate that COLICIN application results in rapid bactericidal control of pathogenic *E. coli* applied to samples of red meat and that the technical effect observed is transient, lasting for approximately 1 h post application and perhaps up to 24 h, but not longer, depending on the storage

environment. The lower CFU/g in all samples of COLICIN-treated meat relative to controls is due to the reduction in CFU resulting from COLICIN treatment.

COLICIN is a food processing aid

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

COLICIN meets this definition based on the following criteria:

a. COLICIN provides temporary antibacterial effect lasting at most 0-24h and typically 0-1 h post application to several types of meat products;

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

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

As such, COLICIN-treated meat products are exempt from the FSIS labeling requirement.

2.4.4 Compatibility of COLICIN with pressurized spray application equipment

The formulated COLICIN product consists of a solution of antimicrobial colicin proteins. The solution is designed to be compatible with a range of food disinfection practices, including application to meats prior to processing via spray, dip (immersion), wash, or marinade, or as a low-volume package additive to RTE meats. Washing, immersion or dip are low-shear practices that are not expected to unduly denature the colicin proteins. Spraying of a solution onto food using commercial high-pressure spray equipment is a high-shear practice that has the possibility of adversely impacting protein structure and hence potentially diminishing the efficacy of COLICIN.

To assess the compatibility of COLICIN with pressurized spray equipment, various hand-held and pilot-scale sprayers were evaluated, ranging from a simple atomizer to various adjustable-pressure sprayers with defined nozzle apertures. These applicators were used to quantify the impact of spray equipment design on COLICIN activity against *E. coli* tester strain DH10B. Test equipment used is summarized in Figure 2-21.

Experimental design

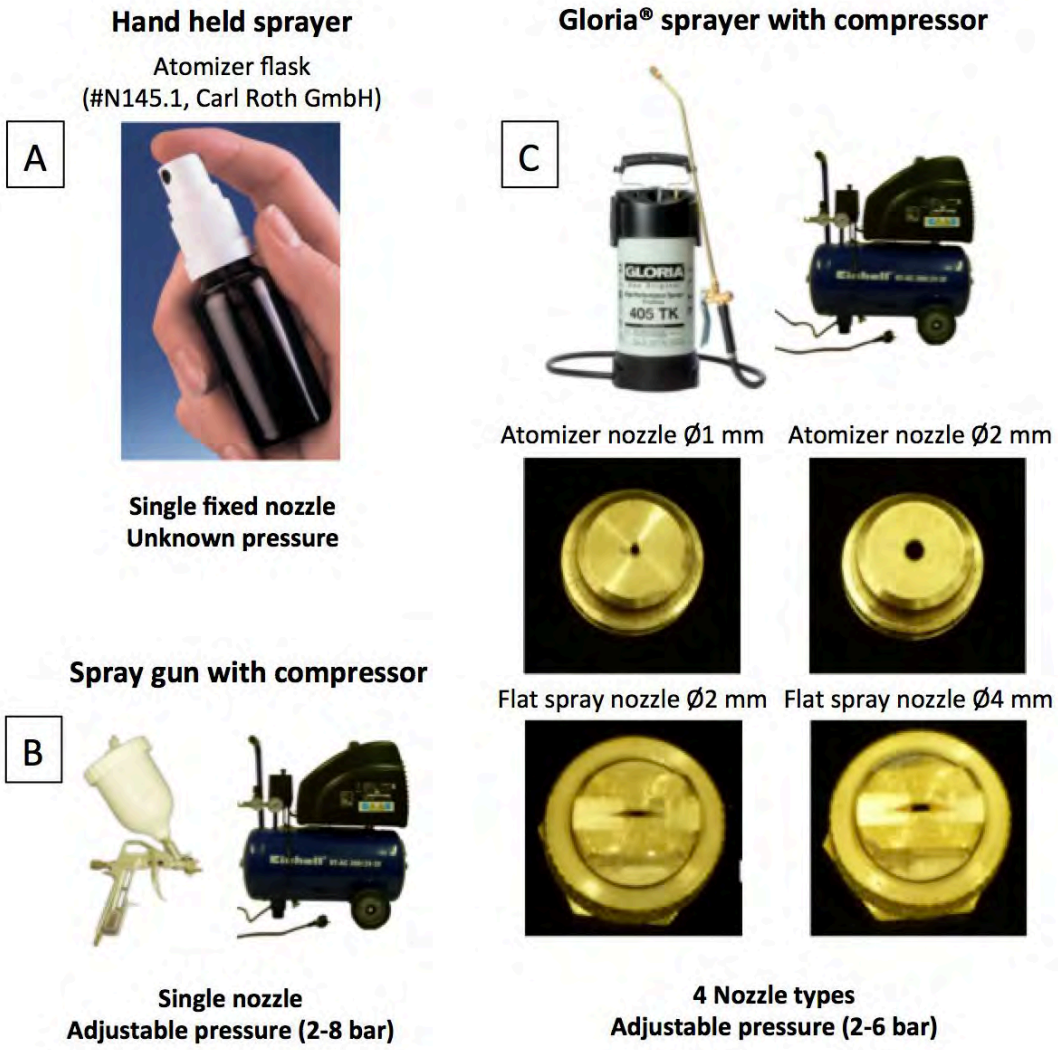
To test compatibility of COLICIN antibacterial solution with spray equipment, individual colicins were produced recombinantly, extracted, mixed to predetermined ratios, sprayed using different applicators (shown in Figure 2-21), collected and assayed for antibacterial activity using non-sprayed COLICIN solution as a reference control.

Methods

Individual colicins were produced as previously described (**GRN 593 APPENDIX B: COLICIN Manufacturing Process; pp 51-57**). For these studies, after accumulation of colicins in plant tissue, plant leaf samples were frozen at -80°C until analysis. Aliquots of frozen tissue were removed from cold storage, supplemented with 5 vol pre-chilled buffer (50 mM HEPES pH7.0, 10 mM K acetate, 5 mM Mg acetate, 10% (v/v) glycerol, 0.05%

(v/v) Tween-20, 300 mM NaCl), mixed, incubated on ice and centrifuged for 10 min, 4000 rpm at 4°C. The supernatants were transferred to new containers for protein analysis. Total soluble protein (TSP) concentration of each isolate was determined by the Bradford assay, and TSP samples containing individual colicins were diluted to the desired concentration for further blending and stored on ice until use.

Figure 2-21. Spray equipment used to determine compatibility with applied COLICIN solution



Three types of sprayers were used to determine their compatibility with applied COLICIN solution and the impact on COLICIN efficacy. **A**. hand-held atomizer flask, single fixed nozzle with non-regulated (unknown) pressure; **B.** spray gun with single nozzle, adjustable pressure from compressor, 2-8 bar; **C.** Gloria® brand sprayer, 2 nozzle types of 2 diameters each (4 combinations), adjustable pressure from compressor, 2-6 bar.

The colicins were then blended into the COLICIN solution at the following ratio: Col M: 3 mg/kg + 1 mg/kg each for Col E7, Col Ia, Col K, Col 5 and Col U. The ratio and strength of the colicins in this solution were the same as those in the COLICIN solution applied to meats to determine efficacy (Section 2.4.2). The COLICIN solution prepared as described above was used to fill various spray containers as shown in Table 2-7. Test Sample 1 in the table was a control comprising the same COLICIN solution that was not sprayed with any application equipment. Test Sample 2 was sprayed 10 times (10 pumps) with a hand sprayer comprising a

fixed, round atomizer nozzle using unknown pressure. Samples 3 and 4 were applied with a spray gun pressurized with a compressor to the indicated pressures (psi/bar). Test Samples 5-12 were sprayed with a Gloria® brand sprayer with compressor, using the nozzle types and pressures indicated. Spray times varied as indicated in the table. NA = not applicable.

Table 2-7. Design of COLICIN compatibility study with various pressurized spray devices

Sample	Equipment/Sprayer	Nozzle/Type	Pressure	Spray Duration
1	No spray (control)	NA	NA	NA
2	Hand atomizer flask	Undefined; atomizer	Undefined	10 pumps
3	Spray gun	Undefined; atomizer	40 psi (2.8 bar)	5 sec
4		Undefined; atomizer	100 psi (6.9 bar)	5 sec
5	Gloria® sprayer	Atomizer; Ø 1mm	40 psi (2.8 bar)	5 sec
6		Atomizer; Ø 2mm	40 psi (2.8 bar)	5 sec
7		Flat spray; Ø 2mm	40 psi (2.8 bar)	1 sec
8		Flat spray; Ø 4mm	40 psi (2.8 bar)	1 sec
9		Atomizer; Ø 1mm	87 psi (6 bar)	5 sec
10		Atomizer; Ø 2mm	87 psi (6 bar)	5 sec
11		Flat spray; Ø 2mm	87 psi (6 bar)	1 sec
12		Flat spray; Ø 4mm	87 psi (6 bar)	1 sec

Analyses

The sample solutions were analyzed for antibacterial activity post application, using the non-sprayed COLICIN solution (Sample 1 in Table 2-7) as the reference standard. A bioassay was used to determine variances in antibacterial activity as a function of mode of application. Briefly, colicin solution was collected in sterile tubes after application and diluted using 50 mM HEPES buffer pH7.0, 10 mM K acetate, 5 mM Mg acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween 20, and 300 mM NaCl. Serial dilutions began with 1:10, 1:100 and 1:1000 followed by 37 1:1 dilution steps. LB plates supplemented with 50 µg/ml streptomycin and 50 µg/ml kanamycin were prepared in advance by pouring ~25 ml melted LB solid medium in sterile quadratic Petri dishes (~120x120x17 mm). On the day of experiment, plates were incubated at 55°C, LB soft agar medium was melted, aliquoted to 25 ml in 50 ml Falcon tubes and incubated at 50°C.

Five (5) ml of LB supplemented with 50 µg/ml streptomycin and 50 µg/ml kanamycin with overnight cultures of indicator *E. coli* strain DH10B (pICH73311) were inoculated from -80°C glycerol stock. A saturated liquid culture grown overnight was diluted to OD_{600}=1.0. Each aliquot of LB soft agar medium (~45°C) was supplemented with 50 µg/ml streptomycin and 50 µg/ml kanamycin and 250 µl bacterial culture of OD_{600}=1.0, mixed and immediately poured on pre-poured LB plates. Soft agar overlay plates were stored at 4°C (max. 4 h) until use.

Five (5) µl of serial 1:1 dilutions of COLICIN test solutions were applied to soft agar overlay and the plates were incubated at 37 °C for 16-20 h. COLICIN activity expressed as AU/mg fresh weight (FW) plant material

was determined by visual inspection of plates for growth inhibition using a background light source. The highest dilution showing a visible difference in opacity between treated to non-treated bacterial growth areas was deemed the minimum inhibitory concentration and correlated to sample treatment.

Results

Table 2-8 summarizes the results obtained in this study, expressed as averages of two independent replicate experiments. Although the bioassay method used is semiquantitative, the relative activity of COLICIN solution samples applied using various types of spray equipment, nozzle designs, pressure conditions, and duration of application, enabled comparison to the reference standard, from which the compatibility and suitability of the equipment for applying COLICIN could be concluded.

There was no degradation of COLICIN's antibacterial activity under any of the application conditions studied. Using the antibacterial activity of the non-sprayed reference standard solution (Sample 1) as 100%, all treatments resulted in maintenance of 100% of the activity regardless of the geometry of the nozzle (circular or flat), pressure applied (2.8 to 6.9 bar = 40 to 100 psi), application time (1 to 5 sec), or equipment scale (hand-held atomizer to pilot-scale Gloria® sprayer).

Table 2-8. Retention of antibacterial activity of COLICIN solution after spray application

Sample	Equipment/Sprayer	Nozzle/Type	Pressure	Spray Duration	Colicin Activity[2]	Percent Activity[3]
1	No spray (control)	NA	NA	NA	$4.1x10^6$	100
2	Hand atomizer flask	Atomizer[1]	Undefined	10 pumps	$4.1x10^6$	100
3	Spray gun	Atomizer[1]	40 psi (2.8 bar)	5 sec	$4.1x10^6$	100
4		Atomizer[1]	100 psi (6.9 bar)	5 sec	$4.1x10^6$	100
5	Gloria® sprayer	Atomizer; Ø 1mm	40 psi (2.8 bar)	5 sec	$4.1x10^6$	100
6		Atomizer; Ø 2mm	40 psi (2.8 bar)	5 sec	$4.1x10^6$	100
7		Flat spray; Ø 2mm	40 psi (2.8 bar)	1 sec	$6.55x10^7$	>100
8		Flat spray; Ø 4mm	40 psi (2.8 bar)	1 sec	$6.55x10^7$	>100
9		Atomizer; Ø 1mm	87 psi (6 bar)	5 sec	$6.55x10^7$	>100
10		Atomizer; Ø 2mm	87 psi (6 bar)	5 sec	$6.55x10^7$	>100
11		Flat spray; Ø 2mm	87 psi (6 bar)	1 sec	$6.55x10^7$	>100
12		Flat spray; Ø 4mm	87 psi (6 bar)	1 sec	$6.55x10^7$	>100

[1] Nozzles in equipment used with Samples 1-3 were round atomizer type with undefined apertures.

[2] Colicin antibacterial activity, expressed as AU/mg fresh weight plant material, was calculated from visual inspection of clearing zones in agar culture of *E. coli* DH10B at highest dilution that produced growth inhibition, per description in Analyses, above.

[3] Antibacterial activity remaining after spray application relative to non-sprayed reference COLICIN solution in Sample 1 (defined as 100%).

Samples sprayed with the pilot sprayer using a flat nozzle at either pressure tested, and round aperture atomizer nozzle at >40 psi (6 bar), showed >100% activity, for reasons that are not clear. Potentially,

residual plant proteins present in the formulation that interfered with COLICIN activity may be denatured or deactivated during some spraying procedures. We plan to repeat these studies with industrial-scale equipment and to optimize the spray conditions to ensure consistent delivery of desired antibacterial effect. Nevertheless, our results using a variety of sprayers show that COLICIN solution is compatible with such equipment and does not lose antibacterial efficacy.

2.5 Effect of COLICIN Application on Organoleptic Properties of Meat

The methods applied to determine COLICIN efficacy and duration of technical effect as reported in this Notice used meat matrices inoculated with a mixture of pathogenic strains (EHEC/STEC/EAEC) of *E. coli*. As such, no organoleptic evaluations were conducted.

Solutions of mixed colicin proteins, formulated as either lower purity COLICIN Concentrate (Table 2-2) or higher purity COLICIN Isolate (Table 2-3) are generally visually clear with only a slight beige or tan tint, and have no objectionable odor. The solubles in the COLICIN product are applied at 8-10 mg/kg meat (8-10 ppm) initial concentration, and become diluted by diffusion into the matrix upon storage. There is no masking of the color of meat after COLICIN application. No organoleptic changes are anticipated after application, although an independent evaluation will be conducted later in product development.

2.6 Non-Interference of COLICIN with Pathogen Detection Methods

The component colicin proteins in the COLICIN product formulation are specific for *E. coli* strains and exert antibacterial effects quickly even at low levels (8-10 ppm). In liquid suspension cultures, multi-log reductions in CFU are seen within minutes of colicin application (Section 2.4.1). Meat matrices (beef, pork, lamb) inoculated at low (1-2 log CFU/g) and high (4-5 log CFU/g) contamination levels with a mixture of pathogenic *E. coli* strains were sampled at 1 h and at various times after storage at three different temperatures. Uniformly, rapid and significant reductions in CFU/g were seen at the earliest time of sampling, followed by normal growth of surviving bacteria in COLICIN-treated samples relative to control treatments.

Inspection of meat products for the presence of pathogens can be done by taking samples from meat surfaces prior to and after COLICIN application. As shown in the studies reported in this Notice, viable bacteria will grow after COLICIN treatment if incubated at a permissive growth temperature. Therefore COLICIN application should not interfere with pathogen determination methods used in meat processing, including protein-protein interactions for ELISAs, or PCR-based amplification reactions. Moreover, since some plates in our studies were lost due to non-specific contaminants naturally present on the meat surface (including fungi), COLICIN application does not appear to interfere with growth of non-target microorganisms and should not interfere with methods used in their detection.

2.7 Occupational Safety Related to Use of COLICIN Product

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

The antibacterial proteins comprising COLICIN are GRAS (GRN 593) and are made endogenously in the human intestine and in the gut of many domestic animals to which we are exposed. The colicin proteins used in the COLICIN product have low allergenic potential. The host plants used to produce colicins are food species including lettuce, red beet and spinach, which can be consumed at unrestricted levels. Therefore,

any residual proteins and plant extractives remaining in the COLICIN formulation are GRAS. In addition, the excipients in the formulation are food grade and approved for food use.

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

2.8 Overall Conclusion

The results of studies we report in this Notice and that we previously described in GRN 593 and in Schulz 2015, corroborate numerous studies on colicins reported by others in the literature. Collectively and uniformly, these studies verify the bactericidal activity of colicins against pathogenic strains of *E. coli* (STEC/EHEC/EAEC) responsible for food-borne outbreaks in much of the world.

In this Notice, as in GRN 593, bactericidal activity of plant-produced colicins was verified using standard microbiological procedures *in vitro*, as well as using food matrices and contamination levels that are relevant to industrial food processing environments. The antimicrobial potency of COLICIN (mixed colicin product) applied to meats was confirmed under a variety of conditions ranging from a low contamination level of 1-2 log CFU/g meat to a high contamination level of 4-5 log CFU/g meat.

Three mammalian meats were used as sample matrices, namely beef, pork and lamb. COLICIN treatment of these various meat products consistently resulted in potent antibacterial effects. Consequently, we believe that COLICIN's bactericidal effect, determined by the methods described, can apply to meat matrices not specifically evaluated for this Notice, such as veal and mutton. Beef and pork were specifically evaluated because together these meats represent the vast majority of total red meat consumed in the USA and many other countries.

In all studies conducted, COLICIN application to the surface of various meat matrices resulted in the rapid decrease of bacterial populations ("knock-down" effect). The reduction in viable bacteria was followed by a period of normal growth of bacteria that survived COLICIN treatment if the meat was incubated at a temperature that was permissive for growth. Because of the lower viability in COLICIN-treated samples, significant reductions in CFU/g between COLICIN-treated and control-treated samples could be documented; these differences in CFU/g lasted from as low as 1 h to as high as 144 h.

In controlled studies *in vitro* using purified materials, COLICIN showed 1-5 log CFU reductions in bacterial populations of various pathogenic *E. coli* strains (e.g. Table 2-6). In studies on several meat matrices contaminated with Big 7+1 pathogen mix under a variety of conditions, COLICIN displayed a net 0.7-4.92 log CFU/g reductions in bacterial populations relative to controls. COLICIN's activity was remarkably consistent when considering the greater heterogeneity, porosity, organic content, resident microflora, and other attributes of the meat matrices not found *in vitro*, plus the greater challenge in ensuring uniform treatment and sampling when working with food products.

COLICIN's bactericidal effect was quantified when contaminated meat treated with COLICIN was stored at temperatures ranging from 4 °C to 15 °C prior to sampling and bacterial enumeration. While refrigeration is important in minimizing microbial growth, COLICIN treatment provides a significant pre-refrigeration safety

advantage by achieving a rapid "knock-down" effect on pathogen CFU. There was no re-growth at 4 °C regardless of storage time.

At higher temperatures such as 10 °C and 15 °C that are suboptimal but permissive for *E. coli* growth, and that represent more typical food processing environments, COLICIN treatment rapidly reduced mixed-pathogen populations during the first 0-24 h (typically 0-1 h), followed by growth of lower levels of surviving bacteria during storage. The short duration of activity was also reported in GRN 593. Here, this effect was observed in whole meat cuts (beef and lamb) as well as in whole beef cuts treated prior to comminution into ground beef.

The short duration of COLICIN's technical effect (1-24 h maximum on meat matrices) define the product as a food processing aid for meats, corroborating the short-duration technical effect found on produce (fruits and vegetables) reported in GRN 593.

COLICIN solution was found compatible with a range of application devices including pressurized spraying equipment, showing no signs of denaturation or loss of potency after application (Table 2-8). While in-plant evaluation using industrial equipment will be conducted later in development, our pilot-scale tests suggest that application of COLICIN can be accomplished using standard equipment.

COLICIN application should not interfere with the organoleptic properties of the meat product. The COLICIN formulation is applied at a very low application rate of 8-10 ppm initially and dissolves or diffuses in the meat matrix post application. There is no color-masking of meat after application, and solutions of COLICIN are generally clear and have no objectionable odor. No organoleptic evaluation was conducted for this Notice because the meat matrices were inoculated with known pathogens.

Although the product has not yet been evaluated at-scale, no special handling procedures or protective measures are anticipated when using the product in industrial settings, and none are indicated from the public literature. The proteins and excipients used in COLICIN formulations are either GRAS (GRN 593) or food-grade. As a precaution, eye (goggles), respiratory (mask) and skin (gloves) protection could be implemented during preparation of solutions of COLICIN if starting with a granular or powdered stock, and during mixing, transfer or disposal of the COLICIN solution.

3 Dietary Exposure

The application rate of COLICIN on meats should not exceed 10 mg COLICIN per kg of treated food. Application rates of 1-10 mg COLICIN/kg meat product have been shown effective in controlling *E. coli* Big Seven plus O104:H4 pathogens on mammalian-sourced red meat cuts, as detailed in Section 2.4.2.

3.1 Estimated dietary intake of selected animal meats

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

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

The National Health and Nutrition Examination Survey (NHANES) by the Centers for Disease Control and Prevention (CDC) reports total yearly red meat consumption as 99.7 lbs/person retail weight for survey years 2003-2004 (analyzed by Daniel 2011). The NHANES is based on interviews of >18,000 individuals and explores what people consume over a 24-hr period, from which yearly figures are projected.

Calculations from the World Agricultural Supply and Demand Estimates (WASDE) database suggest a total U.S. red meat per capita consumption of 99.3 lbs/person retail weight for year ending 2015 and approximately 106 lbs/person retail weight projected for CY2016 (USDA WASDE 2016).

The per capita red meat consumption estimates may be slightly under-represented in many of these surveys because they do not take into account persons who do not consume meats. Results of a 2012 Gallup Poll showed that a consistent 5% of the U.S. population is vegetarian. This percentage remains largely unchanged from results of 1999 and 2001 surveys, which reported a value of 6% (Newport 2012). Hence, assuming that the projected domestic U.S. population in CY2016 is 325,032,763 (projected from US Census 2015; http://www.census.gov/popest/data/national/totals/2015/index.html), this means that 16,251,638 people will not be consuming meat at all and should not be included in per capita consumption estimates in the above-referenced statistics. Therefore exposure estimates may be more accurately calculated based on a U.S. population of 308,781,125 potential meat consumers (325,032,763 – 16,251,638). Using USDA ERS total red meat statistics for 2014 (USDA ERS 2014c) and the adjusted population for the same year, yields an estimated red meat annual consumption of 104.3 lbs/person retail weight or 98.6 lbs/person based on boneless weight. These figures translate to 4.6 oz/day (130.4 g/day) retail weight, and 4.3 oz/day (121.9 g/day) boneless weight.

The Wall Street Journal published a report on meat consumption in 2012 that took into account the meat that is actually consumed, rather than meat that is available for consumption based on weights sold and

population (Molla 2014). Their estimates factored in food loss at each level; for example, carcass to retail weight, loss at retail such as spoilage, and loss at the consumer level, such as plate waste. They reported that about half of the weight of meat is lost from the carcass to actual consumption. When taking such losses into account, the adjusted estimate is about 75 lbs/person-year (3.3 oz/day or 93.2 g/day).

Examples of this variation in reported intake are shown in Table 3-1; all figures are based on retail weights.

Table 3-1. Per capita consumption of red meat in the USA based on various surveys

Survey Source, Database, Year	Consumption (retail wt)			
	Annual		Daily	
	lbs	kg	oz	g
USDA, ERS Livestock meat domestic data, 2014[1]	96.2	43.6	4.2	119.5
USDA, ERS Livestock meat domestic data, 2015[1]	104.8	47.5	4.6	130.2
CDC, NHANES, 2003-04 (analyzed by Daniel 2011)[2]	99.7	45.22	4.37	123.9
CDC, NHANES (DeBruicker 2011; Wang 2009)[2]	120.9	54.8	5.3	150.2
NCI analysis of NHANES (DeBruicker 2011)[3]	59.3	26.9	2.6	73.7
USDA, WASDE, 2015[1]	99.3	45.0	4.35	123.4
USDA, WASDE, March 2016 (projection)[1]	105.9	48.0	4.64	131.6
USDA Dietary Guidelines 8th Edition, 2015-2020[3]	57.0	25.9	2.5	70.8

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

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

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

Although meat consumption trends may vary, with reported reduced domestic consumption in recent years, the totality of data published for the last 3-5 years in the above-referenced surveys suggest that U.S. per capita consumption of all red meat ranges from about 57-120 lbs per year (~26 to 55 kg/yr), or 2.5-5.3 oz/day (~71 to 150 g/day).

Therefore, to err on the side of safety for COLICIN exposure calculations, we assumed a high red meat consumption value of **120 lbs/person-year** or **5.3 oz/person-day** (55 kg/person-year; 150 g/person-day). It is important to note that independent of method of calculation or mode of food preparation, >95% of red meat consumed in the US diet consists of beef (~54%) or pork (~45%), with only about 1-2% consisting of lamb, mutton and veal.

3.2 Dietary intake (exposure) of colicins from COLICIN-treated meat products

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

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

$$\frac{120\ \text{lbs}}{\text{year}} \times \frac{\text{year}}{365\ \text{day}} \times \frac{1000\ \text{g}}{2.2\ \text{lb}} = \frac{150\ \text{g red meat}}{\text{person-day}}$$

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

$$\frac{10\ \text{mg COLICIN (max)}}{1000\ \text{g meat}} \times \frac{150\ \text{g meat}}{\text{person-day}} = \frac{1.5\ \text{mg COLICIN consumed}}{\text{person-day}}$$

The total projected per capita maximum intake of COLICIN active ingredients from consumption of treated red meat products would be about **1.5 mg per day** (0.55 g/year). It bears mentioning that this calculated maximum exposure would be from **uncooked meats**, as cooking meats to recommended temperatures would destroy all colicins, including naturally occurring colicins and as well as colicins applied from Notifier's product (see Table 3-3 and Section 6 for additional detail).

Using the same formula (above), the potential colicin intake levels from various COLICIN-treated red meats were calculated. The potential maximum intake was estimated by assuming 10 mg COLICIN/kg meat, and corresponding red meat consumption in grams per person-day. Results are summarized in Table 3-2.

Table 3-2. Estimated human daily exposure to colicins from consumption of various red meats

Meat type[1]		**Estimated daily per capita colicin exposure**[2]	
	Consumption (g/person-day)	**If meat is not cooked**	**If meat is cooked**
Beef	81	0.810 mg	nil
Pork	67.5	0.675 mg	nil
Lamb and mutton	1.2	0.012 mg	nil
Veal	0.3	0.003 mg	nil
Total	150	1.5 mg	nil

[1]Total per type of meat; includes whole cuts and ground meats. [2]COLICIN applied at maximum of 10 mg/kg meat.

3.3 Additional, natural exposure to colicins (intake not related to COLICIN product)

In GNR 593, we provided extensive documentation of the potential exposure to colicins from natural sources separate from exposure via ingestion of the COLICIN product. These natural sources included:

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

The levels of colicin from use of Notifier's product were also estimated in GRN 593. Table 3-3 summarizes exposure to colicins and estimates the intake by food source. A maximum application rate of 10 mg total colicins per kg meat and other treated food products (10 ppm; worst case) was used in the calculations.

A worst-case intake estimate for meats is provided and assumes exposure to COLICIN from consumption of treated uncooked meat (a rarity in the USA), as colicins are destroyed by heating (cooking) as well as by the gastric environment and upper-intestinal proteases. Cooking meats to recommended temperatures would essentially reduce colicin levels to zero, as also shown in the table.

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

Source of exposure	**Estimated daily per capita colicin exposure**	
	If food is not cooked	**If food is cooked**
COLICIN treatment, total of all red meat products consumed (detailed in this Notification and in Table 3-2)	**1.5 mg**	**nil**
COLICIN treatment, total of all produce consumed (fruits and vegetables; from GRN 593)	**4.1 mg**	**nil**
Meat (ingestion of naturally present colicins in meats)	<0.3 mg	nil
Produce (ingestion of naturally present colicins in fruits, vegetables)	<0.1 mg	nil
Total (maximum, all food sources)	6 mg	nil

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

4 Information on Any Self-Limiting Levels of Use

None.

5 Experience Based on Common Use in Food Before 1958

None and not applicable. COLICIN has not been used in foods before. However, it is noted that naturally occurring colicins are present on meats and vegetables that are a part of the human diet, and are produced at significant levels by commensal bacteria.

6 Basis for Conclusion of COLICIN's GRAS Status

Notifier has used scientific procedures to conclude that its COLICIN food antimicrobial product is GRAS under the conditions of intended use. The detailed scientific basis for Notifier's original determination of COLICIN as GRAS for use on produce (fruits and vegetables), including antibacterial efficacy results on produce, was provided in **GRN 593, APPENDIX A: Information to Establish the Safety of COLICIN and the Suitability of Use (pp. 19-50).** For brevity and convenience, the specific sections of GRN 593 pertinent to this discussion are listed in Section 7 of this Notice; specifically in Table 7-1.

New information supporting our determination of COLICIN as **GRAS for use as an antimicrobial processing aid on meat food products** is summarized in this current Notice. Methods specifically used to assess (1) efficacy, suitability, residual technical effect after application to meat products, and (2) safety upon ingestion of treated meats, are described separately in this Notice, specifically in Section 2 and Section 3, respectively.

Historical and technical bases were used to support the conclusion that colicin proteins (singly and/or in combination in a COLICIN blend) are GRAS. It is widely accepted that humans have a very long history of exposure to colicins from various natural sources, including exposure from human commensal and domestic animal microflora. 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, and to low levels of colicins from foods and the environment. The endogenous steady state level of colicin biosynthesis is estimated at about 3 mg/day. Through traditional practices used in food cultivation, preparation and consumption, humans have likely been chronically exposed to colicins from food and environmental sources for millenia. The published studies cited above 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.

There exists the possibility that colicins could be consumed from multiple sources, including from COLICIN-treated meats, COLICIN-treated produce, plus residues from natural sources in various foods. This would increase the total daily exposure to colicins from food consumption. Estimates of potential exposure from all sources are included in Table 3-3. For produce, some vegetables and fruits would be cooked (e.g. boiled or baked) while others would be consumed raw (fresh).

Therefore the estimates in Table 3-3 include a worst-case situation where none of the COLICIN-treated produce is cooked, as well as more limited exposure from cooked produce. From these exposure scenarios, the maximum daily intake of colicins from the COLICIN processing aid can be estimated to be on the order of 1.5 mg from treated meats and 4.1 mg from treated produce.

For perspective, natural, endogenous colicins produced through intestinal biosynthesis by colicinogenic strains of enteric bacteria in humans is estimated to be on the order of ~3 mg per day, chronically (**GRN 593, Section A.2.1; pp 21-25)**. Because of the localization of endogenous colicin synthesis in the colon and the average human daily fecal output (0.030 kg), the intestinal concentration of colicins is estimated to be 100 ppm (3 mg/0.030 kg).

The endogenous value is one half of the maximum amount (6 mg) of colicins potentially consumed if all produce and all red meats were treated with COLICIN yet consumed raw (uncooked), plus intake from natural sources, or two times the maximum amount (1.5 mg) ingested through consumption of all red meats treated with COLICIN yet consumed raw (uncooked). Because the daily human colicin endogenous biosynthetic rate was calculated from multiple estimates based on analyses of colicinogenic strains in human fecal samples, worst-case exposure to food-applied colicins (i.e. if no treated food is cooked) would

range from 0.5-times to 2.0-times the endogenous level, or approximately the same range of concentrations that humans are naturally exposed to daily from their gut microflora.

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 the specificity of these molecules for bacterial target structures, plus the evolutionary adaptation by humans and animals to colicin protein exposure, including immune tolerance.

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

Meats are typically cooked domestically and in most other countries; therefore, the level of intake of colicins from the use of the COLICIN product in meat processing is expected to be less than the intake from treated fresh and minimally processed fruits and vegetables.

The full scientific rationale supporting our original conclusion that colicins are GRAS was presented **in GRN 593 APPENDIX A (pp. 19-50).** This conclusion is further supported in this Notice, by our evaluation of scenarios where the COLICIN product is applied to red meats as an antimicrobial processing aid.

7 Supporting Data and Information

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

The COLICIN product applied to meats as an antibacterial is the same composition that is applied to produce (fruits and vegetables) and is used at the same application rates described in GRN 593. Therefore, for brevity, extensive reference is made to specific sections of GRN 593 that have already documented the safety attributes of colicin proteins.

Table 7-1. Information supporting COLICIN GRAS determination

Topic	Document	Location	Source	Availability
Colicins' mode of action; specificity	GRN 593	Table A-1, pg 20	Yang 2014	Public
History of human exposure to colicins	GRN 593	Section A.2, pp 21-25	Multiple references	Public
Human exposure from commensal and animal microflora	GRN 593	Section A.2.1, pp 21-23	Multiple references	Public
Human exposure from meat (beef)	GRN 593	Section A.2.1, pg 24	Lange 2008 USDA ERS 2006	Public
Human exposure from produce	GRN 593	Section A.2.1, pg 24	Mukherjee 2004, 2006 USDA ERS 2014b	Public
Nature-identical composition of colicins	GRN 593	Section A.2.2, pg 25 Section 2.3, pp 8-14	Nomad Bioscience GmbH	Public
Safety of colicin production organism	GRN 593	Section A.2.2, pp 25-26 Appendix B, pp 51-57	Nomad Bioscience GmbH	Public
Safety of production host species	GRN 593	Section A.2.2, pp 26-28	Multiple references	Public
Safety of manufacturing process	GRN 593	Appendix B, pp 51-57	Nomad Bioscience GmbH	Public
Safety of host and process impurities	GRN 593	Section A.2.2, pp 27-28	Nomad Bioscience GmbH	Public
Anticipated levels of colicin exposure	GRN 593	Section A.2.3, pg 28	Nomad Bioscience GmbH	Public
Digestibility of colicin proteins	GRN 593	Section A.2.3, pp 28-31 Table A-2, pg 30 Table A-3, pg 31	Nomad Bioscience GmbH	Public

Topic	Document	Location	Source	Availability
Safety determined in feeding studies with monogastric animals	GRN 593	Section A.2.3, pg 32	Cutler 2007a, 2007b	Public
Safety determined in human and primate cellular exposure studies *in vitro*	GRN 593	Section A.2.3, pp 32-33	Murinda 2003 Farkas-Himsley 1976, 1995	Public
Low potential for development of resistance	GRN 593	Section A.2.4, pg 34	Nomad Bioscience GmbH	Public
Low potential for development of allergenicity or immunogenicity	GRN 593	Section A.2.5, pp 34-37 Table A-4, pg 35	AllergenOnline 2015; calculated by Nomad Bioscience GmbH	Public
Safe ingestion estimates of colicins applied to meat products	This Notice	Section 3, pp 38-41 Table 3-1, pg 39 Table 3-2, pg 40	Nomad Bioscience GmbH calculations of intake based on colicin application rates and public data on meat consumption, including: USDA ERS 2014a, 2015 CDC NHANES (Daniel 2011; DeBruicker 2011; Wang 2009) Newport 2012 USDA ERS 2014c USDA WASDE 2016 USDA 2015 Dietary Guidelines	Public
Safety from additive consumption of colicins from applied COLICIN product and from natural sources	This Notice	Section 3.2, pg 40 Table 3-2, pg 40 Section 3.3, pp 40-41 Table 3-3, pg 41	Nomad Bioscience GmbH	To be made public through this GRN
Safety of colicin ingestion from all sources relative to colicin levels synthesized *in situ* by endogenous intestinal microflora	GRN 593 and this Notice	GRN 593 Section A.2.1, pp 21-25 This Notice Section 3.3, pp 40-41; Table 3-3, pg 41; Section 6, pp 41-42	Nomad Bioscience GmbH	To be made public through this GRN
Anticipated occupational safety of COLICIN usage	This Notice	Section 2.7, pg 37 Section 2.8, pg 39	Nomad Bioscience GmbH	To be made public through this GRN

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APPENDIX A – Nomad SOP NMD 901-01

Determination of Efficacy and Duration of Bactericidal Effect of COLICIN (Colicin Mixtures) on Pathogenic Strains of *Escherichia coli* Applied to Meat Matrices.

Following this page is SOP NMD 901-01.

	Standard Operating Procedure	NMD 901-01
NOMAD	Determination of Efficacy and Duration of Bactericidal Effect of COLICIN (Colicin Mixtures) on Pathogenic Strains of *Escherichia coli* Applied to Meat Matrices	Page A1 of A10

Author Date of draft	Simone Hahn 2016-09-26	
Last modified by Date	2016-10-21	
Valid from	2016-05-24	

1. PURPOSE

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

2. SCOPE

Assays for COLICIN's efficacy and continued technical effect

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

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

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

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

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

The effect of COLICIN on the bacterial populations upon storage periods of 1-144 h are analyzed at two different temperatures (10 °C and 15 °C). The lower (10 °C) temperature may represent typical industrial processing environments while being suboptimal for bacterial growth. To analyze the duration of COLICIN's technical effect under more stringent conditions, a higher temperature (15 °C) was studied for comparison, as higher temperatures are more favorable for bacterial growth.

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

3. REFERENCE

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

4. DEFINITIONS

LB Luria Bertani medium
OD_{600} Optical density of bacterial solution at 600 nm
RT Room temperature
TSP Total soluble protein
CFU Colony forming unit
SMAC Sorbitol MacConkey Agar

5. CONSUMABLES

Culture vials (e.g. 20 mL plastic or glass vial) for cultivation of *E. coli* in liquid culture
Disposable plastic cuvettes for spectrophotometric measurement of OD_{600}
Sterile Petri dish, ~94x16 mm
Sterile Petri dish, quadratic, ~120x120x17 mm
Sterile 5 ml disposable plastic syringes with Luer lock connector
Sterile disposable 50 ml centrifuge (Falcon) tubes
Sterile disposable 15 ml centrifuge (Falcon) tubes
Sterile 2.0 ml disposable reaction tubes
Sterile disposable forceps 25 cm (RMP-med Steffen Roßberg, cat.# 720183)
Sterile disposable plastic spatulas
Sterile disposable 25 ml, 10 ml, 2 ml serological pipettes
Sterile lateral filter bags BagFilter® 400 P (Interscience, cat.# 111 425)
Atomizer flasks (Carl Roth GmbH & Co. KG, cat.#N145.1)
Sterile tissue paper

6. EQUIPMENT

Sterile 100 ml wide neck Erlenmeyer flasks for cultivation of *E. coli* in liquid culture
Incubator shaker (150 rpm, 37 °C) for cultivation of EHEC strains
Spectrophotometer for measurement of OD_{600} of bacterial culture
Table top centrifuge
Pipetting aid for serological pipettes
100 ml sterile measuring cylinder
250 ml sterile measuring cylinder
500 ml sterile measuring cylinder
5 L sterile beaker
3 L sterile beaker
FW735 meat grinder (Beeketal Lebensmitteltechnik GmbH & Co. KG, Rastdorf, Germany)
Laminar flow cabinet
Microwave oven
Closing clip BagClip® 400 (Interscience, cat.# 231 040)
Lab blender BagMixer® 400 CC® (Interscience, cat.# 024 230)
Incubator (37 °C)
Autoclave
Refrigerators (10 °C and 15 °C)
Freezer -80 °C
Freezer -20 °C
Personal protective equipment

7. CHEMICALS /MEDIA/SOLUTIONS

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

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

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

1% (w/v) Bacto-tryptone (pancreatic digest of casein; Duchefa T1332)
0.5% (w/v) Yeast extract (Duchefa Biochemie, cat. #Y1333)
1% (w/v) NaCl (AppliChem GmbH, cat. #A4661)
pH 7.5, Autoclaved
1.5% (w/v) Agar, bacteriology grade (AppliChem, cat. #0949)
0.8% (w/v) Agar, bacteriology grade (AppliChem, cat. #0949) for LB soft agar medium

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

48.5 g/l, Autoclaved

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

20 g/l, Autoclaved

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

50 mg/mL stock solution

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

Rifampicin: For selection for *Agrobacterium* strain

20 mg/mL Stock solution

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

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

25 mg/mL Stock solution

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

Cefixime (sterile): For selection for EHEC strains

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

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

50 mg/ml stock solution

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

8. BIOLOGICALS

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

The *E. coli* strains used in the experiments conducted within this SOP are shown in Table 8-1. These target strains are included because they represent the most predominant serotypes of *E. coli* responsible for food-borne outbreaks, including Shiga toxin producing (STEC), enterohemorrhagic (EHEC) and enteroaggregative (EAEC) strains, which vary in their mechanisms of pathogenesis.

Table 8-1. *E. coli* pathotypes STEC/EHEC and STEC/EHEC/EAEC used in these studies

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

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

Comparable susceptibility of original strains and nalidixic acid resistant mutants thereof towards colicins are confirmed (Table 8-2). These strains are stored in liquid nitrogen in LB broth supplemented with 15% glycerol and 25 µg/ml nalidixic acid. When using nalidixic acid resistant derivatives of STEC/EHEC strains, all media employed for bacterial growth are supplemented with 25 µg/ml nalidixic acid.

Table 8-2. Relative sensitivity to colicins of wild type and nalidixic acid resistant mutants of corresponding *E. coli* strains

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

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

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

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

9. PRECAUTIONS

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

10. PROCEDURE FOR DETERMINING EFFICACY AND DURATION OF TECHNICAL EFFECT

10.1.1. Colicins

Colicin proteins are produced in plants as described in GRN 593. The intended product contains individual plant-made colicins or a blend of several colicin proteins selected from the list that includes colicins E2 (#AAA23068.1), E7 (#AAA98054.1), K (#Q47502.1), 5 (#CAA61102.1), Ia (#WP_001283344.1), M (#AAA23589.1), E3 (#AAA88416), E6 (#AAA23080.1), D (#P17998.1), N (#P08083.1), U (#CAA72509.1), and B (#P05819.3).

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

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

10.1.2. Verification of basic functionality of colicin (blend) solution

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

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

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

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

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

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

10.2. Preparation of meat test matrix

10.2.1. Purchase of meat in local retail outlets

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

The meat is stored at 4 °C, the packaging is disinfected with 70% ethanol before opening, and the meat is not washed or pre-treated before experimental exposures. A matrix summarizing how meat samples are prepared for these experiments is shown in Table 10-1.

Table 10-1. Matrix for preparation of beef and pork meat cuts

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

10.3. Preparation of bacterial cultures used to experimentally contaminate meat

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

Before inoculation the strains are thawed, individually grown on LB agar medium, and individually inoculated to LB broth. Individual saturated LB broth overnight cultures (37 °C, 150 rpm) are diluted to OD_{600}=0.05 with fresh LB broth and grown to OD_{600}~0.3 which corresponds to ~$3x10^7$ CFU/ml (~7.5 log CFU/ml). Individual cultures are diluted with LB broth to OD_{600}=0.3 and mixed 1:1:1:1:1:1:1:1. The strain mix is further diluted to the desired cell number (see Table 10-2) with LB broth for use as meat contamination suspension.

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

10.4. Contamination of meat

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

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

A summary matrix of the process is shown in Table 10-2.

Table 10-2. Matrix for experimental contamination of meats with mixed pathogen suspension

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

10.5. Application of colicin (blend) solution

Contaminated meat is either treated with carrier or colicin blend solution by low-pressure spraying (2-4 bar) using atomizer flasks, as shown in Table 10-3. Reasonably even coverage of the entire surface is ensured. Proposed application rates are 3 mg/kg for colicin M and 1 mg/kg for any other colicin used in the blend. The meat is further incubated for 30 min at RT while steaks are inverted and meat cubes are tumbled every 15 min.

Table 10-3. Matrix for application of COLICIN to meat samples

Test matrix - general	**Whole cuts**		**Ground meat**
	Raw pork steak	Raw beef steak	Raw beef prior to grinding
Test matrix - specific	Individual steaks of ~85 g weight (~10x6x1.2 cm)	Individual steaks of ~85 g weight (~10x6x1.2 cm)	Pool of meat cubes of ~100 g weight (~5x5x5 cm)
Containment	Sterile Petri dishes, ~120x120x17 mm	Sterile Petri dishes, ~120x120x17 mm	5 L beaker, sterile
Application of COLICIN/carrier solution	Spraying from both sides	Spraying from both sides	Spraying, equal distribution by tumbling, hand mixing

Application rate	~47 ml/kg ~56.5 mg total protein/kg 3+1 up to 3+1+1+1+1+1+1+1 mg colicin/kg of Col M, E7, Ia, K, E6, E2, 5 and U	~42 ml/kg ~50.8 mg total protein/kg 3+1+1+1+1+1 mg colicin/kg of Col M, E7, Ia, K, 5 and U	~42 ml/kg ~50.8 mg total protein/kg 3+1+1+1+1+1 mg colicin/kg of Col M, E7, Ia, K, 5 and U

10.6. Aliquoting and packaging of meat samples

Thirty (30) min after COLICIN application, whole meat is cut into aliquots and beef cubes are ground. Replicate meat samples are placed into sterile sample bags (BagFilter®400 P), the exact weight of each sample is recorded, and sample bags are closed using a closing clip (BagClip®400). A summary matrix of these steps in the process is shown in Table 10-4.

Table 10-4. Matrix for aliquoting and packaging of COLICIN-treated meat samples

Test matrix - general	**whole cuts**		**ground meat**
	Raw pork steak	Raw beef steak	Raw beef prior to grinding
Test matrix - specific	Individual steaks of ~85 g weight (~10x6x1.2 cm)	Individual steaks of ~85 g weight (~10x6x1.2 cm)	Pool of meat cubes of ~100 g weight (~5x5x5 cm)
Containment	Sterile Petri dishes, ~120x120x17 mm	Sterile Petri dishes, ~120x120x17 mm	5 L Beaker, sterile
Aliquoting	Cut each steak into 4 pieces of similar size (~20 g weight) using a sterile scalpel	Cut each steak into 4 pieces of similar size (~20 g weight) using a sterile scalpel	Grind meat using FW735 meat grinder (Beeketal Lebensmitteltechnik GmbH & Co. KG, Rastdorf, Germany) – first with a ∅ 6 mm then with a ∅ 3 mm die, hand mix ground meat
Packaging	One steak piece of ~20 g placed into a sterile bag BagFilter®400 P using a sterile forceps; for each treatment group, 4 replicates are prepared	Each one steak piece of ~20 g from 2 steaks placed into a sterile bag BagFilter®400 P using a sterile forceps; for each treatment group, 4 replicates are prepared	~40 g ground meat placed into a sterile bag BagFilter®400 P using a sterile spoon; for each treatment group, 3 replicates are prepared
Incubation at RT upon COLICIN application	1 h (including time for aliquoting and packaging)	1 h (including time for aliquoting and packaging)	2 h (including time for grinding, aliquoting and packaging)

10.7. Storage of meat samples

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

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

10.8.1. Preparation of sample homogenates

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

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

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

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

For pork meat at high contamination Level II, microbial suspensions are not concentrated; instead, a 1:10 dilution series of microbial suspension from the filtered part of the storage bag (100 µl microbial suspension + 900 µl peptone water) is prepared. 100 µl aliquots of undiluted or diluted microbial suspensions are plated on Sorbitol-MacConkey agar (SMAC) supplemented with 0.05 µg/ml cefixime and 100 µg/ml X-Gluc. For samples without COLICIN treatment, 5 aliquots are plated. For samples with COLICIN treatment, 10 aliquots are plated. The plates are incubated for 18-24 h at 37 °C and the CFU are enumerated.

The CFU number per g sample is calculated as follows:

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

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

10.9. Statistical analysis

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

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