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intralytix
SAFETY BY NATURE™

#834

December 20, 2018

Office of Food Additive Safety (HFS-200)
Center for Food Safety & Applied Nutrition
U.S. Food & Drug Administration
5100 Campus Drive
College Park, MD 20740

Reference: Intralytix GRAS Notification for EcoShield PX™

To Whom It May Concern:

In accordance with the Federal Register [81 Fed. Reg. 159 (17 August 2016)] issuance on GRAS notifications (21 CFR Part 170), Intralytix is pleased to submit a notice that we have concluded, through scientific procedures, the bacteriophage cocktail, EcoShield PX™, is generally recognized as safe and is not subject to the pre-market approval requirements for the use in foods, generally, as a processing aid to control Shiga toxin-producing *E. coli*.

We also request that a copy of the notification be shared with the United States Department of Agriculture's Food Safety and Inspection Service, regarding the use of EcoShield PX™ as a safe and suitable antimicrobial used in the production of meat and poultry products as a processing aid. EcoShield PX™ is substantially equivalent to the several other bacteriophage products also listed in FSIS Directive 7120.1 as processing aids.

If there are any questions or concerns, please contact us.

Sincerely,

[redacted]

Alexander Sulakvelidze
Vice President & Chief Scientist
Intralytix, Inc.

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1 SIGNED STATEMENTS AND CERTIFICATION

1.1 STATEMENT OF INTENT

In accordance with the 21 CFR 170 Subpart E, regulations for GRAS notifications, Intralytix is pleased to submit a notice that we have concluded, through scientific procedures, that the bacteriophage preparation EcoShield PX™, is generally recognized as safe and is not subject to the premarket approval requirements for the use in foods, generally, as a processing aid to control *Escherichia coli* (*E. coli*) under the intended use conditions described within this notification.

1.2 NAME & ADDRESS OF NOTIFIER

Intralytix, Inc.
701 E Pratt St.
Baltimore, MD 21202
Tel: 877-489-7424
Fax: 410-625-2506

1.3 COMMON OR USUAL NAME

Intralytix produces a lytic bacteriophage preparation with potent lytic activity against the Gram-negative bacterium *E. coli* under the trade name EcoShield PX™.

1.4 CONDITIONS OF USE

EcoShield PX™ is intended for use as an antimicrobial to control *E. coli* on food when applied to food surfaces up to $1x10^8$ PFU/gram of food, including the following food categories:

- Ground and whole meat and poultry, including whole carcasses, primals, subprimals, trimmings, and organs
- Ready-to-eat (RTE) meats and poultry
- Fresh and processed fruits
- Fresh and processed vegetables
- Dairy products (including cheese)
- Fish and other seafood

1.5 BASIS FOR THE GRAS CONCLUSION

Pursuant to the GRAS rule, Intralytix has concluded that EcoShield PX™ is GRAS through scientific procedures, in accordance with 21 CFR 170.30 (a) and (b).

1.6 ECOSHIELD IS NOT SUBJECT TO PREMARKET APPROVAL

Because Intralytix has concluded that EcoShield PX™ is GRAS, it is not subject to the premarket approval requirements for the use in foods, generally, as a processing aid to control *E. coli* under the intended use conditions described within this notification.

1.7 AVAILABILITY OF INFORMATION

The data and information that are the basis for Intralytix's conclusion that EcoShield PX™ is GRAS are available for review and copying by FDA during customary business hours, at the location below, or will be sent to FDA upon request, made to:

Intralytix
Joelle Woolston
701 E Pratt St.
Baltimore, MD 21202
jwoolston@intralytix.com

1.8 FREEDOM OF INFORMATION ACT

It is our view that the information contained in this notification is not exempt from disclosure under the Freedom of Information Act.

1.9 CERTIFICATION

To the best of our knowledge, this GRAS notification is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of EcoShield PX™.

1.10 SIGNATURE

12 / 20 / 2018

Alexander Sulakvelidze
VP / Chief Scientific Officer
asulakvelidze@intralytix.com

Date

1.11 FSIS AUTHORIZATION

We also request that a copy of the notification be shared with the United States Department of Agriculture's Food Safety and Inspection Service, regarding the use of EcoShield PX™ as a safe and suitable antimicrobial used in the production of meat and poultry products as a processing aid. EcoShield PX™ is substantially equivalent to the several other bacteriophage products also listed in FSIS Directive 7120.1 as processing aids.

2 IDENTITY AND SPECIFICATIONS OF ECOSHIELD PX™

2.1 IDENTITY

EcoShield PX is a cocktail of three to eight bacteriophages (phages or monophages) targeting Shiga toxin-producing *E. coli* (STEC). The use of multiple phages helps increase the spectrum of lytic activity against the various pathogenic serotypes of *E. coli* (e.g. Shiga toxin-producing *E. coli* O157:H7 and/or non-O157:H7 STEC).

The product may be safely used as an antimicrobial in accordance with the following conditions:

1) The phages are produced on host *E. coli* strains grown in animal product free media.
2) The titer of each monophage in the cocktail is ≥ 9.0 $\log_{10}$ PFU/mL and the titer of the cocktail is ≥ 10.0 $\log_{10}$ PFU/mL.
3) The phages do not contain a functional portion of any of the toxin-encoding sequences described in 40 CFR 725.421(d).
4) The phages do not contain sequences derived from genes encoding bacterial 16S ribosomal RNA.
5) The cocktail consists of a mixture of approximately equal proportions of three to eight different individually purified lytic bacteriophages lytic against Shiga Toxin-producing *E. coli*.
6) The *E. coli* production host strains do not encode functional *stx* genes and/or there is no detectable Shiga toxin in the final product.
7) The cocktail achieves positive lytic results by a spot titer assay against one or more STEC strains available in reference collections (e.g. ATCC).
8) The cocktail contains ≤ 25,000EU/mL of endotoxin at a concentration of bacteriophages at ≥ 9.0 $\log_{10}$ PFU/mL.
9) The cocktail is determined to be bacteriologically sterile.
10) The phage cocktail is used in accordance with the conditions of use outlines in Section 1.4.

The current EcoShield PX™ is a concentrate that is normally diluted with water at the application site to form the EcoShield PX™ working solution, typically with a lytic titer of ca. 9.0 $\log_{10}$ PFU/mL. It is applied at a rate that ensures the final concentration of phage on the food articles is at or below $1x10^8$ PFU/g of food.

2.2 METHOD OF MANUFACTURE

The component monophages of EcoShield PX™ are prepared using Intralytix's well-established phage production protocols. These procedures have been reviewed by the FDA for manufacturing of Intralytix's bacteriophage food safety products, most recently in GRN No. 000672. Each

monophage is produced individually, in separate monophage production runs. Production seed stocks of each host strain and monophage, which have passed quality control tests that confirm the purity and identity of each, are used.

The component monophages of EcoShield PX™ are prepared using an aerobic fermentation process in animal-product free media. For each monophage, the host *E. coli* strain is grown to a target OD_{600}, at which point the culture is infected with the monophage at a previously determined MOI (multiplicity of infection; the ratio of phage to bacteria) and the combination is incubated with aeration and mixing. The suspension is clarified by removal of bacteria by tangential-flow filtration. Following the initial filtration, the monophage is concentrated, washed with 0.1M sodium chloride, then sterilized using filtration. After all component monophages have each passed quality control specifications, proper volumes of each monophage, and sterile 0.1M sodium chloride as necessary, are combined, and final filtration is carried out using a sterilizing grade filter. The EcoShield PX™ article of commerce is prepared so that:

Each monophage is approximately equally represented

AND

The lytic titer is ≥10.0 $\log_{10}$ PFU/mL

The EcoShield PX™ article of commerce is diluted with clean water at the application site, to form the "working solution" or "working concentration" of EcoShield PX™ with a lytic titer of 9.0 $\log_{10}$ PFU/mL.

The filters used in the production of EcoShield PX™ are all constructed of component materials that are non-toxic and are compliant with the criteria of USP <88> for Biological Reactivity for USP Class VI plastics. The component materials are listed by the FDA as appropriate for use in articles intended for repeated food contact. Additionally the filters comply with 21 CFR § 210.3(b)(6) as non-fiber releasing. The final fill containers are made of food-grade materials and are compliant with 21 CFR § 177.1315 (bottle) and 21 CFR § 177.1520 (closure).

Figure 1 provides an overall schematic of the process.

2.3 SPECIFICATIONS

Due to the two-step manufacturing process, there are two levels of quality control. First, each individual monophage lot is analyzed to ensure it meets the release specifications listed in Table 1 before it can be used to prepare a lot of EcoShield PX™.

Table 1 Product specifications for individual monophage lots

Parameter	Specification
Potency (PFU/mL)	≥10.0 log_{10} PFU/mL
Microbial purity	No growth
Identity	Matches reference

Only after all component monophages have met the release specifications can a lot of EcoShield PX™ be produced. Each lot of EcoShield PX™ is analyzed to ensure it meets the following release specifications listed in Table 2.

Table 2 Product specifications for EcoShield PX™

Parameter	Specification
Potency (PFU/mL)	≥10.0 log_{10} PFU/mL
Microbial purity	No growth
Endotoxin Content (EU/mL)	≤25,000 EU/mL (at ca. 9.0 log_{10} PFU/mL)
Identity Test	All component phages are present

2.4 CHARACTERISTIC PROPERTIES

EcoShield PX™ is a clear to opalescent odorless liquid. The phage component of EcoShield PX™ (typical working concentration of ca. $1x10^9$ PFU/mL) is roughly estimated to be 0.0000358% by weight and the remainder is 0.1M sodium chloride. Typical chemical analysis of EcoShield PX™ (at the typical working concentration of ca. $1x10^9$ PFU/mL) is shown below. The values shown are derived (averages) from the chemical analysis of three separate EcoShield PX™ lots.

Table 3 Typical chemical analysis of EcoShield PX (at standard working concentration of $1x10^9$ PFU/mL)

Property / analysis / composition	Reporting Detection Limit	EcoShield PX Lot# [redacted]	EcoShield PX Lot# [redacted]	EcoShield PX Lot# [redacted]	EcoShield PX average
pH	n/a	6.91	6.89	6.89	6.90
Arsenic (mg/L)	0.02	0.0290	ND	0.0299	0.0263
Barium (mg/L)	0.02	ND	ND	ND	ND
Cadmium (mg/L)	0.005	ND	ND	ND	ND
Calcium (mg/L)	2.0	ND	ND	ND	ND
Chromium (mg/L)	0.04	ND	ND	ND	ND
Cobalt (mg/L)	0.02	ND	ND	ND	ND
Copper (mg/L))	0.02	ND	ND	ND	ND
Iron (mg/L)	0.02	ND	ND	ND	ND
Lead (mg/L)	0.02	ND	ND	ND	ND
Magnesium (mg/L)	0.1	ND	ND	ND	ND
Manganese (mg/L)	0.005	ND	ND	ND	ND
Molybdenum (mg/L)	0.1	ND	ND	ND	ND
Nickel (mg/L)	0.02	ND	ND	ND	ND
Potassium (mg/L)	0.05	0.12	0.13	0.13	0.13
Silicon (mg/L)	0.04	ND	ND	ND	ND
Sodium (mg/L)	1.0	210	210	220	213
Tin (mg/L)	0.1	ND	ND	ND	ND
Zinc (mg/L)	0.1	0.173	0.234	0.191	0.199
Chloride (mg/L)	5	316	316	330	321
Nitrate (as N) (mg/L)	1.8	ND	ND	ND	ND
Nitrite (as N) (mg/L)	1.2	ND	ND	ND	ND
Total Organic Carbon (mg/L)	1	3.24	2.22	1.57	2.34
Total Kjeldahl Nitrogen (mg/L)	0.4	2.24	2.52	2.24	2.33
Total Dissolved Solids (mg/L)	10	470	490	690	550
Total Phosphorous (mg/L)	0.01	0.082	0.048	0.036	0.055
Volatile Solids (mg/L)	100	ND	ND	ND	ND
Total Suspended Solids (mg/L)	4	ND	4.4	ND	4.1

ND = none detected

2.5 PHAGE CLASSIFICATION

The current component phages in EcoShield PX™ were fully characterized by a variety of methods, including pulse-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), electron microscopy (EM), full-genome sequence analysis, lytic activity against *E. coli* strains, and lytic activity against non-*E. coli* strains.

The three component bacteriophages currently included in EcoShield PX™ are listed below:

Name:	ECML-117
ATCC #:	PTA-7950
Order:	Caudovirales[1]
Family:	Myoviridae
Properties:	Double-stranded DNA, Lytic

Name:	ECML-359
ATCC #:	PTA-121407
Order:	Caudovirales
Family:	Myoviridae
Properties:	Double-stranded DNA, Lytic

Name:	ECML-363
ATCC #:	PTA-121406
Order:	Caudovirales
Family:	Myoviridae
Properties:	Double-stranded DNA, Lytic

The monophages have not been genetically manipulated (i.e., not GMO).

2.6 POTENTIAL HUMAN TOXICANTS

As with all Gram-negative bacteria, the *E. coli* host strains produce bacterial endotoxin or lipopolysaccharide (LPS). Intralytix tests every lot of EcoShield PX™ for LPS. Endotoxins are further discussed below, in Sections 3.1.2.3, 6.1.3, and 6.2.1.3.

Some *E. coli* strains are known to carry enterotoxins. Even though great care is taken to remove media products, processing enzymes, and host material – including nucleic acids – from phage lysates, bacterial strains that may be used for phage propagation are routinely screened for enterotoxins. *E. coli* toxins are further discussed in Section 6.2.1.3.

2.7 STABILITY

The proposed shelf life of EcoShield PX™ is one year when stored at 2–8°C in a dark, UV-protected area.

[1] as defined by the International Committee on the Taxonomy of Viruses (ICTV) and by 1.Ackermann, H.-W. and L. Berthiaume, *A Summary of Virus Classification*, in *Atlas of Virus Diagrams*. 1995, CRC Press: Boca Raton, FL. p. 3-6..

2.8 ALLERGENS

The component phages in EcoShield PX™ are grown in a soy-based peptone medium. During the initial fermentation, the media components are hydrolyzed by the growing bacterial culture, as the bacteria break down the proteins into amino acids . The media and its components are removed during the subsequent filtration and washing steps of each individual monophage. Moreover, previous testing of similarly produced Intralytix products, SalmoFresh and ShigaShield, by an independent third party laboratory showed no detectable levels of soy protein,

Because EcoShield PX™ is produced using the same growth medium and same production method as all Intralytix bacteriophage products (including SalmoFresh and ShigaShield), there should be no residual soy protein in EcoShield PX™ lots either. Out of an abundance of caution, the product was tested to confirm no soy protein is present in the final product. Two lots of EcoShield PX™ (1817K2930A88 and 1818B2730A09) were tested for the presence of soy allergens. There was no detectable soy present, at a detection limit of 2ppm.

3 DIETARY EXPOSURE

3.1 APPLICATION RATES AND DIETARY INTAKE

3.1.1 Application rates

The current EcoShield PX™ article of commerce is a concentrate that is typically diluted with water at the application site to form the EcoShield PX™ working solution. It is applied at a rate that ensures the final concentration of phage on the food articles is at or below $1x10^8$ PFU/g of food. Future preparations may be sold in more concentrated form, but the accompanying instructions for dilution and application rate will be appropriately adjusted to ensure the final concentration of phage on the food articles is always at or below $1x10^8$ PFU/g of food.

3.1.2 Dietary intakes

EcoShield PX™ is envisioned to be used upon foods, including those in the following food categories:

- Ground and whole meat and poultry, including whole carcasses, primals, subprimals, trimmings, and organs
- Ready-to-eat (RTE) meats and poultry
- Fresh and processed fruits
- Fresh and processed vegetables
- Dairy products (including cheese)
- Fish and other seafood

The calculations described in the subsequent sections were performed to estimate the dietary intake of EcoShield PX™ when used at the maximum application of $1x10^8$ PFU/g for each of the above food categories.

To determine the daily intake of each of the food categories for the US population as a whole, the Food Availability (Per Capita) Data System, provided by the United States Department of Agriculture's Economic Research Services was used [2]. The per capita usage is a measure of food disappearance that is calculated by dividing the total supply available by the US population and does not account for spoilage and waste. Because losses are not taken into consideration, the per capita estimations are most likely higher than actual consumption.

All calculations below are based on a maximum (worst-case scenario) consumption of EcoShield PX™. This worst-case scenario assumes 100% market saturation (i.e. that the entire

food supply is treated with EcoShield PX™), there are no losses from the food supply, and that the maximum application rate of $1x10^8$ PFU/g is used. Even with the added margin of safety added by these overestimations, the amounts of EcoShield PX™, and its constituents, that would be consumed via the five food categories are very small, as shown in the following calculations.

3.1.2.1 Dietary intakes for EcoShield PX™

The following calculation to determine the maximum (worst-case scenario) consumption of EcoShield PX™ by the average American uses the highest rate of EcoShield PX™ application ($1x10^8$ PFU/g):

The concentration recommended for the working solution of EcoShield PX™ is $1x10^9$ PFU/mL. Using that concentration, the volume of EcoShield PX™ that would be applied per gram treated food can be calculated as follows:

$$\frac{1x10^8 \text{ PFU}}{\text{g food}} \times \frac{1 \text{ mL EcoShield PX™}}{1x10^9 \text{ PFU}} = \frac{0.1 \text{ mL EcoShield PX™}}{\text{g food}}$$

Using 0.1mL EcoShield PX™ applied per gram of food, the volume of EcoShield PX™ that would be consumed per day via each food category can be calculated and is presented in Table 4. Assuming the worst case scenario, where 100% of the foods in the five food groups were treated at the maximum application ($1x10^8$ PFU/g), the combined total amount of EcoShield PX™ consumed per day would be about 195.2 mL or the equivalent of about ¾ cup.

Table 4 Volume of EcoShield PX™ consumed per day when applied at $1x10^8$ PFU/g food

	Consumed per American per year (lbs)	Consumed per American per day* (g)	EcoShield PX™ consumed per person per day (mL)
Poultry / Red meat	265.9	331	33.1
Fish/Shellfish	14.9	19	1.9
Fruits	255.8	319	31.9
Vegetables	385.1	480	48.0
Dairy	645.9	804	80.4
Total of all categories	1567.6	1952	195.2

*The ERS per capita usage data is given as lbs/year [2]. This column simply converts lbs/year to grams/day (lbs/year x 1000g ÷ 2.2lbs ÷ 365days).

The majority of the 195.2 mL of EcoShield PX™ would constitute water; the phages, sodium, and potassium contained within that approximate ¾ cup would be negligible, as evidenced by the dietary calculations presented below.

3.1.2.2 Dietary intakes for EcoShield PX™ phages

The following calculation determines the approximate weight of phages consumed per day, again assuming the maximum rate ($1x10^8$ PFU/g) of EcoShield PX™ application:

Total phages (PFU) consumed per day:

$$\frac{1x10^8 \text{ PFU}}{\text{g food}} \times \frac{1952 \text{ g food}}{\text{day}} = \frac{1.95x10^{11} \text{ PFU}}{\text{day}}$$

Weight of total phages consumed/day (in micrograms):

$$\frac{1.95x10^{11} \text{ PFU}}{\text{day}} \times \frac{3.60x10^{-16} \text{ g}}{\text{phage}} \times \frac{1x10^6 \text{ µg}}{\text{g}} = \frac{70.2 \text{ µg}}{\text{day}}$$

Where $3.60x10^{-16}$ g = approximate mass of one phage

Assuming the average diet is 3 kg/day, the dietary concentration of phages is:

$$\frac{70.2 \text{ µg}}{\text{day}} \times \frac{\text{day}}{3 \text{ kg}} = 23.4 \text{ ppb}$$

The weight of phages consumed per day via EcoShield PX™ would be 70.2 µg, or 23.4 ppb in a 3 kg diet. This is insignificant.

3.1.2.3 Dietary intake of endotoxin

Normal saliva contains approximately 1 mg endotoxin per mL [3]. For endotoxin, 1 EU/mL is approximately equal to 1 ng/mL. This means that the 1 mg/mL of endotoxin in saliva is equivalent to approximately $1x10^6$ EU/mL. Specification for EcoShield PX™ lots for endotoxin is ≤ 25,000 EU/mL at 9.0 $\log_{10}$ PFU/mL.

The approximate daily volume of EcoShield PX™ consumed is 195.2 mL (see Section 3.1.2.1). Again using the worst case scenario (maximum allowable endotoxin level by specification), the maximum amount of endotoxin consumed via EcoShield PX™ is thus:

$$\frac{195.2\ \text{mL EcoShield PX™}}{\text{day}} * \frac{2.5\text{x}10^{4}\ \text{EU}}{\text{mL EcoShield PX™}} = \frac{4.9\text{x}10^{6}\ \text{EU}}{\text{day}}$$

Humans produce approximately 500 to 750 mL of saliva per day. Using the lower, more conservative number, healthy humans consume from saliva:

$$\frac{500\ \text{mL saliva}}{\text{day}} * \frac{1\text{x}10^{6}\ \text{EU}}{\text{mL saliva}} = \frac{5\text{x}10^{6}\ \text{EU}}{\text{day}}$$

The maximal amount contributed by EcoShield PX™ would thus constitute 0.98% of the daily load of endotoxin from saliva. The level of endotoxin found in EcoShield PX™ is therefore considered safe.

3.1.2.4 Sodium and potassium content

From Section 2.4, the highest value obtained for sodium content in an EcoShield PX™ lot was 220 mg/L. From this value and using the worst-case scenario value from Table 4 (all foods from each food category are treated with EcoShield PX™), the amount of sodium contributed to the daily diet via EcoShield PX™ can be calculated as follows:

$$\frac{220\ \text{mg sodium}}{1000\ \text{mL EcoShield PX™}} \text{ x } \frac{195.2\ \text{mL EcoShield PX™}}{\text{day}} = \frac{42.9\ \text{mg sodium}}{\text{day}}$$

The recommended daily allowance of sodium is 2,300 mg (21 CFR § 101.9(c)(9)). The amount of sodium per day contributed by EcoShield PX™ thus represents 1.87% of the RDA and is negligible. The amount of sodium per day contributed by EcoShield PX™, 42.9 mg, would be spread across several servings and meals. The amount of sodium consumed per serving would be below the level that would change nutritional content labeling by the end-user.

From Section 2.4, highest value obtained for potassium content an EcoShield PX™ lot was 0.13 mg/L. From this value, the amount of potassium contributed to the daily diet via EcoShield PX™ on the five food categories can be calculated as follows:

$$\frac{0.13\ \text{mg potassium}}{1000\ \text{mL EcoShield PX™}} \text{ x } \frac{195.2\ \text{mL EcoShield PX™}}{\text{day}} = \frac{0.03\ \text{mg potassium}}{\text{day}}$$

Assuming the potassium levels of EcoShield PX™ are just below the detection limit, then the amount of potassium per day contributed by EcoShield PX™, 0.03 mg, is well below the level that

would change nutritional content labeling by the end-user. The recommended daily allowance of potassium is 4,700 mg (21 CFR § 101.9(c)(9)). The amount of potassium per day contributed by EcoShield PX™ thus represents 0.0006% of the RDA and is negligible.

4 SELF-LIMITING LEVELS OF USE

The proposed use for EcoShield PX™ is as an antimicrobial processing aid for foods that are at high risk to be contaminated with *E. coli*.

The self-limiting levels of use are:

Due to the cost of the product, the end-user would use the minimum dose required to achieve a significant reduction or elimination of pathogenic *E. coli*.

Once the *E. coli* contamination is depleted, the phage will slowly decrease in number due to a lack of host.

Phages are susceptible to many environmental factors, including sunlight, heat, and UV light. Exposure to these will cause the number of phages to decrease.

5 EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

This section is not applicable to this notification.

6 NARRATIVE

In the following sections, the data and information providing the basis for our conclusion that EcoShield PX™ is GRAS, through scientific procedures, under the conditions of its intended use is presented. The information provided below, and elsewhere in this document, that is generally available has been properly cited. The list of references is presented in Part 7.

6.1 COMPONENTS OF ECOSHIELD PX™

EcoShield PX™ is a mixture of component bacteriophages together with added sodium chloride; due to the method of production, there may also be small amounts of residual production by-products. The primary active ingredient is not a single chemical substance but a mixture of naturally-occurring bacteriophages. In the appropriate sections below, we consider separately the safety of the:

- Phages (active component)
- Added salts
- Manufacturing by-products

6.1.1 Monophages

The safety and ubiquity of bacteriophages have been well established. The pertinent safety data on bacteriophages is reviewed below. The published literature on phages and other information developed by Intralytix show that:

Bacteriophages are the most ubiquitous organisms on earth. For example, one milliliter of non-polluted stream water has been reported [4] to contain approximately $2x10^8$ PFU of phages/mL, and the total number of phages on this planet has been estimated to be in the range of $10^{30} - 10^{32}$ (see http://www.asm.org/division/m/M.html and [5]). This abundance of phages in the environment, and the continuous exposure of animals to them, explains the extremely good tolerance of mammalian organisms to phages.

Phages have been used therapeutically in humans for almost 100 years, without any serious side effects [6, 7]. During the long history of using phages as therapeutic agents in Eastern Europe and the former Soviet Union (and, before the antibiotic era, in the United States, France, Australia, and other countries), phages have been administered to humans:

- orally, in tablet or liquid formulations,
- rectally,
- locally (skin, eye, ear, nasal mucosa, etc).; in tampons, rinses and creams,
- as aerosols or intrapleural injections, and

- intravenously

There have been virtually no reports of serious complications associated with their use. A number of reviews summarize the results of some of the human therapy studies involving bacteriophages [8-12].

Phages have also been administered to humans for non-therapeutic purposes without any recorded illness or death. To give just a few examples, phage preparations have been used extensively to monitor humoral immune function in humans in the United States in the 1970s-1990s, including in patients with Down's syndrome, the Wiskott-Aldrich syndrome, and immunodeficient patients [13, 14]. In some of the studies (including several studies performed by the FDA), the purified phages were injected intravenously into HIV-infected patients or other immunodeficient individuals without any apparent side effects [15-17].

Phages have also been administered to humans via various sera and FDA-approved vaccines commercially available in the United States [18-20].

The biology of phages has been exhaustively studied. These studies have clearly shown that phages are obligate intracellular parasites of bacteria and are not infectious in humans or other mammals.

Bacteriophages are common populace/commensals of the human gut, and they are likely to play an important role in regulating the diversity and population structure of various bacteria in human gastrointestinal (GI) tracts. For example, phages capable of infecting *E. coli*, *Bacteroides fragilis* and various *Salmonella* serotypes have been isolated from human fecal specimens in concentrations as high as 10^5 PFU/100 g of feces [21-23]. The recent data based on metagenomic analyses (using partial shotgun sequencing) of an uncultured viral community from human feces suggested that bacteriophages are the second most abundant category after bacteria in the uncultured fecal library [24, 25]. There are an estimated 10^{15} phages [26] typically present in the human gastrointestinal tract.

No serious adverse immunologic or allergic sequelae have ever been reported because of human or animal exposure to phages [6, 9].

Bacteriophages are commonly consumed via drinking water [27-29].

Bacteriophages are natural components of all fresh, unprocessed foods and are commonly consumed via various foods. For example, bacteriophages have been readily isolated from a wide range of food products, including ground beef, pork sausage, chicken, farmed freshwater fish, common carp and marine fish, oil sardine, raw skim milk, and cheese [30-39]. Several studies have suggested that 100% of the ground beef and chicken meat sold at retail contain various levels of a number of bacteriophages. To give just a few examples, bacteriophages were recovered from 100% of examined fresh chicken and pork sausage samples and from 33% of delicatessen meat samples analyzed by Kennedy, Oblinger [39]. The levels ranged from 3.3 to 4.4×10^{10} PFU/100 g of fresh chicken, up to 3.5×10^{10} PFU/100 g of fresh pork, and up to 2.7×10^{10} PFU/100 g of roast turkey breast samples. Additionally,

E. coli- and *Shigella*-specific bacteriophages were recently isolated from 100% of beef and 68% of mixed salad purchased in a variety of markets [40].

Because of the (1) highly specific nature of bacteriophages and (2) extremely common exposure of humans and animals to bacteriophages (including daily consumption of bacteriophages with various foods and drinking water), bacteriophages do not deleteriously affect the GI microflora. For example:

- When *E. coli*-specific phage T4 was administered orally to 15 healthy adult volunteers, it did not cause a decrease in total fecal *E. coli* counts. In addition, no substantial phage T4 replication on the commensal *E. coli* population was identified, and no adverse events related to phage application were observed in any of the volunteers [41].
- A pharmacokinetic and toxicological study using mice and guinea pigs did not show any signs of acute toxicity or histological changes, even when the dose administered was 3500-fold higher than the human dose projected in the course of the study [42].
- High doses of *Listeria* phage preparations (i.e. ListShield™ and P100) were administered to laboratory animals (mice and rats) without any adverse effects [43, 44].
- A long-term toxicity study with ShigaShield™ (under the tradename ShigActive™) in mice, showed no significant effect on any health or toxicity markers in the mice. Additionally, the phage preparation did not significantly affect the microbiota of the treated mice [45].

Bacteriophages are commonly consumed by animals (including agriculturally-important species) via various foods. For example, in a recent study from Texas A&M University, male-specific and somatic *E. coli* targeting phages were detected in all animal feeds, feed ingredients, and poultry diets examined, even after the samples were stored at -20°C for 14 months [46].

6.1.1.1 Lytic phages are GRAS

All lytic phages are, by nature, GRAS. There are two major types of phages: "virulent" (also called "lytic") and "temperate" (often mistakenly called "lysogenic"). Lytic phages lyse host bacteria without integrating into the host genome. In contrast, temperate phages may integrate into the host genome and a small subset of these may theoretically transduce undesirable bacterial genes, such as those encoding toxins or antibiotic resistance. Both lytic and temperate phages are extremely common in the environment, the human and animal gut, the human oral cavity, foods sold at retail, sewage, and many other places that we encounter daily. Humans shed large numbers of both lytic and temperate phages into the environment every day – estimated to be on the order of $4x10^9$ phages daily per person [7]. Temperate phages have been found in almost all

bacterial genera, including *Staphylococcus*, *Vibrio*, *Pseudomonas*, *Salmonella*, *Shigella*, *Bacillus*, *Corynebacterium*, *Listeria,* and *Streptococcus* [47-50]. Indeed, some strains can release as many as five different types of temperate phages. Although the possibility of added gene transfer events is highly unlikely to bring danger to any individual consuming temperate phages, the use of such phages on an industrial scale could increase the overall risk of potentially harmful genes being acquired by new bacterial strains. Therefore, Intralytix identifies and uses only lytic phages in its phage preparations (including EcoShield PX™).

6.1.1.2 EcoShield PX™ monophages are GRAS

The component phages in EcoShield PX™ were characterized by Intralytix's scientists. Each was characterized by various approaches, including electron microscopy, genotypic fingerprinting, and full genome sequence analysis. The component phages in EcoShield PX™ are members of the *Myoviridae* double-stranded DNA phage families,

Intralytix will fully sequence any and all component monophages included in EcoShield PX™. This approach is used to exclude bacteriophages carrying sequences encoding undesirable genes, and phages displaying prior evidence of transduction (e.g., bacterial 16S RNA genes).

Intralytix excludes all bacteriophages carrying sequences encoding any undesirable genes. Undesirable genes include genes encoding bacterial toxins (including genes listed in 40 CFR § 725.421), other known toxin genes, and genes associated with drug resistance. Undesirable genes are identified by comparing a complete bacteriophage sequence to all sequences contained in GenBank and other databases available through the National Center for Biotechnology Information website of the National Library of Medicine using the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/).

The cut-off *e*-value level for the latter analysis is $1x10^{-4}$, which detects virtually all undesirable genes in the phages' genomes. In practice, significant matches are considered to be those with *e*-values of $\leq 10^{-5}$ [51]. Therefore, our proposed cut-off value provides a very strong (10-fold higher than the proposed 10^{-5} cut-off) assurance that undesirable genes are not missed during the analysis.

Intralytix will sequence the complete genome of each phage incorporated into EcoShield PX™. Table 5 summarizes the current three phages genomes properties. Analysis of the sequences yielded the following results:

> No toxin genes have been identified among the open reading frames of the annotated genomes of any of the current three monophages.

No 16S ribosomal RNA genes have been identified among annotated genomes of any of the current three monophages.

No antibiotic resistance genes have been identified among annotated genomes of any of the current three monophages.

Summary: The approach of obtaining the full nucleotide sequence for each commercialized phage and complete bioinformatics analysis of all open reading frames will insure that no detrimental genes will be present in any of the phages which will be used. This provides the fullest assurance of the phage safety as can presently be obtained by any method.

Table 5 Genome size and composition of phages contained in EcoShield PX™

Phage	ATCC #	GC%	Size (bp)	Number of Open Reading Frames (ORFs)	Undesirable genes
ECML-117	PTA-7950	46.1	66,854	103	None
ECML-359	PTA-121407	40.4	169,468	282	None
ECML-363	PTA-121406	35.4	167,029	272	None

6.1.1.3 EcoShield PX™ is specific to *E. coli*

Lytic activity of EcoShield PX™ is targeted against *E. coli* strains. EcoShield PX™ has been screened for its lytic activity against just over 160 *E. coli* O157 isolates in the Intralytix collection. As shown in Table 6, EcoShield PX™ is very effective against the collection.

Table 6 *E. coli* O157:H7 in Intralytix's collection and the percent susceptible to EcoShield PX™ at 1x10^9 PFU/mL.

Species	# isolates in Intralytix collection	Percent kill (1x10^9 PFU/mL EcoShield PX™)
O157:H7	161	97%

EcoShield PX™ is also highly specific. Table 7 shows that EcoShield PX™ does not lyse any of the non-targeted isolates examined. These strains include Gram positive strains, five each of *Staphylococcus aureus* and *Listeria* species and 13 strains of *Enterococcus* species. EcoShield PX™ also does not lyse several non-*Escherichia* Gram negative strains, including 5 strains each of *Acinetobacter baumannii* and six strains of *Pseudomonas* species.

Table 7 Lytic activity of EcoShield PX™ against strains of common bacteria

Non-*E. coli* isolates		Species	Susceptibility to EcoShield PX™ ($1x10^9$ PFU/mL)
Intralytix ID	Original ID		
Sa36	ATCC25923	*Staphylococcus aureus*	-
Sa37	ATCC29213	*Staphylococcus aureus*	-
Sa211	ATCC700699	*Staphylococcus aureus*	-
Sa298	ATCC49775	*Staphylococcus aureus*	-
Sa299	ATCC14458	*Staphylococcus aureus*	-
Lm 314	ATCC19117	*Listeria monocytogenes*	-
Lm 315	ATCC19118	*Listeria monocytogenes*	-
L. innocua 316	ATCC51724	*Listeria innocua*	-
Lm 317	ATCC19116	*Listeria monocytogenes*	-
L. innocua 318	ATCC33090	*Listeria innocua*	-
Ab3	ATCC19606	*Acinetobacter baumannii*	-
Ab4	HER1401	*Acinetobacter baumannii*	-
Ab5	4308-2	*Acinetobacter baumannii*	-
Ab6	3247-1	*Acinetobacter baumannii*	-
Ab7	1673-2	*Acinetobacter baumannii*	-
E102	WCC188	*Enterococcus* spp.	-
E402	ATCC11823	*Enterococcus* spp.	-
E403	ATCC19433	*Enterococcus* spp.	-
E404	1133455	*Enterococcus* spp.	-
E405	1126611	*Enterococcus* spp.	-
E610	ERV99	*Enterococcus* spp.	-
E611	503	*Enterococcus* spp.	-
E612	513	*Enterococcus* spp.	-
E613	TX1330	*Enterococcus* spp.	-
E614	TX1322	*Enterococcus* spp.	-
E615	Y16-1	*Enterococcus* spp.	-
E616	BAA-2820	*Enterococcus* spp.	-
E617	51299-MINI-PACK	*Enterococcus* spp.	-
Pa76	ATCC10145	*Pseudomonas aeruginosa*	-
Pa161	ATCC15692	*Pseudomonas aeruginosa*	-
Pa162	ATCC51674	*Pseudomonas aeruginosa*	-
Pa163	ATCC43390	*Pseudomonas aeruginosa*	-
Pa164	ATCC39324	*Pseudomonas aeruginosa*	-
Ps579	HM-214/2_1_26	*Pseudomonas* spp.	-

+ Lysed by phage preparation - Not lysed by phage preparation

6.1.2 Sodium chloride

Sodium chloride "table salt" is the prototype in 21 CFR § 182.1 (a) of an ingredient that is so obviously GRAS that the FDA has not listed it as GRAS.

6.1.3 By-products

Even though great care is taken to remove media products, processing enzymes, and host material – including nucleic acids – from phage lysates, bacterial strains that may be used for phage propagation are routinely screened for enterotoxins. The most commonly known *E. coli* enterotoxin are Shiga toxins I and II [52, 53]. The current host strains have been determined lack a complete gene for these enterotoxins. The enterotoxins are further discussed in Section 6.2.1.3.

As with all Gram-negative bacteria, the *E. coli* host strains produce bacterial endotoxin or LPS. Intralytix tests every lot of EcoShield PX™ to ensure its LPS levels fall below the established release criteria. Endotoxins are further discussed in Sections 6.2.1.3 and 6.2.2.2.

6.2 MANUFACTURING OF ECOSHIELD PX™

EcoShield PX™ is manufactured using Intralytix's standard procedures. These procedures have been reviewed by the FDA for manufacturing of Intralytix's bacteriophage food safety products, ListShield™ (21 CFR §172.785), EcoShield™ (FCN No. 1018), SalmoFresh™ (GRAS Notice No. 435), and ShigaShield (GRN No. 000672) and are currently used to manufacture commercial lots of these products.

EcoShield PX™ is prepared by cultivation of individual host *E. coli* strain/phage combinations followed by filtration, concentration, wash, and final sterile filtration. After each monophage passes quality control, the monophages are combined with 0.1M sodium chloride to form the EcoShield PX™ concentrate. Final filtration is then carried out with a sterilizing grade filter.

6.2.1 Starting materials

There are four starting materials for manufacture of EcoShield PX™ component monophages:

Animal-product free media

Antifoam

Host strain

Monophages

The safety of each is considered separately below.

6.2.1.1 Animal-product free media

The animal-product free media is a vegan custom blend. The main components are described here and have an existing regulatory status as regulated GRAS ingredients or additives.

Phytone Peptone and *Soytone:* Peptones are GRAS affirmed at 21 CFR § 184.1553 for use as processing aids, among other uses, at levels not to exceed good manufacturing practice. Peptones are protein hydrolysates consisting of free amino acids and short peptides in an aqueous salt solution.

Yeast Extract: Yeast extract is a commonly used food ingredient. For example, baker's yeast extract is GRAS affirmed as a flavoring agent or adjuvant at up to 5% in foods generally. 21 CFR § 184.1983.

Sodium Chloride: Sodium chloride "table salt" is the prototype in 21 CFR § 182.1 (a) of an ingredient that is so obviously GRAS that FDA has not listed it as GRAS.

Magnesium Sulfate: Magnesium sulfate salt is GRAS affirmed at 21 CFR § 184.1443 for use as a processing aid, among other uses, at levels not to exceed good manufacturing practice.

6.2.1.2 Antifoaming agent

P2000 antifoam is polypropylene glycol-based, Kosher-certified product, approved for a variety of food additive uses, both direct and indirect (The Dow Chemical Company, Midland, Michigan; http://www.dow.com). Small amounts of the P2000 antifoam may be used in the initial fermentation of the individual monophages. The antifoam is listed in GRAS notification in 21 CFR § 173.340.

6.2.1.3 Host strains

The component monophages are produced on *Escherichia coli* isolates from Intralytix's collection of *Escherichia* strain. These *E. coli* host strains were characterized at Intralytix. Their biochemical properties were examined using the bioMérieux API testing kit. Their background genomic composition/type was examined through the standard PFGE protocol for bacteria. They were also examined for the presence of endogenous phage(s) and its susceptibility to seven commonly prescribed antibiotics (amoxicillin / clavulanic acid, azithromycin, ceftriaxone, cephalothin, ciprofloxacin, levofloxacin, and sulfamethoxazole / trimethoprim).

The *E. coli* host strains are not known to produce any enterotoxins that could compromise the safety of the final product. *E. coli* strains are known to produce enterotoxins, the host strains have been PCR screened to be free of the enterotoxin genes.

The only production host strain-related toxins that are relevant for EcoShield PX™ safety is endotoxin (also known as LPS). As with all Gram-negative bacteria, the outer membrane of *E.coli* contains lipopolysaccharide or LPS [54]. Due to the lysis of host cells during the fermentation process (as the result of phage lytic cycle), *E. coli* LPS is present in the resulting phage lysates. Most of the endotoxin is expected to be removed during phage purification process.

LPS is of concern if sufficiently high amounts enter the human bloodstream, where it can trigger the signaling cascade for macrophage/endothelial cells to secrete pro-inflammatory cytokines and nitric oxide that may lead to "endotoxic shock." However, LPS has not been shown to cross the intestinal mucosa and oral administration of LPS shows no negative effects and may even elicit beneficial responses in the GI system [55]. Additionally, there is no FDA specification for levels of endotoxin in oral products. Still, as a standard quality control protocol, Intralytix analyzes every EcoShield PX™ batch for the presence and levels of the LPS endotoxin in the final product. All product lots must be at or below 25,000 endotoxin unit (EU)/mL at 9.0 $\log_{10}$ PFU/mL level in order to pass the release criteria for LPS. This level is very safe and is based upon the levels of endotoxins that are found naturally in healthy human saliva [3]. See Section 3.1.2.3 for discussion of dietary intake.

6.2.1.4 Monophages

Each monophage is produced and purified in the same manner as the monophages included in the GRAS-listed bacteriophage based products EcoShield™, ShigaShield™, SalmoFresh™, and ListShield™.

The safety of monophages is discussed in Section 6.1.1.2.

6.2.2 Quality Control

6.2.2.1 Monophages

The following tests are performed upon each monophage lot:

Lytic titer

The lytic titer test measures the lytic titer of each monophage lot, by determining the number of plaque forming units per milliliter (PFU/mL). The specification for each monophage lot is the titer is ≥10.0 $\log_{10}$ PFU/mL. Lots failing to meet the specification due to a low titer may be appropriately concentrated and retested.

Microbial purity

The microbial purity test confirms that the monophage solution does not contain viable microbes. Briefly, samples of each monophage solution are tested by a) direct plating onto non-selective agar and b) after enrichment. The specification is that each monophage lot must be bacteriologically sterile. Lots failing the test may be re-filtered and retested. Lots repeatedly failing to meet the specification will be discarded.

Identity

Currently, genotypic fingerprinting, through restriction fragment length polymorphism (RFLP), is used to confirm the identity of each monophage lot. The specification for RFLP is that the bands should visually match those in the reference pattern. Lots repeatedly failing the RFLP test will be discarded.

6.2.2.2 EcoShield PX™

The following tests are performed upon each batch of EcoShield PX™:

Lytic titer test

The lytic titer test method confirms the titer (PFU/mL) of the EcoShield PX™ preparation. The specification for this test is EcoShield PX™ has a lytic titer of ≥10.0 $\log_{10}$ PFU/mL. Lots failing to meet the specification due to a low titer may be appropriately concentrated and retested.

Microbial purity

The microbial purity test is a determination of the viable microbial contamination in a phage solution. Briefly, a 1% representative sample of each lot of EcoShield PX™ is tested by combining with a concentrated growth media and incubating for 14 days. Growth is monitored visually and by plating, if growth is not visually detectable. Both positive and negative controls are included. The specification for this test is that EcoShield PX™ must be bacteriologically sterile. Lots failing

the test may be re-filtered and retested. Lots repeatedly failing to meet the specification will be discarded.

Endotoxin content test

Endotoxins are toxins associated with host bacteria, of which a residual amount could be present in the phage preparations. A commercially available quantitative Limulus amebocyte lysate-based test specifically for measurement of endotoxin is currently used by Intralytix. The specification for this test is each lot of EcoShield PX™ must contain ≤ 25,000 EU/mL (at standard working concentration ca. 9.0 $\log_{10}$ PFU/mL). Lots failing to meet the specification may be washed with sterile 0.1M saline and subjected to the full panel of quality control tests.

Identity test

The identity test verifies that all phages claimed to be present in EcoShield PX™ are actually present. There are currently three methods available to confirm this; any one can be used alone or in combination with the others. The first method uses RT-PCR to confirm the presence of each monophage. In this case, three sets of primer pairs, each specific to a single EcoShield PX™ component monophage, are screened against EcoShield PX™. The specification is that all expected amplicons are present. The second method uses the spot test method. Briefly *E. coli* strains, each of which is susceptible to only one component monophage, are screened for lysis by EcoShield PX™. The specification for this test is that all reference bacterial strains are lysed by the preparation (e.g., if one of the strains is not lysed, it is because the phage specifically lytic for that strain was not included in the phage preparation). The third method uses visual, signature-based confirmation that all monophages were included in the EcoShield PX™ lot during manufacturing. Briefly, as the lot is mixed, a second employee must be present to observe and confirm that each and every component monophage is actually added. At least two employees must sign the preparation mixing worksheet, which is archived by the QC department for a minimum of 2 years. Lots that fail to meet the specification may be retested. Lots repeatedly failing the specification may be supplemented with the missing component monophage and retested for all QC tests.

6.3 SUBSTANTIAL EQUIVALENCE TO APPROVED PRODUCTS

6.3.1 Previously approved bacteriophage preparations

Several lytic bacteriophage products targeting various bacterial pathogens have already been designated GRAS and/or cleared for food safety usage and other applications by a number of regulatory agencies:

EcoShield™ (formerly ECP-100) a phage preparation containing three lytic *E. coli* O157:H7-specific phages (one of which is also contained in EcoShield PX™), is FDA-cleared for use as a food contact substance (FCN No. 1018).

EcoShield™ is also listed by the FSIS for use as processing aid on red meat parts and trim prior to grinding (FSIS Directive 7120.1).

PhageGuard E™, a phage preparation containing two *E. coli*-specific lytic phages is GRAS (GRAS Notice 000757).

ListShield™ (formerly known as LMP-102,) a phage preparation containing six lytic *Listeria monocytogenes*-specific phages, is FDA-cleared as a food additive (21 CFR §172.785).

ListShield™ is also GRAS (GRAS Notice No. 000528).

ListShield™ is also listed by the FSIS for use on various RTE meats and poultry products (FSIS Directive 7120.1).

ListShield™ is also EPA-registered for use on non-food surfaces in food processing plants to prevent or significantly reduce contamination of *Listeria monocytogenes* (EPA registration #74234-1).

Listex™[2], a phage preparation containing a single *Listeria monocytogenes* lytic phage, P100, is GRAS (GRAS Notice No. 000218). (Now marketed as PhageGuard L)

Listex™ is also listed by the FSIS for use as processing aid when applied at a level of $1x10^7$ to $1x10^9$ PFU/g food product (FSIS Directive 7120.1).

SalmoFresh™, a phage preparation containing six *Salmonella*-specific lytic phages is GRAS (GRAS Notice No. 435).

SalmoFresh™ is also listed by the FSIS for use on various poultry products (FSIS Directive 7120.1).

Salmonelex™[3], a phage preparation containing two *Salmonella*-specific lytic phages is GRAS (GRAS Notice 000468).

SalmPro®, a phage preparation containing two *Salmonella*-specific lytic phages is GRAS (GRAS Notice 000752).

AgriPhage™, a phage preparation targeting *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. Tomato, is EPA-registered for use on tomatoes and peppers (EPA Reg. No. 67986-1).

[2] Currently marketed as PhageGuard L.

[3] Currently marketed as PhageGuard S.

Two bacteriophage preparations – one *Salmonella* targeting and one *E. coli* O157:H7 targeting – are listed by the FSIS for use as processing aids on the hides and feathers of live animals before slaughter (FSIS Directive 7120.1).

ShigaShield™, a phage preparation containing five *Shigella*-specific lytic phages is GRAS (GRAS Notice No. 000672).

An *E.* coli specific phage preparation containing six of 12 *E. coli* specific bacteriophages is GRAS (GRAS Notice No. 000724).

Several regulatory agencies are represented in the preceding list, each of which separately concluded that a different bacteriophage preparation was safe and effective. The variety of these previously cleared or registered bacteriophage preparations attests to the general safety of bacteriophages and therefore supports their natural GRAS status. EcoShield PX™ is substantially equivalent to the above bacteriophage preparations and therefore is also GRAS.

6.4 SUMMARY AND BASIS FOR GRAS

EcoShield PX™ is an all-natural product made of three to eight *E. coli*-specific lytic bacteriophages. All phages included in EcoShield PX™ are lytic phages; each phage is rigorously characterized (including full genome sequencing) prior to inclusion in the cocktail.

Phages are omnipresent in the environment. Bacteriophages are the oldest, most ubiquitous organisms on earth, with their numbers estimated to be between 10^{30} and 10^{32}. Phages are present everywhere – including in our mouths, on our skin, and within our gastrointestinal tracks. They are also common and natural ingredients of all fresh, unprocessed foods. The omnipresence of phages (including in foods) and their daily consumption by humans makes them naturally GRAS.

In further recognition of their safety, several lytic bacteriophage products targeting various bacterial pathogens have already been designated GRAS and/or cleared for food safety usage and other applications by a number of regulatory agencies.

Although all lytic bacteriophages are, by nature, GRAS, the phages in EcoShield PX™ must be verified to be lytic and to not contain any undesirable genes listed in 40 CFR § 725.421.

The genomes of the three bacteriophages in EcoShield PX™ have been sequenced. Bioinformatic analysis of the component phages' sequences shows none contain any undesirable genes listed in 40 CFR §725.421. Furthermore, no antibiotic resistance gene, no 16S RNA sequences, or other known toxin genes were identified in any of the phage genomes.

EcoShield PX™ is manufactured using Intralytix's standard procedures. These procedures have been reviewed by the FDA for manufacturing of Intralytix's bacteriophage food safety products, ListShield™ (21 CFR §172.785), EcoShield™ (FCN No. 1018), SalmoFresh™ (GRAS Notice No. 435), and ShigaShield™ (GRAS Notice No. 672) and are currently used to manufacture commercial lots of these products.

The only manufacturing byproduct of potential concern during EcoShield PX™ manufacturing is LPS. Intralytix tests every lot of EcoShield PX™ for LPS ensure it meets the release criteria. The LPS levels of the EcoShield PX™ must be ≤ 25,000 EU/mL (at standard working concentration ca. 9.0 $\log_{10}$ PFU/mL) for the lot to be released. This standard is the same as the maximum LPS level previously cleared by the FDA for EcoShield™ (per FCN 1018).

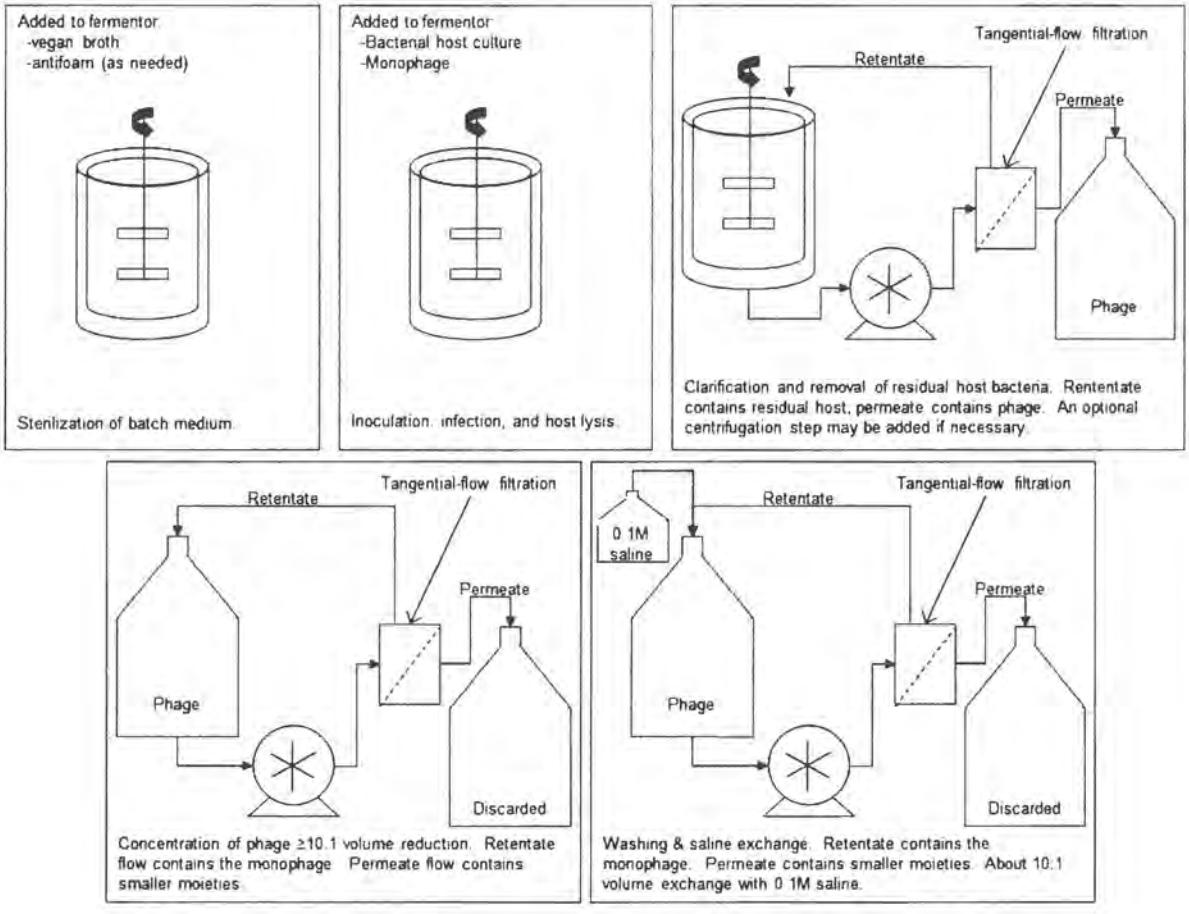
EcoShield PX™ is produced on animal-product free media. The final EcoShield PX™ product contains no preservatives, known allergenic substances, or additives. EcoShield PX™ is eligible for certification as both Kosher and Halal, as the manufacturing process has previously been certified for both ListShield™ and SalmoFresh™. EcoShield PX™ is also eligible for OMRI-listing, to certify it is suitable for use in organic production. These approvals will be pursued dependent upon market demands.

The proposed application rate for EcoShield PX™ is up to $1x10^8$ PFU per gram of food article. Assuming the maximum application rate of $1x10^8$ PFU/g of all five target food groups, the average daily consumption of these foods would contain a mere 70.2 μg of phage particles, 42.9 mg of added sodium, and 0.03 mg of added potassium. This consumption would be spread out across several servings and meals, so the added sodium and potassium levels per serving would be so low as to not require any changes to labeling. The weight of added phage is negligible.

EcoShield PX™ is substantially equivalent to the lytic bacteriophage preparations that have been previously designated GRAS and/or cleared by other regulatory agencies. Furthermore, with the proposed maximum application rate for EcoShield PX™ of up to $1x10^8$ PFU per gram of food article, even in the worst case scenario ($1x10^8$ PFU/g) the rate is equal to or lower than the rates previously cleared for those other preparations as safe and effective. For instance, the maximum proposed application rate of EcoShield PX™ is 10 times lower than that of the previously GRAS-listed Listex P100 bacteriophage preparation.

In summary, the data presented in this document fully supports our designation of EcoShield PX™ as GRAS. The basis for our conclusion is five-fold. First, the scientific literature extensively documents that lytic bacteriophages pose no safety concerns to humans. Second, all bacteriophages in EcoShield PX™ are lytic, non-genetically modified, and free of any and all undesirable genes. Third, Intralytix's manufacturing process ensures the safety and quality of the final EcoShield PX™ product. Fourth, the estimated daily intake of the EcoShield PX™ phage

preparation is so low it is negligible. And, fifth, the bacteriophage product is substantially equivalent to several bacteriophage products already receiving regulatory clearance. Based on this information, it is evident that EcoShield PX™ is GRAS.


Added to fermentor:
-vegan broth
-antifoam (as needed)
Sterilization of batch medium
Added to fermentor:
-Bacterial host culture
-Monophage
Inoculation, infection, and host lysis
Tangential-flow filtration
Retentate
Permeate
Phage
Clarification and removal of residual host bacteria. Rententate contains residual host, permeate contains phage. An optional centrifugation step may be added if necessary.
Retentate
Tangential-flow filtration
Permeate
Phage
Discarded
Concentration of phage ≥10.1 volume reduction. Retentate flow contains the monophage. Permeate flow contains smaller moieties.
0.1M saline
Retentate
Tangential-flow filtration
Permeate
Phage
Discarded
Washing & saline exchange. Retentate contains the monophage. Permeate contains smaller moieties. About 10.1 volume exchange with 0.1M saline.

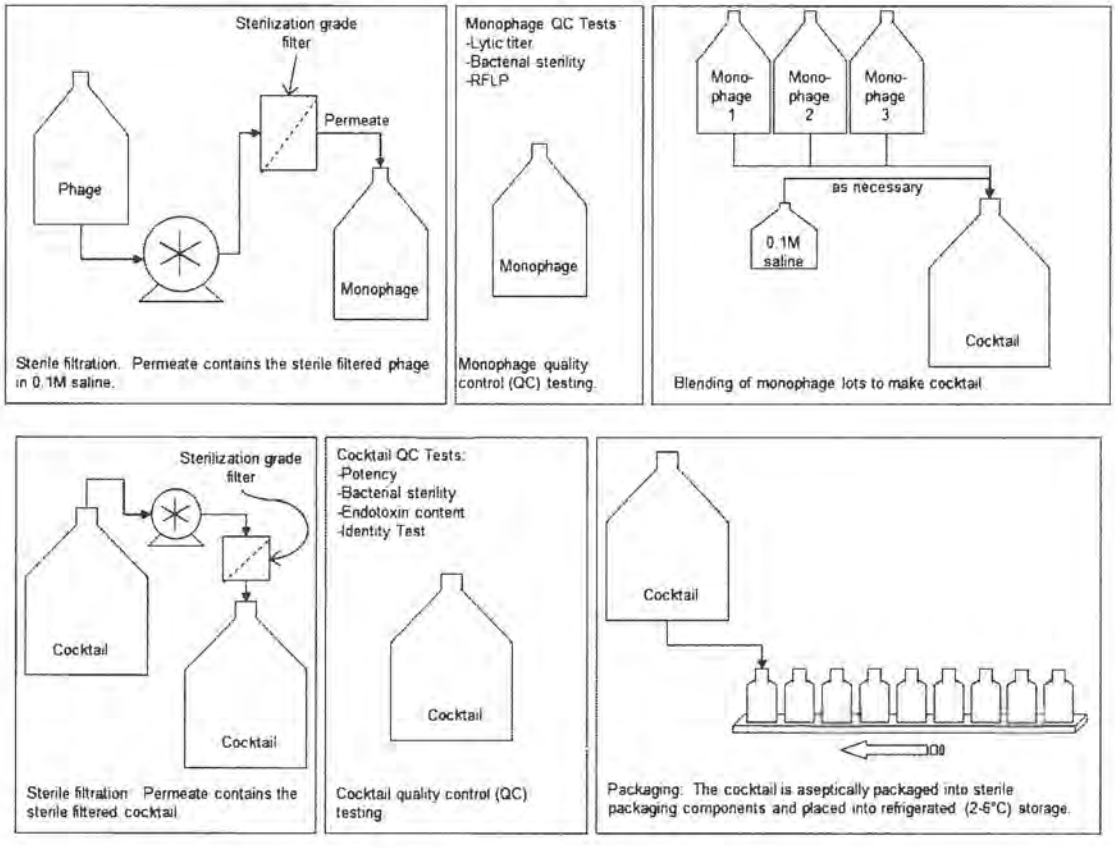


Figure 1 Overview of EcoShield PX™ manufacturing process

7 LIST OF SUPPORTING DATA AND INFORMATION

7.1 APPENDICES (INCLUDES NOT GENERALLY AVAILABLE DATA)

Appendix 1 Efficacy of EcoShield PX™ on Foods

7.2 REFERENCES (FOR GENERALLY AVAILABLE DATA)

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APPENDIX 1: EFFICACY STUDIES

Substance: Bacteriophage preparation (Shiga toxin-producing *Escherichia coli* targeted)

Product:

- Ground and whole meat and poultry, including whole carcasses, primals, subprimals, trimmings, and organs
- Ready-to-eat (RTE) meats and poultry
- Fresh and processed fruits
- Fresh and processed vegetables
- Dairy products (including cheese)
- Fish and other seafood

Amount: Applied as a spray to the surface of the product at a level of ca. ≤ $1x10^8$ plaque forming units (PFU) per gram of product

Reference: Acceptability determination

Labeling Requirements: None under the accepted conditions of use

EcoShield PX™ is an all-natural product comprised of *E. coli*-specific lytic bacteriophages. All phages included in EcoShield PX™ are lytic phages that have not been genetically manipulated in any way. The component phages of EcoShield PX™ are rigorously characterized, including full genome sequencing, prior to inclusion in the product.

The EcoShield PX™ preparation is intended for use in food products to control Shiga toxin-producing *E. coli* (STEC) when added at ≤ $1x10^8$ PFU per gram of food. Intralytix, Inc. has concluded that EcoShield PX™ is generally recognized as safe (GRAS), and therefore, we believe it is not subject to the requirement of pre-market approval, under the conditions of its intended use.

ECOSHIELD PX™ IS EFFECTIVE.

Target range

EcoShield PX™ has been screened for its lytic activity against 161 *E. coli* O157:H7 strains. At the standard "working concentration" of $1x10^9$ PFU/mL, it lyses 156 (97%) of the *E. coli* O157:H7 strains in our collection.

Effect on *E. coli* levels in foods

EcoShield PX™ is intended to produce a statistically significant reduction of *E. coli* O157:H7 contamination vs. a water control when applied as directed to food products.

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Efficacy study summary

EcoShield PX™ was examined for its ability to reduce *E. coli* O157:H7 contamination when applied to various foods. Detailed reports of the studies are included in Appendix 1.1 - Appendix 1.9. A summary of the results is given below.

Description of the test system

For each food tested, portions were inoculated with *E. coli* Ec229, a nalidixic acid resistant isolate. After allowing the bacteria to colonize, the food was then treated with water or EcoShield PX™. The EcoShield PX™ contact time was 15 minutes at room temperature, after which the samples were analyzed for populations of *E. coli*.

Summary of results

Whole meat

Study Ec18J12JT and Study Ec18K06JT examined the efficacy of EcoShield PX™ in reducing *E. coli* levels on chuck roast pieces. In both studies, three concentrations of EcoShield PX™ ($1x10^6$, $5x10^6$, and $1x10^7$ PFU/g in Ec18J12JT and $1x10^6$, $1x10^7$, and $1x10^8$ PFU/g) were applied. After 15 minutes at room temperature, each concentration significantly reduced the number of viable *E. coli*. In Ec18J12JT, the reductions were 45%, 57%, and 67%, respectively. In Ec18K06JT, the reductions were 44%, 82%, and 96%, respectively. The complete details of these studies can be seen in Appendix 1.2 and Appendix 1.7.

Whole poultry

Study Ec18K01JT examined the efficacy of EcoShield PX™ on reducing *E. coli* levels on chicken breast. Three concentrations of EcoShield PX™ ($1x10^6$, $5x10^6$, and $1x10^7$ PFU/g) were applied. After 15 minutes at room temperature, each concentration reduced the number of viable *E. coli* by ca. 34%, 69%, and 80%, respectively. The complete details of this study can be seen in Appendix 1.5.

Ground meat and poultry

Study Ec18J29JT examined the efficacy of EcoShield PX™ on reducing *E. coli* levels in ground beef. Three concentrations of EcoShield PX™ ($1x10^6$, $5x10^6$, and $1x10^7$ PFU/g) were applied. After 15 minutes at room temperature, each concentration reduced the number of viable *E. coli* by ca. 16%, 27%, and 49%, respectively. The complete details of this study can be seen in Appendix 1.4

Ready-to-eat poultry and red meat

Study Ec18K09JT examined the efficacy of EcoShield PX™ in reducing *E. coli* levels on pre-cooked (ready-to-eat) roast chicken. Three concentrations of EcoShield PX™ ($1x10^6$, $1x10^7$, and $1x10^8$ PFU/g) were applied. After 15 minutes at room temperature, each concentration

significantly reduced the number of viable *E. coli*. The reductions were 32%, 77%, and 99%, respectively. The complete details of these studies can be seen in Appendix 1.8.

Fish and shellfish

Study Ec18K20JT examined the efficacy of EcoShield PX™ on reducing *E. coli* levels on salmon. Three concentrations of EcoShield PX™ ($1x10^6$, $1x10^7$, and $1x10^8$ PFU/g) were applied. After 15 minutes at room temperature, each concentration significantly reduced the number of viable *E. coli* by 58%, 89%, and 98%, respectively. The complete details of this study can be seen in Appendix 1.9.

Fresh and processed fruits

Study Ec18J22JT examined the efficacy of EcoShield PX™ on reducing *E. coli* levels on cantaloupe. Three concentrations of EcoShield PX™ ($1x10^6$, $5x10^6$, and $1x10^7$ PFU/g) were applied. After 15 minutes at room temperature, each concentration significantly reduced the number of viable *E. coli* by 36%, 42%, and 65%, respectively. The complete details of this study can be seen in Appendix 1.3.

Fresh and processed vegetables

Study Ec18J04JT examined the efficacy of EcoShield PX™ on reducing *E. coli* levels on lettuce. Three concentrations of EcoShield Px™ ($1x10^6$, $5x10^6$, and $1x10^7$ PFU/g) were applied. After 15 minutes at room temperature, each concentration reduced the number of viable *E. coli* by ca. 29%, 44%, and 76%, respectively. The complete details of this study can be seen in Appendix 1.1.

Dairy

Study Ec18K15JT examined the efficacy of EcoShield PX™ on reducing *E. coli* levels on cheese slices. Three concentrations of EcoShield PX™ ($1x10^6$, $5x10^6$, and $1x10^7$ PFU/g) were spread on the cheddar cheese slices. After 15 minutes at room temperature, each concentration significantly reduced the number of viable *E. coli* by 64%, 95%, and 97%, respectively. The complete details of this study can be seen in Appendix 1.6.

Summary

We believe the data summarized here fully supports our conclusion that EcoShield PX™ is GRAS and our request for EcoShield PX™ to be included in FSIS directive 7120.1 as a safe and suitable ingredient used in the production of red meat, poultry, fruits, vegetables, dairy, fish, and seafood products as a processing aid. Its intended use is as a spray applied to significantly reduce levels of *E. coli* when applied at ≤$1x10^8$ PFU/g. Additionally, no foods treated to product specifications should require EcoShield PX™ as a listed ingredient on product labels.

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Appendices

Appendix 1.1	**Report Ec18J04JT**
	Lettuce
Appendix 1.2	**Report Ec18J12JT**
	Beef Pieces
Appendix 1.3	**Report Ec18J22JT**
	Cantaloupe
Appendix 1.4	**Report Ec18J29JT**
	Ground Beef
Appendix 1.5	**Report Ec18K01JT**
	Raw Chicken Breast
Appendix 1.6	**Report Ec18K15JT**
	Cheese Slices
Appendix 1.7	**Report Ec18K06JT**
	Beef Pieces, High Titer
Appendix 1.8	**Report Ec18K09JT**
	Cooked Chicken
Appendix 1.9	**Report Ec18K20JT**
	Salmon

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated lettuce

Study # Ec18J04JT

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Table of Contents

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1 STUDY TITLE

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated lettuce.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Jeffrey Tokman, MS	Research Scientist	Hands-on-research / Report assembly
Joelle Woolston, MS	Director of Laboratory Operations	Data review / Report assembly

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
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5 STUDY OBJECTIVE

To determine whether the application of EcoShield PX™ reduces the number of viable *E. coli* O157:H7 on lettuce when applied at the rate of $1x10^6$ – $1x10^7$ PFU/g.

6 Test Matrix

Lettuce was obtained from a local Baltimore grocery store. It was not washed or pre-treated prior to our studies.

7 EcoShield PX™ Lot and Application

- EcoShield PX™ Lot 1817K2830A88
- Titer: approx. $3x10^{10}$ PFU/mL
- EcoShield PX™ was diluted as necessary with water just prior to application
- The application rate was ca. 1.05mL EcoShield PX™ per 25g lettuce (19mL/lb)
- EcoShield PX™ was applied using a spray bottle with pump vaporizer

8 Bacterial Strains Used to Experimentally Contaminate Lettuce

The lettuce test matrix was experimentally contaminated with *Escherichia coli* strain:

- Ec229: A nalidixic acid resistant mutant developed from Intralytix strain Ec133

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25µg/mL. Intralytix strain designation was assigned (i.e., Ec229). The strain was stored at –80°C, at Intralytix, in 70% LB broth/30% glycerol supplemented with 25µg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown (37±2°C, 16-24h) in LB broth supplemented with nalidixic acid (25µg/mL). Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

The lettuce was experimentally contaminated with ca. $3x10^3$ CFU/g of lettuce.

9 Media and Reagents

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- PBS (Phosphate buffered saline, pH 7.4)(Life Technologies, Grand Island, NY; catalog # 10010031)

- MacConkey Agar (BD, Sparks, MD; catalog # 212123)

10 General Outline of Study

1) Eighteen 25g portions of lettuce were divided into six treatments A, B, C, D, E, F.
2) The challenge dose of bacteria was applied onto the lettuce surface of treatments A, B, C, and E.
3) The samples were placed in sterile filter bags and the bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
4) Water (control) or EcoShield PX™ was applied as described in Section 7. Treatments were evenly applied to the lettuce samples' surfaces as follows:
    - Group A = 1.05mL $2.4x10^8$ PFU/mL EcoShield PX/25g = $1x10^7$ PFU/g
    - Group B = 1.05mL $1.2x10^8$ PFU/mL EcoShield PX/25g = $5x10^6$ PFU/g
    - Group C = 1.05mL $2.4x10^7$ PFU/mL EcoShield PX/25g = $1x10^6$ PFU/g
    - Group D = 1.05mL $2.4x10^8$ PFU/mL EcoShield PX/25g = $1x10^7$ PFU/g bacteria added to bag containing sample after treatment (phage control)
    - Group E = 1.05mL water/25g (positive control)
    - Group F = 1.05mL water/25g (negative control)
5) The samples were covered and incubated at room temperature for ca. 15 minutes.
6) At 15 minutes post-treatment with water or EcoShield PX™, 225mL of sterile PBS was added. The bags were stomached for a minimum of 30 seconds at 230rpm.
7) The number of viable *E. coli* in the samples was determined by plating aliquots (0.1mL and 0.5mL) of the stomached lettuce/PBS mixture onto separate MacConkey plates supplemented with nalidixic acid (25µg/mL) in duplicate. The plates were incubated (37±2°C, 24±2hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated lettuce}} = \frac{\textit{CFU}}{\textit{0.1 and 0.5mL plating}} \times \frac{\textit{225mL PBS}}{\textit{25g sample}}$$

Counts were used from both 0.1 and 0.5 mL platings during analysis, unless there was an uncountable (>330) number of colonies on the plates.

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study # Ec18J04JT

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU/g
A ($1x10^7$ PFU/g)	Yes	25	$1x10^7$ PFU/g EcoShield PX	3	796.5, 693, 801
B ($5x10^6$ PFU/g)	Yes	25	$5x10^6$ PFU/g EcoShield PX	3	1557, 2142, 1680
C ($1x10^6$ PFU/g)	Yes	25	$1x10^6$ PFU/g EcoShield PX	3	1984.5, 2065.5, 2767.5
D ($1x10^7$ PFU/g Control)	No (bacteria added later)	25	$1x10^7$ PFU/g EcoShield PX	3	2106, 2610, 4275
E (+ Control)	Yes	25	Water	3	3861, 3000, 2700
F (- Control)	No	25	Water	3	0

11.2 Tabular presentation of results

Table 2 Reduction of *E. coli* counts on lettuce treated with EcoShield PX when applied at ca. $1x10^6$ – $1x10^7$ PFU/g (1.05mL per 25g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^7$ PFU/g)	Yes	$1x10^7$ PFU/g EcoShield PX	*n* =3	763.5	76%	0.62	Yes
B ($5x10^6$ PFU/g)	Yes	$5x10^6$ PFU/g EcoShield PX	*n* = 3	1793	44%	0.25	No
C ($1x10^6$ PFU/g)	Yes	$1x10^6$ PFU/g EcoShield PX	*n* =3	2272.5	29%	0.15	No
D ($1x10^7$ PFU/g Control)	No (bacteria added later)	$1x10^7$ PFU/g EcoShield PX	*n* =3	2997	6%	0.03	No
E (+ Control)	Yes	Water	*n* =3	3187	N/A	N/A	N/A
F (- Control)	No	Water	*n* =3	0	N/A	N/A	N/A

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

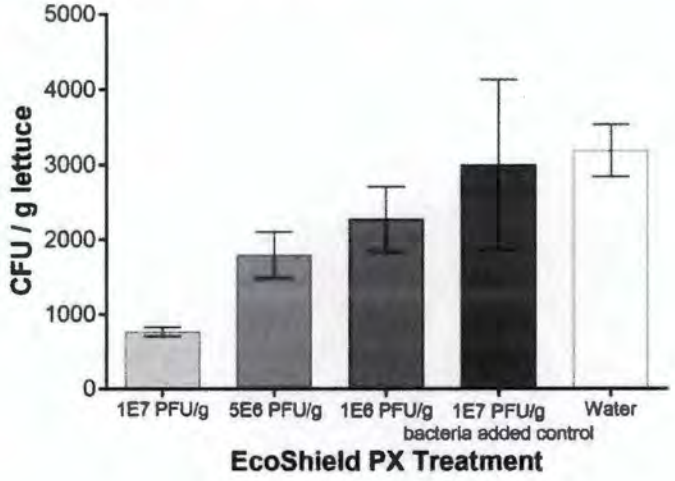
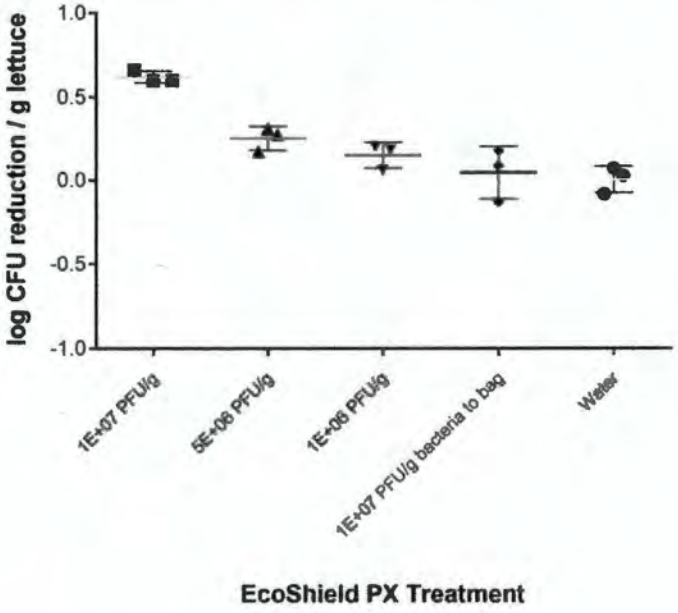


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the EcoShield PX™ treatment in reducing the number of viable *E. coli* in the experimentally contaminated lettuce was evaluated by comparing the data obtained with the water-treated control samples and the EcoShield PX™-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 7.04 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value, <0.01, is considered significant. Variation between the means is significantly greater than expected by chance.

Comparison	Mean Difference	q value	p value
1E+07 PFU/g vs Water	-2424	6.746	** P<0.01
5E+06 PFU/g vs Water	-1394	3.88	ns P>0.05
1E+06 PFU/g vs Water	-914.5	2.546	ns P>0.05
1E+07 PFU/g bacteria added vs Water	-190	0.5289	ns P>0.05
1E+07 PFU/g vs 5E+06 PFU/g	-1030	2.866	ns P>0.05
1E+07 PFU/g vs 1E+06 PFU/g	-1509	4.2	ns P>0.05
1E+07 PFU/g vs 1E+07 PFU/g bacteria added	-2234	6.217	** P<0.01
5E+06 PFU/g vs 1E+06 PFU/g	-479.5	1.335	ns P>0.05
5E+06 PFU/g vs 1E+07 PFU/g bacteria added	-1204	3.351	ns P>0.05
1E+06 PFU/g vs 1E+07 PFU/g bacteria added	-724.5	2.017	ns P>0.05

ns = not significant

11.5 Brief discussion of results and study's conclusions

- Applying EcoShield PX™ at ca. $1x10^7$ PFU/g lettuce reduced the number of viable *E. coli* by ca. 76% after 15 minutes of incubation at RT. The observed reduction was statistically significant ($P<0.01$).

- Applying EcoShield PX™ at ca. $5x10^6$ PFU/g lettuce reduced the number of viable *E. coli* by ca. 44% after 15 minutes of incubation at RT. The observed reduction was not statistically significant ($P>0.05$).

- Applying EcoShield PX™ at ca. $1x10^6$ PFU/g lettuce reduced the number of viable *E. coli* by ca. 29% after 15 minutes of incubation at RT. The observed reduction was not statistically significant ($P>0.05$).

- Reduction in *E. coli* levels achieved by using more concentrated EcoShield PX™ was higher compared to those obtained with more dilute EcoShield PX™ (76% vs. 44% vs. 29% when using ca. $1x10^7$ PFU/g, $5x10^6$ PFU/g, and $1x10^6$ PFU/g, respectively), but the reductions were not statistically different from each other ($P>0.05$).

- The *E. coli* levels observed in the phage control (bacteria added to the bag after phage treatment on the food) were 6% lower than the water control. The difference was not statistically significant.

12 SUMMARY CONCLUSION OF THE STUDY

EcoShield PX™ can reduce viable *E. coli* levels in experimentally contaminated lettuce by ca. 29-76% after a 15 minute contact time, when applied at ca. $1x10^6$ – $1x10^7$ PFU/g.

Using the higher EcoShield PX™ application rate (ca. $1x10^7$ PFU/g) resulted in statistically significantly reduction of *E. coli* levels when compared to water.

There was no significant difference between positive control samples (water) and phage control samples ($1x10^7$ PFU/g bacteria added control), showing the lytic activity of the phages occurred on the food, not during sampling (e.g. in the bag or on the plate).

13 SIGNATURES

[redacted]

Jeffrey Tokman
Research Scientist

[redacted]

Joelle Woolston
Director of Laboratory Operations

[redacted]

Alexander Sulakvelidze, Ph.D.
Study Director

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated beef pieces

Study # Ec18J12JT

Intralytix

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1 STUDY TITLE

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated beef pieces.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Jeffrey Tokman, MS	Research Scientist	Hands-on-research / Report assembly
Joelle Woolston, MS	Director of Laboratory Operations	Data review / Report assembly

4 PERFORMING LABORATORY

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5 STUDY OBJECTIVE

To determine whether the application of EcoShield PX™ reduces the number of viable *E. coli* O157:H7 on beef pieces when applied at the rate of $1x10^6 – 1x10^7$ PFU/g.

6 TEST MATRIX

Chuck roast was obtained from a local Baltimore grocery store. It was not washed or pre-treated prior to our studies.

7 ECOSHIELD PX™ LOT AND APPLICATION

- EcoShield PX™ Lot 1817K2830A88
- Titer: approx. $3x10^{10}$ PFU/mL
- EcoShield PX™ was diluted as necessary with water just prior to application.
- The application rate was ca. 0.21mL EcoShield PX™ per 25g beef (3.8mL/lb).
- EcoShield PX™ was applied using a spray bottle with pump vaporizer

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATED BEEF

The beef test matrix was experimentally contaminated with *Escherichia coli* strain:

- Ec229: A nalidixic acid resistant mutant developed from Intralytix strain Ec133

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25µg/mL. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Ec229). The strain was stored at -80°C, at Intralytix, in 70% LB broth/30% glycerol supplemented with 25 µg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown (37±2°C, 16-24h) in LB broth supplemented with nalidixic acid (25µg/mL). Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

The beef was experimentally contaminated with ca. $1x10^3$ CFU/g of beef.

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- PBS (Phosphate buffered saline, pH 7.4) (Life Technologies, Grand Island, NY; catalog # 10010031)

- CR-Sorbitol MacConkey Agar (OXOID, Basingstoke, UK; catalog # CM1005)

10 General Outline of Study

1) Fifteen 25g portions of beef were divided into five treatments A, B, C, D, E.
2) The challenge dose of bacteria was applied onto the lettuce surface of treatments A, B, C, and D.
3) The samples were placed in sterile filter bags and the bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60min.
4) Water (control) or EcoShield PX™ was applied as described in Section 7. Treatments were evenly applied to the beef samples' surfaces as follows:
    - Group A = 0.21mL $1.2x10^9$ PFU/mL EcoShield PX/25g = $1x10^7$ PFU/g
    - Group B = 0.21mL $6.0x10^8$ PFU/mL EcoShield PX/25g = $5x10^6$ PFU/g
    - Group C = 0.21mL $1.2x10^8$ PFU/mL EcoShield PX/25g = $1x10^6$ PFU/g
    - Group D = 0.21mL water/25g (positive control)
    - Group E = 0.21mL water/25g (negative control)
5) The samples were covered and incubated at room temperature for ca. 15 minutes.
6) At 15 minutes post-treatment with water or EcoShield PX™, 225mL of sterile PBS was added. The bags were stomached for a minimum of 30 seconds at 230rpm.
7) The number of viable *E. coli* in the samples was determined by plating aliquots (0.1mL and 0.5mL) of the stomached beef pieces/PBS mixture onto separate CR-SMAC plates supplemented with nalidixic acid (25µg/mL) in duplicate. The plates were incubated (37±2°C, 24±2hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated beef}} = \frac{\textit{CFU}}{\textit{0.1 and 0.5mL plating}} \times \frac{\textit{225mL PBS}}{\textit{25g sample}}$$

Counts were used from both 0.1 and 0.5 mL platings during analysis, unless there was an uncountable (>330) number of colonies on the plates.

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study # Ec18J12JT

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU/g
A ($1x10^7$ PFU/g)	Yes	25	$1x10^7$ PFU/g EcoShield PX	3	396, 328.5, 382.5
B ($5x10^6$ PFU/g)	Yes	25	$5x10^6$ PFU/g EcoShield PX	3	675, 364.5, 387
C ($1x10^6$ PFU/g)	Yes	25	$1x10^6$ PFU/g EcoShield PX	3	594, 612, 652.5
D (+ Control)	Yes	25	Water	3	1048.5, 1431, 873
E (- Control)	No	25	Water	3	0

11.2 Tabular presentation of results

Table 2 Reduction of *E. coli* counts on beef treated with EcoShield PX when applied at ca. $1x10^6$ – $1x10^7$ PFU/g (0.21mL per 25g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^7$ PFU/g)	Yes	$1x10^7$ PFU/g EcoShield PX	*n* =3	369	67%	0.48	Yes
B ($5x10^6$ PFU/g)	Yes	$5x10^6$ PFU/g EcoShield PX	*n* = 3	475.5	57%	0.37	Yes
C ($1x10^6$ PFU/g)	Yes	$1x10^6$ PFU/g EcoShield PX	*n* =3	619.5	45%	0.26	Yes
D (+ Control)	Yes	Water	*n* =3	1117.5	N/A	N/A	N/A
E (- Control)	No	Water	*n* =3	0	N/A	N/A	N/A

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

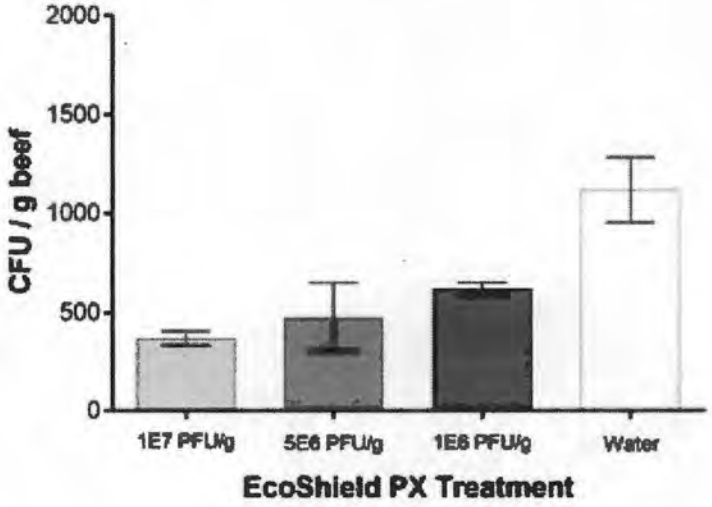
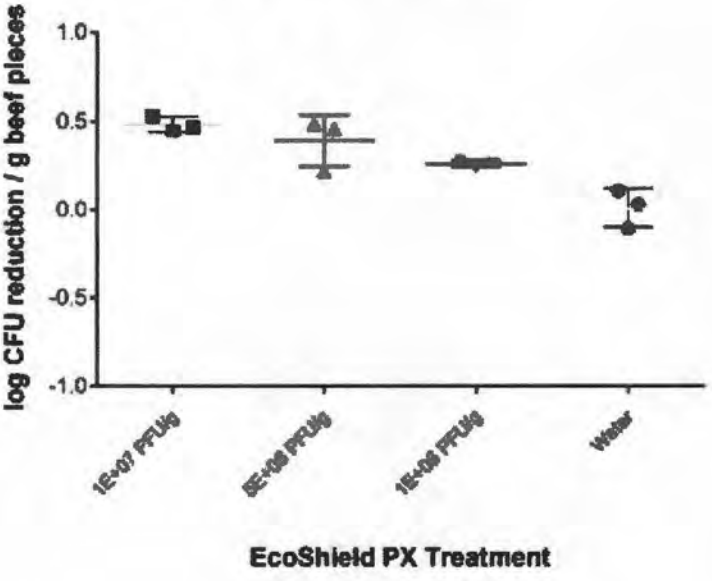


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the EcoShield PX™ treatment in reducing the number of viable *E. coli* in the experimentally contaminated beef was evaluated by comparing the data obtained with the water-treated control samples and the EcoShield PX™-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 7.04 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is <0.01, is considered significant. Variation between the means is significantly greater than expected by chance.

Comparison	Mean Difference	q value	p value
1E+07 PFU/g vs Water	-748.5	7.694	** P<0.01
5E+06 PFU/g vs Water	-642	6.599	** P<0.01
1E+06 PFU/g vs Water	-498	5.119	* P>0.05
1E+07 PFU/g vs 5E+06 PFU/g	-106.5	1.095	ns P>0.05
1E+07 PFU/g vs 1E+06 PFU/g	-250.5	0.3311	ns P>0.05
5E+06 PFU/g vs 1E+06 PFU/g	-144	0.7287	ns P>0.05

ns = not significant

11.5 Brief discussion of results and study's conclusions

- Applying EcoShield PX™ at ca. $1x10^7$ PFU/g beef reduced the number of viable *E. coli* by ca. 67% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.05).
- Applying EcoShield PX™ at ca. $5x10^6$ PFU/g beef reduced the number of viable *E. coli* by ca. 57% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.05).
- Applying EcoShield PX™ at ca. $1x10^6$ PFU/g beef reduced the number of viable *E. coli* by ca. 45% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P>0.05).

- Reduction in *E. coli* levels achieved by using more concentrated EcoShield PX™ was higher compared to those obtained with more dilute EcoShield PX™ (67% vs. 57% vs. 45% when using ca. $1x10^7$ PFU/g, $5x10^6$ PFU/g, and $1x10^6$ PFU/g, respectively), but the reductions were not statistically different from each other ($P>0.05$).

12 SUMMARY CONCLUSION OF THE STUDY

EcoShield PX™ can significantly reduce viable *E. coli* levels in experimentally contaminated beef by ca. 45-67% after a 15 minute contact time, when applied at ca. $1x10^6 – 1x10^7$ PFU/g.

Using the higher EcoShield PX™ application rates (ca. $1x10^7$ PFU/g or $5x10^6$ PFU/g) resulted in better reduction of *E. coli* levels compared to lower EcoShield PX™ application rate (ca. $1x10^6$ PFU/g).

13 SIGNATURES

[redacted]

Jeffrey Tokman
Research Scientist

[redacted]

Joelle Woolston
Director of Laboratory Operations

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Alexander Sulakvelidze, Ph.D.
Study Director

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated cantaloupe

Study # Ec18J22JT

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1 STUDY TITLE

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated cantaloupe.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Jeffrey Tokman, MS	Research Scientist	Hands-on-research / Report assembly
Joelle Woolston, MS	Director of Laboratory Operations	Data review / Report assembly

4 PERFORMING LABORATORY

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5 STUDY OBJECTIVE

To determine whether the application of EcoShield PX™ reduces the number of viable *E. coli* O157:H7 on cantaloupe pieces when applied at the rate of $1x10^6$ – $1x10^7$ PFU/g.

6 Test Matrix

Whole cantaloupe was obtained from a local Baltimore grocery store. It was not washed or pre-treated prior to our studies. It was cut by hand into 25g wedges.

7 EcoShield PX™ Lot and Application

- EcoShield PX™ Lot 1817K2830A88
- Titer: approx. $3x10^{10}$ PFU/mL
- EcoShield PX™ was diluted as necessary with water just prior to application.
- The application rate was ca. 0.42mL EcoShield PX™ per 25g cantaloupe (7.6mL/lb).
- EcoShield PX™ was applied using a spray bottle with pump vaporizer

8 Bacterial Strains Used to Experimentally Contaminate Cantaloupe

The cantaloupe test matrix was experimentally contaminated with *Escherichia coli* strain:

- Ec229: A nalidixic acid resistant mutant developed from Intralytix strain Ec133

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25µg/mL. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Ec229). The strain was stored at –80°C, at Intralytix, in 70% LB broth/30% glycerol supplemented with 25µg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown (37±2°C, 16-24h) in LB broth supplemented with nalidixic acid (25µg/mL). Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

The cantaloupe was experimentally contaminated with ca. $4x10^3$ CFU/g of cantaloupe.

9 Media and Reagents

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)

- PBS (Phosphate buffered saline, pH 7.4) (Life Technologies, Grand Island, NY; catalog # 10010031)
- MacConkey Agar (BD, Sparks, MD; catalog # 212123)

10 General Outline of Study

1) Fifteen 25g portions of cantaloupe were divided into five treatments A, B, C, D, E.
2) The challenge dose of bacteria was applied onto the cantaloupe surface of treatments A, B, C, and D.
3) The samples were placed in sterile filter bags and the bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
4) Water (control) or EcoShield PX™ was applied as described in Section 7. Treatments were evenly applied to the canteloupe samples' surfaces as follows:
    - Group A = 0.42mL $6.0x10^8$ PFU/mL EcoShield PX/25g = $1x10^7$ PFU/g
    - Group B = 0.42mL $3.0x10^8$ PFU/mL EcoShield PX/25g = $5x10^6$ PFU/g
    - Group C = 0.42mL $6.0x10^7$ PFU/mL EcoShield PX/25g = $1x10^6$ PFU/g
    - Group D = 0.42mL water/25g (positive control)
    - Group E = 0.42mL water/25g (negative control)
5) The samples were covered and incubated at room temperature for ca. 15 minutes.
6) At 15 minutes post-treatment with water or EcoShield PX™, 225mL of sterile PBS was added. The bags were stomached for a minimum of 30 seconds at 230rpm.
7) The number of viable *E. coli* in the samples was determined by plating aliquots (0.1mL and 0.5mL) of the stomached cantaloupe/PBS mixture onto separate MacConkey plates supplemented with nalidixic acid (25µg/mL) in duplicate. The plates were incubated (37±2°C, 24±2hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated cantaloupe}} = \frac{\textit{CFU}}{\textit{0.1 and 0.5mL plating}} \times \frac{\textit{225mL PBS}}{\textit{25g sample}}$$

Counts were used from both 0.1 and 0.5 mL platings during analysis, unless there was an uncountable (>330) number of colonies on the plates.

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study # Ec18J22JT

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU/g
A ($1x10^7$ PFU/g)	Yes	25	$1x10^7$ PFU/g EcoShield PX	3	1458, 855, 1770
B ($5x10^6$ PFU/g)	Yes	25	$5x10^6$ PFU/g EcoShield PX	3	1876.5, 2272.5, 2668.5
C ($1x10^6$ PFU/g)	Yes	25	$1x10^6$ PFU/g EcoShield PX	3	1917, 2961, 2664
D (+ Control)	Yes	25	Water	3	3577.5, 4099.5, 4032
E (- Control)	No	25	Water	3	0

11.2 Tabular presentation of results

Table 2 Reduction of *E. coli* counts on cantaloupe treated with EcoShield PX when applied at ca. $1x10^6$ – $1x10^7$ PFU/g (0.42mL per 25g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^7$ PFU/g)	Yes	$1x10^7$ PFU/g EcoShield PX	*n* =3	1361	65%	0.46	Yes
B ($5x10^6$ PFU/g)	Yes	$5x10^6$ PFU/g EcoShield PX	*n* = 3	2272.5	42%	0.23	Yes
C ($1x10^6$ PFU/g)	Yes	$1x10^6$ PFU/g EcoShield PX	*n* =3	2514	36%	0.19	Yes
D (+ Control)	Yes	Water	*n* =3	3903	N/A	N/A	N/A
E (- Control)	No	Water	*n* =3	0	N/A	N/A	N/A

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

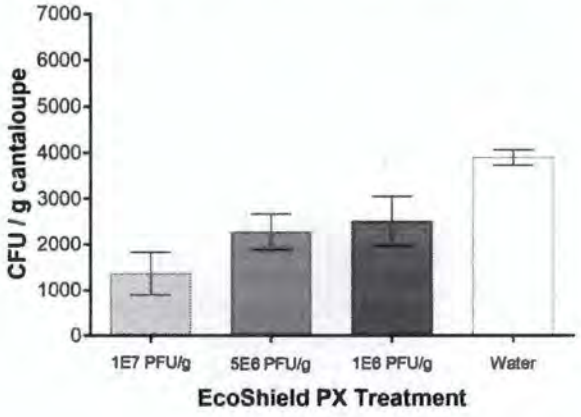
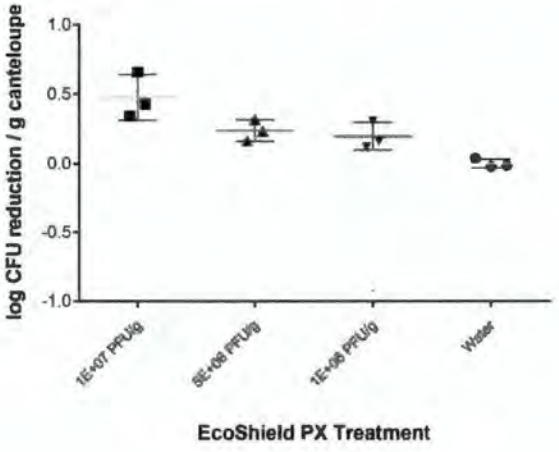


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the EcoShield PX™ treatment in reducing the number of viable *E. coli* in the experimentally contaminated cantaloupe was evaluated by comparing the data obtained with the water-treated control samples and the EcoShield PX™-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 7.04 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is <0.001, is considered significant. Variation between the means is significantly greater than expected by chance.

Comparison	Mean Difference	q value	p value
1E+07 PFU/g vs Water	-2542	10.21	*** P<0.001
5E+06 PFU/g vs Water	-1631	6.552	** P<0.01
1E+06 PFU/g vs Water	-1389	5.582	* P<0.05
1E+07 PFU/g vs 5E+06 PFU/g	-911.5	3.663	ns P>0.05
1E+07 PFU/g vs 1E+06 PFU/g	-1153	4.633	* P<0.05
5E+06 PFU/g vs 1E+06 PFU/g	-241.5	0.9704	ns P>0.05

ns = not significant

11.5 Brief discussion of results and study's conclusions

- Applying EcoShield PX™ at ca. $1x10^7$ PFU/g cantaloupe reduced the number of viable E. coli by ca. 65% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).
- Applying EcoShield PX™ at ca. $5x10^6$ PFU/g cantaloupe reduced the number of viable E. coli by ca. 42% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.01).
- Applying EcoShield PX™ at ca. $1x10^6$ PFU/g cantaloupe reduced the number of viable E. coli by ca. 36% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.05).
- Reduction in E. coli levels achieved by using more concentrated EcoShield PX™ was higher compared to those obtained with more dilute EcoShield PX™ (65% vs. 42% vs. 36% when using ca. $1x10^7$ PFU/g, $5x10^6$ PFU/g, and $1x10^6$ PFU/g, respectively).
- The difference in E. coli recovered when EcoShield PX™ was applied in the two most concentrated forms (application rates $1x10^7$ PFU/g vs. $5x10^6$ PFU/g) was not statistically significant (P>0.05).

- The differences in E. coli recovered when EcoShield PX™ was applied in the most concentrated form vs the least concentrated (application rates $1x10^7$ PFU/g vs. $1x10^6$ PFU/g) was statistically significant ($P<0.05$).

12 SUMMARY CONCLUSION OF THE STUDY

EcoShield PX™ can significantly reduce viable *E. coli* levels in experimentally contaminated canteloupe by ca. 36-65% after a 15 minute contact time, when applied at ca. $1x10^6$ – $1x10^7$ PFU/g.

Using the higher EcoShield PX™ application rates (ca. $1x10^7$ PFU/g or $5x10^6$ PFU/g) resulted in better reduction of *E. coli* levels compared to lower EcoShield PX™ application rate (ca. $1x10^6$ PFU/g).

13 SIGNATURES

[redacted]

Jeffrey Tokman
Research Scientist

[redacted]

Joelle Woolston
Director of Laboratory Operations

[redacted]

Alexander Sulakvelidze, Ph.D.
Study Director

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated ground beef

Study # Ec18J29JT

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1 STUDY TITLE

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated ground beef.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Jeffrey Tokman, MS	Research Scientist	Hands-on-research / Report assembly
Joelle Woolston, MS	Director of Laboratory Operations	Data review / Report assembly

4 PERFORMING LABORATORY

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5 STUDY OBJECTIVE

To determine whether the application of EcoShield PX™ reduces the number of viable *E. coli* O157:H7 on ground beef when applied at the rate of $1x10^6$ – $1x10^7$ PFU/g.

6 Test Matrix

Ground beef was obtained from a local Baltimore grocery store. It was not pre-treated prior to our studies.

7 EcoShield PX™ Lot and Application

- EcoShield PX™ Lot 1817K2830A88
- Titer: approx. $3x10^{10}$ PFU/mL
- EcoShield PX™ was diluted as necessary with water just prior to application.
- The application rate was ca. 0.21mL EcoShield PX™ per 25g ground beef (3.8mL/lb).
- EcoShield PX™ was applied using a spray bottle with pump vaporizer

8 Bacterial Strains Used to Experimentally Contaminate Ground Beef

The ground beef test matrix was experimentally contaminated with *Escherichia coli* strain:

- Ec229: A nalidixic acid resistant mutant developed from Intralytix strain Ec133

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25µg/mL. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Ec229). The strain was stored at –80°C, at Intralytix, in 70% LB broth/30% glycerol supplemented with 25µg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown (37±2°C, 16-24h) in LB broth supplemented with nalidixic acid (25µg/mL). Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

The ground beef was experimentally contaminated with ca. $4x10^3$ CFU/g of beef.

9 Media and Reagents

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)

- PBS (Phosphate buffered saline, pH 7.4) (Life Technologies, Grand Island, NY; catalog # 10010031)
- CR-Sorbitol MacConkey Agar (OXOID, Basingstoke, UK; catalog # CM1005)

10 General Outline of Study

1) Fifteen 25g portions of ground beef were divided into five treatments A, B, C, D, E.
2) The challenge dose of bacteria was applied onto ground beef and mixed throughout the beef of treatments A, B, C, and D.
3) The samples were placed in sterile petri dishes and spread using sterile cell spreaders over the surface of the petri dish, in order to have a larger surface area to treat, and the bacteria were allowed to colonize the matrix samples' surfaces at approximately 4°C for 60 min.
4) Water (control) or EcoShield PX™ was applied as described in Section 7. Treatments were evenly applied to the beef samples' spread over the petri dish as follows:
    - Group A = 0.21mL $1.2x10^9$ PFU/mL EcoShield PX/25g = $1x10^7$ PFU/g
    - Group B = 0.21mL $6.0x10^8$ PFU/mL EcoShield PX/25g = $5x10^6$ PFU/g
    - Group C = 0.21mL $1.2x10^8$ PFU/mL EcoShield PX/25g = $1x10^6$ PFU/g
    - Group D = 0.21mL water/25g (positive control)
    - Group E = 0.21mL water/25g (negative control)
5) The samples were placed in sterile filter bags and incubated at room temperature for ca. 15 minutes.
6) At 15 minutes post-treatment with water or EcoShield PX™, 225mL of sterile PBS was added. The bags were stomached for a minimum of 30 seconds at 230rpm.
7) The number of viable *E. coli* in the samples was determined by plating aliquots (0.1mL and 0.5mL) of the stomached ground beef/PBS mixture onto separate CR-SMAC plates supplemented with nalidixic acid (25µg/mL) in duplicate. The plates were incubated (37±2°C, 24±2hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated beef}} = \frac{\textit{CFU}}{\textit{0.1 and 0.5mL plating}} \times \frac{\textit{225mL PBS}}{\textit{25g sample}}$$

Counts were used from both 0.1 and 0.5 mL platings during analysis, unless there was an uncountable (>330) number of colonies on the plates.

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #Ec18J29JT

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU/g
A ($1x10^7$ PFU/g)	Yes	25	$1x10^7$ PFU/g EcoShield PX	3	2070, 1444.5, 1902
B ($5x10^6$ PFU/g)	Yes	25	$5x10^6$ PFU/g EcoShield PX	3	3015, 2686.5, 2148
C ($1x10^6$ PFU/g)	Yes	25	$1x10^6$ PFU/g EcoShield PX	3	3240, 3001.5, 2745
D (+ Control)	Yes	25	Water	3	3780, 3195, 3735
E (- Control)	No	25	Water	3	0

11.2 Tabular presentation of results

Table 2 Reduction of *E. coli* counts on ground beef treated with EcoShield PX when applied at ca. $1x10^6$ – $1x10^7$ PFU/g (0.21mL per 25g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^7$ PFU/g)	Yes	$1x10^7$ PFU/g EcoShield PX	*n* =3	1805.5	49%	0.30	Yes
B ($5x10^6$ PFU/g)	Yes	$5x10^6$ PFU/g EcoShield PX	*n* = 3	2616.5	27%	0.13	Yes
C ($1x10^6$ PFU/g)	Yes	$1x10^6$ PFU/g EcoShield PX	*n* =3	2995.5	16%	0.08	No
D (+ Control)	Yes	Water	*n* =3	3570	N/A	N/A	N/A
E (- Control)	No	Water	*n* =3	0	N/A	N/A	N/A

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

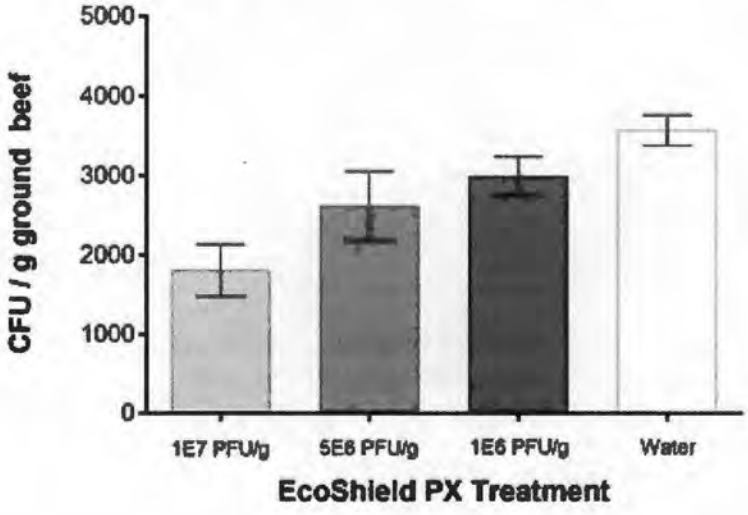
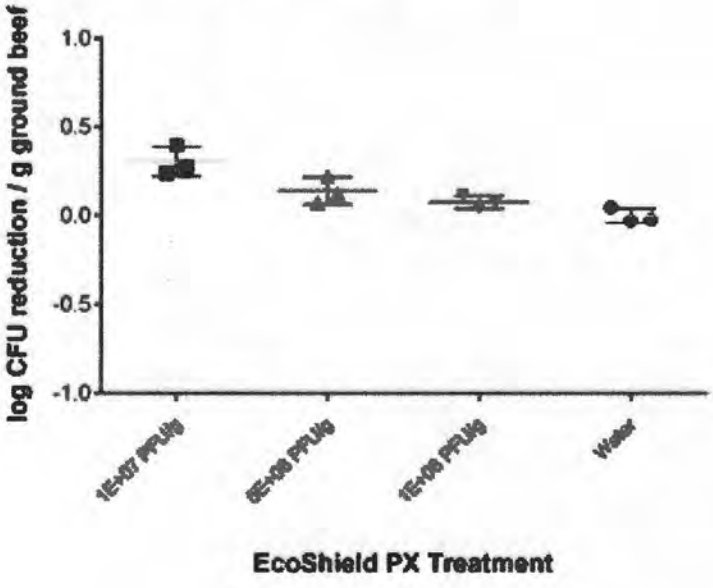


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the EcoShield PX™ treatment in reducing the number of viable *E. coli* in the experimentally contaminated beef was evaluated by comparing the data obtained with the water-treated control samples and the EcoShield PX-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 7.04 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is <0.01, is considered extremely significant. Variation between the means is significantly greater than expected by chance.

Comparison	Mean Difference	q value	p value
1E+07 PFU/g vs Water	-1765	8.977	** P<0.01
5E+06 PFU/g vs Water	-953.5	4.851	* P<0.05
1E+06 PFU/g vs Water	-574.5	2.923	ns P>0.05
1E+07 PFU/g vs 5E+06 PFU/g	-811	4.126	ns P>0.05
1E+07 PFU/g vs 1E+06 PFU/g	-1190	6.054	* P<0.05
5E+06 PFU/g vs 1E+06 PFU/g	-379	1.928	ns P>0.05

ns = not significant

11.5 Brief discussion of results and study's conclusions

- Applying EcoShield PX™ at ca. $1x10^7$ PFU/g ground beef reduced the number of viable *E. coli* by ca. 49% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.01).
- Applying EcoShield PX™ at ca. $5x10^6$ PFU/g beef reduced the number of viable *E. coli* by ca. 27% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.05).
- Applying EcoShield PX™ at ca. $1x10^6$ PFU/g beef reduced the number of viable *E. coli* by ca. 16% after 15 minutes of incubation at RT. The observed reduction was not statistically significant (P>0.05).

- Reduction in *E. coli* levels achieved by using more concentrated EcoShield PX™ was higher compared to those obtained with more dilute EcoShield PX™ (49% vs. 27% vs. 16% when using ca. $1x10^7$ PFU/g, $5x10^6$ PFU/g, and $1x10^6$ PFU/g, respectively).
- The difference in *E. coli* recovered when EcoShield PX™ was applied in the two most concentrated forms (application rates $1x10^7$ PFU/g vs. $5x10^6$ PFU/g) was not statistically significant ($P>0.05$).
- The differences in *E. coli* recovered when EcoShield PX™ was applied in the most concentrated forms vs the least concentrated (application rates $1x10^7$ PFU/g vs. $1x10^6$ PFU/g) was statistically significant ($P<0.05$).

12 SUMMARY CONCLUSION OF THE STUDY

EcoShield PX™ can significantly reduce viable *E. coli* levels in experimentally contaminated beef by ca. 27-49% after a 15 minute contact time, when applied at ca. $5x10^6 – 1x10^7$ PFU/g.

Using the higher EcoShield PX™ application rate (ca. $1x10^7$ PFU/g) resulted in statistically significantly better reduction of *E. coli* levels compared to lower EcoShield PX™ application rate (ca. $1x10^6$ PFU/g).

13 SIGNATURES

[redacted]

Jeffrey Tokman
Research Scientist

[redacted]

Joelle Woolston
Director of Laboratory Operations

[redacted]

Alexander Sulakvelidze, Ph.D.
Study Director

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated raw chicken breast

Study # Ec18K01JT

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1 STUDY TITLE

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated chicken.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Jeffrey Tokman, MS	Research Scientist	Hands-on-research / Report assembly
Joelle Woolston, MS	Director of Laboratory Operations	Data review / Report assembly

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of EcoShield PX™ reduces the number of viable *E. coli* O157:H7 on raw chicken breast when applied at the rate of $1x10^6$ – $1x10^7$ PFU/g.

6 TEST MATRIX

Chicken breasts were obtained from a local Baltimore grocery store. They were not washed or pre-treated prior to our studies.

7 ECOSHIELD PX™ LOT AND APPLICATION

- EcoShield PX™ Lot 1817K2830A88
- Titer: approx. $3x10^{10}$ PFU/mL
- EcoShield PX™ was diluted as necessary with water just prior to application.
- The application rate was ca. 0.21mL EcoShield PX™ per 25g chicken (3.8mL/lb).
- EcoShield PX™ was applied using a spray bottle with pump vaporizer

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATED CHICKEN

The chicken test matrix was experimentally contaminated with *Escherichia coli* strain:

- Ec229: A nalidixic acid resistant mutant developed from Intralytix strain Ec133

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25µg/mL. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Ec229). The strain was stored at –80°C, at Intralytix, in 70% LB broth/30% glycerol supplemented with 25µg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown (37±2°C, 16-24 h) in LB broth supplemented with nalidixic acid (25µg/mL). Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

The chicken was experimentally contaminated with ca. $3x10^3$ CFU/g of chicken.

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- PBS (Phosphate buffered saline, pH 7.4) (Life Technologies, Grand Island, NY; catalog # 10010031)

- MacConkey Agar (BD, Sparks, MD; catalog # 212123)

10 General Outline of Study

1) Fifteen 25g portions of chicken breast were divided into five treatments A, B, C, D, E.
2) The challenge dose of bacteria was applied onto the chicken surface of treatments A, B, C, and D.
3) The samples were placed in sterile filter bags and the bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60min.
4) Water (control) or EcoShield PX™ was applied as described in Section 7. Treatments were evenly applied to the chicken samples' surfaces as follows:
    - Group A = 0.21mL $1.2x10^9$ PFU/mL EcoShield PX/25g = $1x10^7$ PFU/g
    - Group B = 0.21mL $6.0x10^8$ PFU/mL EcoShield PX/25g = $5x10^6$ PFU/g
    - Group C = 0.21mL $1.2x10^8$ PFU/mL EcoShield PX/25g = $1x10^6$ PFU/g
    - Group D = 0.21mL water/25g (positive control)
    - Group E = 0.21mL water/25g (negative control)
5) The samples were covered and incubated at room temperature for ca. 15 minutes.
6) At 15 minutes post-treatment with water or EcoShield PX™, 225mL of sterile PBS was added. The bags were stomached for a minimum of 30 seconds at 230rpm.
7) The number of viable *E. coli* in the samples was determined by plating aliquots (0.1mL and 0.5mL) of the stomached chicken/PBS mixture onto separate MacConkey plates supplemented with nalidixic acid (25µg/mL) in duplicate. The plates were incubated (37±2°C, 24±2hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated chicken}} = \frac{\textit{CFU}}{\textit{0.1 and 0.5mL plating}} \times \frac{\textit{225mL PBS}}{\textit{25g sample}}$$

Counts were used from both 0.1 and 0.5 mL platings during analysis, unless there was an uncountable (>330) number of colonies on the plates

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study # Ec18K01JT

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU/g
A ($1x10^7$ PFU/g)	Yes	25	$1x10^7$ PFU/g EcoShield PX	3	540, 328.5, 657
B ($5x10^6$ PFU/g)	Yes	25	$5x10^6$ PFU/g EcoShield PX	3	405, 630, 1291.5
C ($1x10^6$ PFU/g)	Yes	25	$1x10^6$ PFU/g EcoShield PX	3	1669.5, 1377, 1950
D (+ Control)	Yes	25	Water	3	2205, 2385, 2970
E (- Control)	No	25	Water	3	0

11.2 Tabular presentation of results

Table 2 Reduction of *E. coli* counts on chicken treated with EcoShield PX when applied at ca. $1x10^6$ – $1x10^7$ PFU/g (0.21mL per 25g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^7$ PFU/g)	Yes	$1x10^7$ PFU/g EcoShield PX	*n* =3	508.5	80%	0.70	Yes
B ($5x10^6$ PFU/g)	Yes	$5x10^6$ PFU/g EcoShield PX	*n* = 3	775.5	69%	0.51	Yes
C ($1x10^6$ PFU/g)	Yes	$1x10^6$ PFU/g EcoShield PX	*n* =3	1665.5	34%	0.18	No
D (+ Control)	Yes	Water	*n* =3	2520	N/A	N/A	N/A
E (- Control)	No	Water	*n* =3	0	N/A	N/A	N/A

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

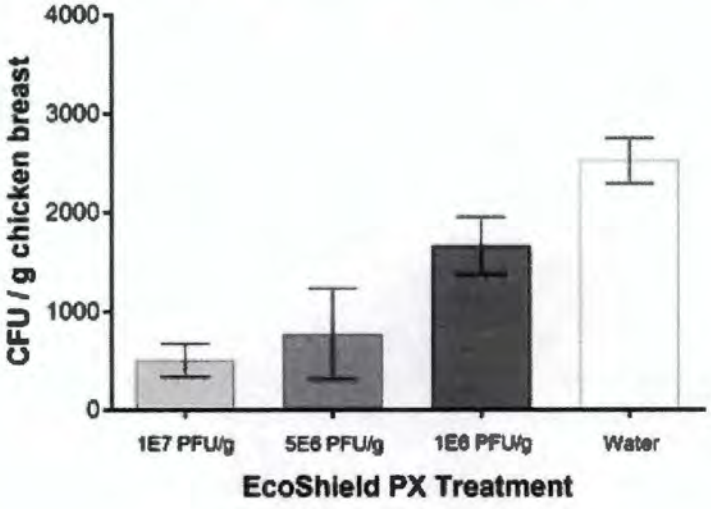
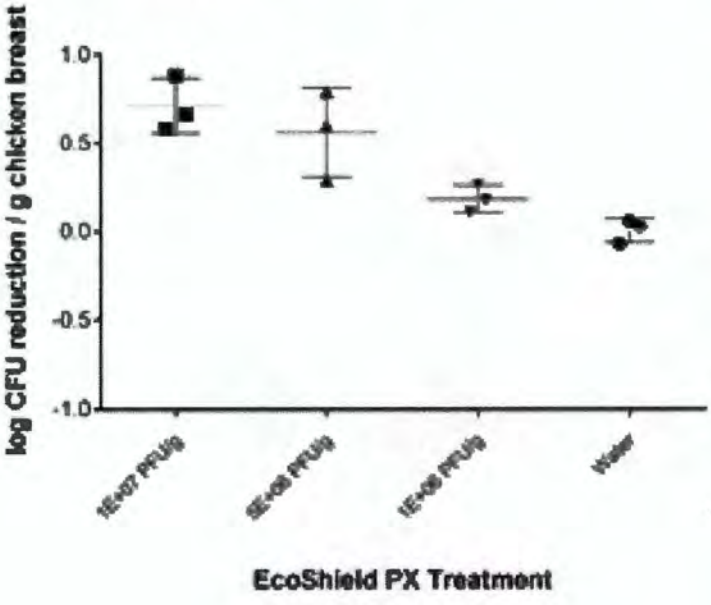


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the EcoShield PX™ treatment in reducing the number of viable *E. coli* in the experimentally contaminated chicken was evaluated by comparing the data obtained with the water-treated control samples and the EcoShield PX-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 7.04 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is <0.001, is considered significant. Variation between the means is significantly greater than expected by chance.

Comparison	Mean Difference	q value	p value
1E+07 PFU/g vs Water	-2012	10.04	*** P<0.001
5E+06 PFU/g vs Water	-1745	8.703	** P<0.01
1E+06 PFU/g vs Water	-854.5	4.263	ns P>0.05
1E+07 PFU/g vs 5E+06 PFU/g	-267	1.332	ns P>0.05
1E+07 PFU/g vs 1E+06 PFU/g	-1157	5.772	* P<0.05
5E+06 PFU/g vs 1E+06 PFU/g	-890	4.44	ns P>0.05

ns = not significant

11.5 Brief discussion of results and study's conclusions

- Applying EcoShield PX™ at ca. $1x10^7$ PFU/g chicken reduced the number of viable *E. coli* by ca. 80% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).

- Applying EcoShield PX™ at ca. $5x10^6$ PFU/g chicken reduced the number of viable *E. coli* by ca. 69% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.01).

- Applying EcoShield PX™ at ca. $1x10^6$ PFU/g chicken reduced the number of viable *E. coli* by ca. 34% after 15 minutes of incubation at RT. The observed reduction was not statistically significant ($P>0.05$).

- Reduction in *E. coli* levels achieved by using more concentrated EcoShield PX™ was higher compared to those obtained with more dilute EcoShield PX™ (80% vs. 69% vs. 34% when using ca. $1x10^7$ PFU/g, $5x10^6$ PFU/g, and $1x10^6$ PFU/g, respectively).

- The difference in *E. coli* recovered when EcoShield PX™ was applied in the two most concentrated forms (application rates $1x10^7$ PFU/g vs. $5x10^6$ PFU/g) was not statistically significant ($P>0.05$).

- The differences in *E. coli* recovered when EcoShield PX™ was applied in the most concentrated form vs the least concentrated (application rates $1x10^7$ PFU/g vs. $1x10^6$ PFU/g) was statistically significant ($P<0.05$).

12 Summary Conclusion of the Study

EcoShield PX™ can significantly reduce viable *E. coli* levels in experimentally contaminated chicken by ca. 69-80% after a 15 minute contact time, when applied at ca. $5x10^6$ – $1x10^7$ PFU/g.

Using the higher EcoShield PX™ application rate (ca. $1x10^7$ PFU/g) resulted in statistically significantly better reduction of *E. coli* levels compared to lower EcoShield PX™ application rate (ca. $1x10^6$ PFU/g).

13 SIGNATURES

[redacted]

Jeffrey Yokman
Research Scientist

[redacted]

Joelle Woolston
Director of Laboratory Operations

[redacted]

Alexander Sulakvelidze, Ph.D.
Study Director

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated cheese slices

Study # Ec18K15JT

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1 STUDY TITLE

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated cheese slices.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Jeffrey Tokman, MS	Research Scientist	Hands-on-research / Report assembly
Joelle Woolston, MS	Director of Laboratory Operations	Data review / Report assembly

4 PERFORMING LABORATORY

Intralytix, Inc.
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5 STUDY OBJECTIVE

To determine whether the application of EcoShield PX™ reduces the number of viable *E. coli* O157:H7 on cheese when applied at the rate of $1x10^6$ – $1x10^7$ PFU/g.

6 Test Matrix

Cheddar cheese slices were obtained from a local Baltimore grocery store. They were not washed or pre-treated prior to our studies.

7 EcoShield PX™ Lot and Application

- EcoShield PX™ Lot 1817K2830A88
- Titer: approx. $3x10^{10}$ PFU/mL
- EcoShield PX™ was diluted as necessary with water just prior to application.
- The application rate was ca. 0.21mL EcoShield PX™ per 25g cheese (3.8mL/lb).
- EcoShield PX™ was applied using a spray bottle with pump vaporizer

8 Bacterial Strains Used to Experimentally Contaminate Cheese

The cheese test matrix was experimentally contaminated with *Escherichia coli* strain:

- Ec229: A nalidixic acid resistant mutant developed from Intralytix strain Ec133

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25µg/mL. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Ec229). The strain was stored at –80°C, at Intralytix, in 70% LB broth/30% glycerol supplemented with 25µg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown (37±2°C, 16-24h) in LB broth supplemented with nalidixic acid (25µg/mL). Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

The cheese was experimentally contaminated with ca. $4x10^3$ CFU/g of cheese.

9 Media and Reagents

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- PBS (Phosphate buffered saline, pH 7.4) (Life Technologies, Grand Island, NY; catalog # 10010031)

- MacConkey Agar (BD, Sparks, MD; catalog # 212123)

10 General Outline of Study

1) Fifteen 25g portions of cheese were divided into five treatments A, B, C, D, E.
2) The challenge dose of bacteria was applied onto the cheese surface of treatments A, B, C, and D.
3) The samples were placed in sterile filter bags and the bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
4) Water (control) or EcoShield PX™ was applied as described in Section 7. Treatments were evenly applied to the cheese samples' surfaces as follows:
    - Group A = 0.21mL $1.2x10^9$ PFU/mL EcoShield PX/25g = $1x10^7$ PFU/g
    - Group B = 0.21mL $6.0x10^8$ PFU/mL EcoShield PX/25g = $5x10^6$ PFU/g
    - Group C = 0.21mL $1.2x10^8$ PFU/mL EcoShield PX/25g = $1x10^6$ PFU/g
    - Group D = 0.21mL water/25g (positive control)
    - Group E = 0.21mL water/25g (negative control)
5) The samples were covered and incubated at room temperature for ca. 15 minutes.
6) At 15 minutes post-treatment with water or EcoShield PX™, 225mL of sterile PBS was added. The bags were stomached for a minimum of 30 seconds at 230rpm.
7) The number of viable *E. coli* in the samples was determined by plating aliquots (0.1mL and 0.5mL) of the stomached cheese/PBS mixture onto separate MacConkey plates supplemented with nalidixic acid (25µg/mL) in duplicate. The plates were incubated (37±2°C, 24±2hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated cheese}} = \frac{\textit{CFU}}{\textit{0.1 and 0.5mL plating}} \times \frac{\textit{225mL PBS}}{\textit{25g sample}}$$

Counts were used from both 0.1 and 0.5 mL platings during analysis, unless there was an uncountable (>330) number of colonies on the plates.

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study # Ec18K15JT

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU/g
A ($1x10^7$ PFU/g)	Yes	25	$1x10^7$ PFU/g EcoShield PX	3	76.5, 121.5, 130.5
B ($5x10^6$ PFU/g)	Yes	25	$5x10^6$ PFU/g EcoShield PX	3	184.5, 180, 157.5
C ($1x10^6$ PFU/g)	Yes	25	$1x10^6$ PFU/g EcoShield PX	3	1219.5, 1296, 1399.5
D (+ Control)	Yes	25	Water	3	3672, 3321, 3892.5
E (- Control)	No	25	Water	3	0

11.2 Tabular presentation of results

Table 2 Reduction of *E. coli* counts on cheese slices treated with EcoShield PX when applied at ca. $1x10^6$ – $1x10^7$ PFU/g (0.21mL per 25g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^7$ PFU/g)	Yes	$1x10^7$ PFU/g EcoShield PX	*n* =3	109.5	97%	1.52	Yes
B ($5x10^6$ PFU/g)	Yes	$5x10^6$ PFU/g EcoShield PX	*n* = 3	174	95%	1.32	Yes
C ($1x10^6$ PFU/g)	Yes	$1x10^6$ PFU/g EcoShield PX	*n* =3	1305	64%	0.44	Yes
E (+ Control)	Yes	Water	*n* =3	3626.5	N/A	N/A	N/A
F (- Control)	No	Water	*n* =3	0	N/A	N/A	N/A

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

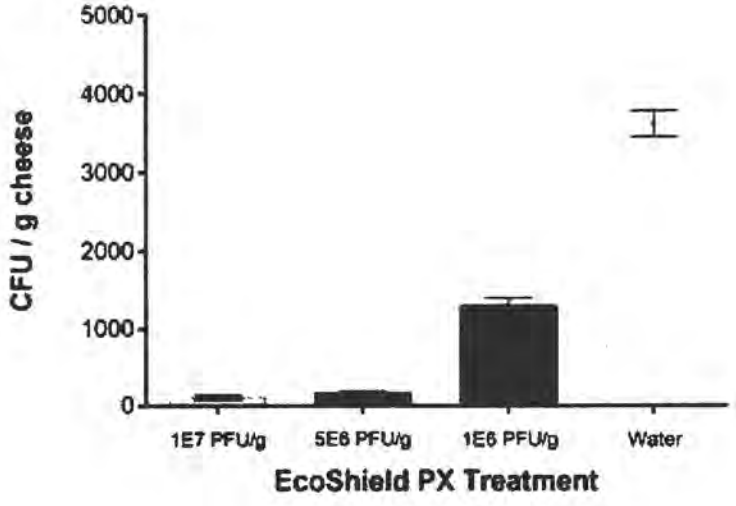
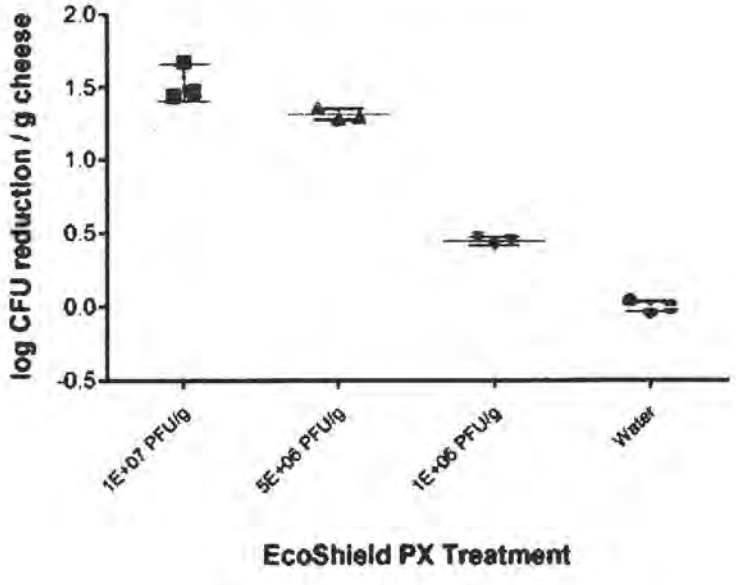


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the EcoShield PX™ treatment in reducing the number of viable *E. coli* in the experimentally contaminated cheese was evaluated by comparing the data obtained with the water-treated control samples and the EcoShield PX-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 7.04 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is <0.0001, is considered extremely significant. Variation between the means is significantly greater than expected by chance.

Comparison	Mean Difference	q value	p value
1E+07 PFU/g vs Water	-3519	40.13	**** P<0.0001
5E+06 PFU/g vs Water	-3455	39.39	**** P<0.0001
1E+06 PFU/g vs Water	-2324	26.50	**** P<0.0001
1E+07 PFU/g vs 5E+06 PFU/g	-64.5	0.7355	ns P>0.05
1E+07 PFU/g vs 1E+06 PFU/g	-1196	13.63	**** P<0.0001
5E+06 PFU/g vs 1E+06 PFU/g	-1131	12.90	**** P<0.0001

ns = not significant

11.5 Brief discussion of results and study's conclusions

- Applying EcoShield PX™ at ca. $1x10^7$ PFU/g cheese reduced the number of viable *E. coli* by ca. 97% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.0001).
- Applying EcoShield PX™ at ca. $5x10^6$ PFU/g cheese reduced the number of viable *E. coli* by ca. 95% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.0001).
- Applying EcoShield PX™ at ca. $1x10^6$ PFU/g cheese reduced the number of viable *E. coli* by ca. 64% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P>0.0001).

- Reduction in *E. coli* levels achieved by using more concentrated EcoShield PX™ was higher compared to those obtained with more dilute EcoShield PX™ (97% vs. 95% vs. 64% when using ca. $1x10^7$ PFU/g, $5x10^6$ PFU/g, and $1x10^6$ PFU/g, respectively).

- The difference in *E. coli* recovered when EcoShield PX™ was applied in the two most concentrated forms (application rates $1x10^7$ PFU/g vs. $5x10^6$ PFU/g) was not statistically significant ($P>0.05$).

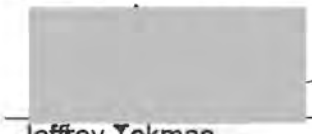
- The differences in *E. coli* recovered when EcoShield PX™ was applied in the two most concentrated forms vs the least concentrated (application rates $1x10^7$ PFU/g vs. $1x10^6$ PFU/g OR $5x10^6$ PFU/g vs. $1x10^6$ PFU/g) were statistically significant ($P<0.0001$).

12 SUMMARY CONCLUSION OF THE STUDY

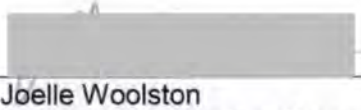
EcoShield PX™ can significantly reduce viable *E. coli* levels on experimentally contaminated cheese by ca. 64-97% after a 15 minute contact time, when applied at ca. $1x10^6$ – $1x10^7$ PFU/g.

Using the higher EcoShield PX™ application rates (ca. $1x10^7$ PFU/g or $1x10^6$ PFU/g) resulted in statistically significantly better reduction of *E. coli* levels compared to lower EcoShield PX™ application rate (ca. $1x10^6$ PFU/g).

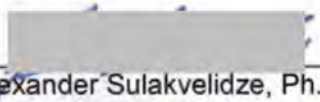
13 SIGNATURES



Jeffrey Tokman
Research Scientist



Joelle Woolston
Director of Laboratory Operations



Alexander Sulakvelidze, Ph.D.
Study Director

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated beef pieces

Study # Ec18K06JT

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1 STUDY TITLE

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated beef pieces.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Jeffrey Tokman, MS	Research Scientist	Hands-on-research / Report assembly
Joelle Woolston, MS	Director of Laboratory Operations	Data review / Report assembly

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether the application of EcoShield PX™ reduces the number of viable *E. coli* O157:H7 on beef when applied at the rate of $1x10^6$ – $1x10^8$ PFU/g.

6 TEST MATRIX

Chuck roast was obtained from a local Baltimore grocery store. It was not washed or pre-treated prior to our studies.

7 ECOSHIELD PX™ LOT AND APPLICATION

- EcoShield PX™ Lot 1817K2830A88
- Titer: approx. $3x10^{10}$ PFU/mL
- EcoShield PX™ was diluted as necessary with water just prior to application.
- The application rate was ca. 0.21mL EcoShield PX™ per 25g beef (3.8mL/lb).
- EcoShield PX™ was applied using a spray bottle with pump vaporizer

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATE BEEF

The beef test matrix was experimentally contaminated with *Escherichia coli* strain:

- Ec229: A nalidixic acid resistant mutant developed from Intralytix strain Ec133

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25µg/mL. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Ec229). The strain was stored at –80°C, at Intralytix, in 70% LB broth/30% glycerol supplemented with 25µg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown (37±2°C, 16-24h) in LB broth supplemented with nalidixic acid (25µg/mL). Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

The beef was experimentally contaminated with ca. $2x10^3$ CFU/g of beef.

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- PBS (Phosphate buffered saline, pH 7.4) (Life Technologies, Grand Island, NY; catalog # 10010031)

- CR-Sorbitol MacConkey Agar (OXOID, Basingstoke, UK; catalog # CM1005)

10 General Outline of Study

1) Fifteen 25g portions of beef were divided into five treatments A, B, C, D, E.
2) The challenge dose of bacteria was applied onto the beef surface of treatments A, B, C, and D.
3) The samples were placed in sterile filter bags and the bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 10 min.
4) Water (control) or EcoShield PX™ was applied as described in section 7. Treatments were evenly applied to the beef samples' surfaces as follows:
    - Group A = 0.21mL $1.2x10^{10}$ PFU/mL EcoShield PX/25g = $1x10^{8}$ PFU/g
    - Group B = 0.21mL $1.2x10^{9}$ PFU/mL EcoShield PX/25g = $1x10^{7}$ PFU/g
    - Group C = 0.21mL $1.2x10^{8}$ PFU/mL EcoShield PX/25g = $1x10^{6}$ PFU/g
    - Group D = 0.21mL water/25g (positive control)
    - Group E = 0.21mL water/25g (negative control)
5) The samples were covered and incubated at room temperature for ca. 15 minutes.
6) At 15 minutes post-treatment with water or EcoShield PX™, 225mL of sterile PBS was added. The bags were stomached for a minimum of 30 seconds at 230rpm.
7) The number of viable *E. coli* in the samples was determined by plating aliquots (0.1mL and 0.5mL) of the stomached beef/PBS mixture onto separate CR-SMAC plates supplemented with nalidixic acid (25µg/mL) in duplicate. The plates were incubated (37±2°C, 24±2hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated beef}} = \frac{\textit{CFU}}{\textit{0.1 and 0.5mL plating}} \times \frac{\textit{225mL PBS}}{\textit{25g sample}}$$

Counts were used from both 0.1 and 0.5 mL platings during analysis, unless there was an uncountable (>330) number of colonies on the plates.

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #Ec18K06JT

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU/g
A ($1x10^8$ PFU/g)	Yes	25	$1x10^8$ PFU/g EcoShield PX	3	121.5, 67.5, 49.5
B ($1x10^7$ PFU/g)	Yes	25	$1x10^7$ PFU/g EcoShield PX	3	274.5, 229.5, 706.5
C ($1x10^6$ PFU/g)	Yes	25	$1x10^6$ PFU/g EcoShield PX	3	1395, 1230, 1080
D (+ Control)	Yes	25	Water	3	2070, 1896, 2610
E (- Control)	No	25	Water	3	0

11.2 Tabular presentation of results

Table 2 Reduction of *E. coli* counts on beef treated with EcoShield PX when applied at ca. $1x10^6$ – $1x10^8$ PFU/g (0.21mL per 25g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^8$ PFU/g)	Yes	$1x10^8$ PFU/g EcoShield PX	*n* =3	79.5	96%	1.44	Yes
B ($1x10^7$ PFU/g)	Yes	$1x10^7$ PFU/g EcoShield PX	*n* = 3	403.5	82%	0.73	Yes
C ($1x10^6$ PFU/g)	Yes	$1x10^6$ PFU/g EcoShield PX	*n* =3	1235	44%	0.25	Yes
D (+ Control)	Yes	Water	*n* =3	2192	N/A	N/A	N/A
E (- Control)	No	Water	*n* =3	0	N/A	N/A	N/A

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

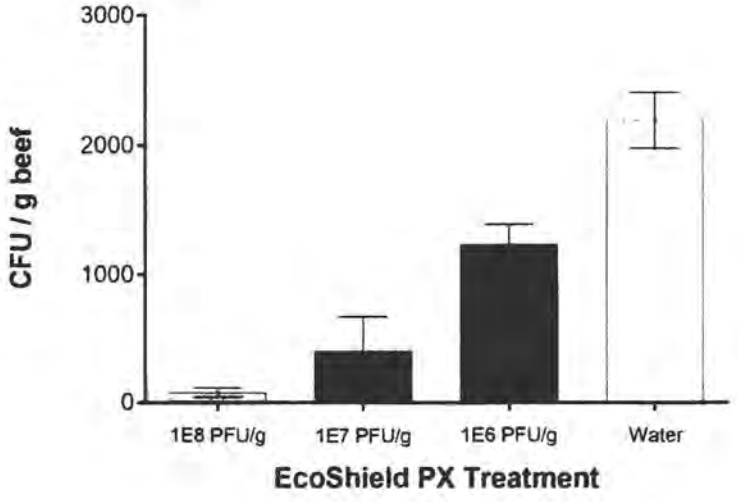
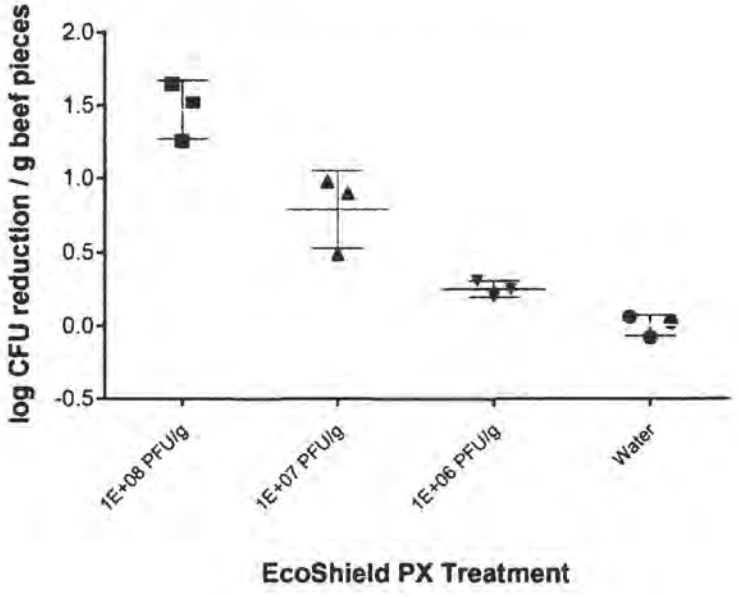


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the EcoShield PX™ treatment in reducing the number of viable *E. coli* in the experimentally contaminated beef was evaluated by comparing the data obtained with the water-treated control samples and the EcoShield PX-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 7.04 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is <0.0001, is considered extremely significant. Variation between the means is significantly greater than expected by chance.

Comparison	Mean Difference	q value	p value
1E+08 PFU/g vs Water	-2113	15.12	**** P<0.0001
1E+07 PFU/g vs Water	-1789	12.80	**** P<0.0001
1E+06 PFU/g vs Water	-957	6.850	** P<0.01
1E+08 PFU/g vs 1E+07 PFU/g	-324	2.319	ns P>0.05
1E+08 PFU/g vs 1E+06 PFU/g	-1156	8.271	** P<0.01
1E+07 PFU/g vs 1E+06 PFU/g	-831.5	5.952	* P<0.05

ns = not significant

11.5 Brief discussion of results and study's conclusions

- Applying EcoShield PX™ at ca. $1x10^8$ PFU/g beef reduced the number of viable *E. coli* by ca. 96% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.0001).
- Applying EcoShield PX™ at ca. $1x10^7$ PFU/g beef reduced the number of viable *E. coli* by ca. 82% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P>0.0001).

- Applying EcoShield PX™ at ca. $1x10^6$ PFU/g beef reduced the number of viable *E. coli* by ca. 44% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P>0.01).

- Reduction in *E. coli* levels achieved by using more concentrated EcoShield PX™ was higher compared to those obtained with more dilute EcoShield PX™ (96% vs. 82% vs. 44% when using ca. $1x10^8$ PFU/g, $1x10^7$ PFU/g, and $1x10^6$ PFU/g, respectively).

- The difference in *E. coli* recovered when EcoShield PX™ was applied in the two most concentrated forms (application rates $1x10^8$ PFU/g vs. $1x10^7$ PFU/g) was not statistically significant (P>0.05).

- The differences in *E. coli* recovered when EcoShield PX™ was applied in the two most concentrated forms vs the least concentrated (application rates $1x10^8$ PFU/g vs. $1x10^6$ PFU/g OR $1x10^7$ PFU/g vs. $1x10^6$ PFU/g) were statistically significant (P<0.01, P<0.05 respectively).

12 SUMMARY CONCLUSION OF THE STUDY

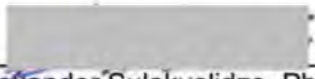
EcoShield PX™ can significantly reduce viable *E. coli* levels in experimentally contaminated beef by ca. 44-96% after a 15 minute contact time, when applied at ca. $1x10^6$ – $1x10^8$ PFU/g.

Using the higher EcoShield PX™ application rates (ca. $1x10^8$ PFU/g or $1x10^7$ PFU/g) resulted in statistically significantly better reduction of *E. coli* levels compared to lower EcoShield PX™ application rate (ca. $1x10^6$ PFU/g).

13 SIGNATURES

Jeffrey Yokman
Research Scientist

Joelle Woolston
Director of Laboratory Operations



Alexander Sulakvelidze, Ph.D.
Study Director

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated cooked chicken

Study # Ec18K09JT

Intralytix

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Table of Contents

1 STUDY TITLE

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination on experimentally contaminated cooked chicken.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Jeffrey Tokman, MS	Research Scientist	Hands-on-research / Report assembly
Joelle Woolston, MS	Director of Laboratory Operations	Data review / Report assembly

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of EcoShield PX™ reduces the number of viable *E. coli* O157:H7 on ready to eat cooked chicken when applied at the rate of $1x10^6$ – $1x10^8$ PFU/g.

6 Test Matrix

Roast chicken was obtained from a local Baltimore grocery store. It was not washed or pre-treated prior to our studies.

7 EcoShield PX™ Lot and Application

- EcoShield PX™ Lot 1817K2830A88
- Titer: approx. $3x10^{10}$ PFU/mL
- EcoShield PX™ was diluted as necessary with water just prior to application
- The application rate was ca. 0.21mL EcoShield PX™ per 25g chicken (3.8mL/lb)
- EcoShield PX™ was applied using a spray bottle with pump vaporizer

8 Bacterial Strains Used to Experimentally Contaminated Chicken

The chicken test matrix was experimentally contaminated with *Escherichia coli* strain:

- Ec229: A nalidixic acid resistant mutant developed from Intralytix strain Ec133

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25µg/mL. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Ec229). The strain was stored at –80°C, at Intralytix, in 70% LB broth/30% glycerol supplemented with 25µg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown (37±2°C, 16-24h) in LB broth supplemented with nalidixic acid (25µg/mL). Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

The chicken was experimentally contaminated with ca. $4x10^3$ CFU/g of chicken.

9 Media and Reagents

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- PBS (Phosphate buffered saline, pH 7.4) (Life Technologies, Grand Island, NY; catalog # 10030031)

- MacConkey Agar (BD, Sparks, MD; catalog # 212123)

10 General Outline of Study

1) Fifteen 25g portions of chicken were divided into five treatments A, B, C, D, E.
2) The challenge dose of bacteria was applied onto the chicken surface of treatments A, B, C, and D.
3) The samples were placed in sterile filter bags and the bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 10min.
4) Water (control) or EcoShield PX™ was applied as described in Section 7. Treatments were evenly applied to the chicken samples' surfaces as follows:
    - Group A = 0.21mL $1.2x10^{10}$ PFU/mL EcoShield PX/25g = $1x10^{8}$ PFU/g
    - Group B = 0.21mL $1.2x10^{9}$ PFU/mL EcoShield PX/25g = $1x10^{7}$ PFU/g
    - Group C = 0.21mL $1.2x10^{8}$ PFU/mL EcoShield PX/25g = $1x10^{6}$ PFU/g
    - Group D = 0.21mL water/25g (positive control)
    - Group E = 0.21mL water/25g (negative control)
5) The samples were covered and incubated at room temperature for ca. 15 minutes.
6) At 15 minutes post-treatment with water or EcoShield PX™, 225mL of sterile PBS was added. The bags were stomached for a minimum of 30 seconds at 230rpm.
7) The number of viable *E. coli* in the samples was determined by plating aliquots (0.1mL and 0.5mL) of the stomached chicken/PBS mixture onto separate MacConkey plates supplemented with nalidixic acid (25µg/mL) in duplicate. The plates were incubated (37±2°C, 24±2hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated chicken}} = \frac{\textit{CFU}}{\textit{0.1 and 0.5mL plating}} \times \frac{\textit{225mL PBS}}{\textit{25g sample}}$$

Counts were used from both 0.1 and 0.5 mL platings during analysis unless there was an uncountable (>330) number of colonies on the plates.

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #Ec18K09JT

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU/g
A ($1x10^8$ PFU/g)	Yes	25	$1x10^8$ PFU/g EcoShield PX	3	13.5, 27, 99
B ($1x10^7$ PFU/g)	Yes	25	$1x10^7$ PFU/g EcoShield PX	3	945, 1309.5, 616.5
C ($1x10^6$ PFU/g)	Yes	25	$1x10^8$ PFU/g EcoShield PX	3	3375, 2340, 2835
D (+ Control)	Yes	25	Water	3	3420, 4455, 4635
E (- Control)	No	25	Water	3	0

11.2 Tabular presentation of results

Table 2 Reduction of *E. coli* counts on chicken treated with EcoShield PX when applied at ca. $1x10^6$ – $1x10^8$ PFU/g (0.21mL per 25g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^8$ PFU/g)	Yes	$1x10^8$ PFU/g EcoShield PX	*n* =3	46.5	99%	1.95	Yes
B ($1x10^7$ PFU/g)	Yes	$1x10^7$ PFU/g EcoShield PX	*n* = 3	957	77%	0.64	Yes
C ($1x10^6$ PFU/g)	Yes	$1x10^6$ PFU/g EcoShield PX	*n* =3	2850	32%	0.17	Yes
D (+ Control)	Yes	Water	*n* =3	4170	N/A	N/A	N/A
E (- Control)	No	Water	*n* =3	0	N/A	N/A	N/A

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

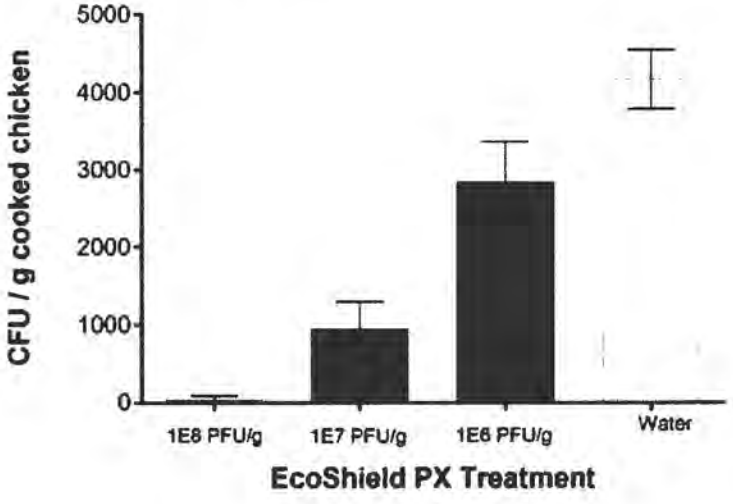
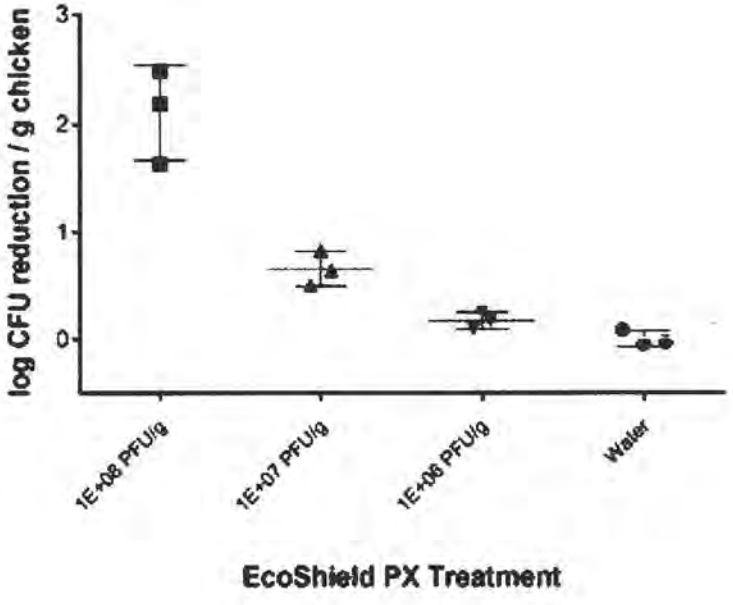


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the EcoShield PX™ treatment in reducing the number of viable *E. coli* in the experimentally contaminated ready to eat chicken was evaluated by comparing the data obtained with the water-treated control samples and the EcoShield PX-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 7.04 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is <0.0001, is considered extremely significant. Variation between the means is significantly greater than expected by chance.

Comparison	Mean Difference	q value	p value
1E+07 PFU/g vs Water	-4124	15.77	**** P<0.0001
5E+06 PFU/g vs Water	-3213	12.29	*** P<0.001
1E+06 PFU/g vs Water	-1320	5.049	* P<0.05
1E+07 PFU/g vs 5E+06 PFU/g	-910.5	3.483	ns P>0.05
1E+07 PFU/g vs 1E+06 PFU/g	-2804	10.72	*** P<0.001
5E+06 PFU/g vs 1E+06 PFU/g	-1893	7.241	** P<0.01

ns = not significant

11.5 Brief discussion of results and study's conclusions

- Applying EcoShield PX™ at ca. $1x10^8$ PFU/g chicken reduced the number of viable *E. coli* by ca. 99% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.0001).
- Applying EcoShield PX™ at ca. $1x10^7$ PFU/g chicken reduced the number of viable *E. coli* by ca. 77% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.001).
- Applying EcoShield PX™ at ca. $1x10^6$ PFU/g chicken reduced the number of viable *E. coli* by ca. 32% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.05).

- Reduction in *E. coli* levels achieved by using more concentrated EcoShield PX™ was higher compared to those obtained with more dilute EcoShield PX™ (99% vs. 77% vs. 32% when using ca. $1x10^8$ PFU/g, $1x10^7$ PFU/g, and $1x10^6$ PFU/g, respectively).

- The difference in *E. coli* recovered when EcoShield PX™ was applied in the two most concentrated forms (application rates $1x10^8$ PFU/g vs. $1x10^7$ PFU/g) was not statistically significant ($P>0.05$).

- The differences in *E. coli* recovered when EcoShield PX™ was applied in the two most concentrated forms vs the least concentrated (application rates $1x10^8$ PFU/g vs. $1x10^6$ PFU/g OR $1x10^8$ PFU/g vs. $1x10^6$ PFU/g) were statistically significant ($P<0.001$, $P<0.01$ respectively).

12 SUMMARY CONCLUSION OF THE STUDY

EcoShield PX™ can significantly reduce viable *E. coli* levels in experimentally contaminated cooked chicken by ca. 32-99% after a 15 minute contact time, when applied at ca. $1x10^6$ – $1x10^8$ PFU/g.

Using the higher EcoShield PX™ application rates (ca. $1x10^8$ PFU/g or $1x10^7$ PFU/g) resulted in statistically significantly better reduction of *E. coli* levels compared to lower EcoShield PX™ application rate (ca. $1x10^6$ PFU/g).

13 SIGNATURES

[redacted]

Jeffrey Tokman
Research Scientist

[redacted]

Joelle Woolston
Director of Laboratory Operations

[redacted]

Alexander Sulakvelidze, Ph.D.
Study Director

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated salmon

Study # Ec18K20JT

Intralytix

The Columbus Center

701 E. Pratt St.

Baltimore, MD 21202

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SAFETY BY NATURE

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1 STUDY TITLE

Evaluation of the ability of EcoShield PX™ to reduce *E. coli* O157:H7 contamination in experimentally contaminated salmon.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Jeffrey Tokman, MS	Research Scientist	Hands-on-research / Report assembly
Joelle Woolston, MS	Director of Laboratory Operations	Data review / Report assembly

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of EcoShield PX™ reduces the number of viable *E. coli* O157:H7 on salmon when applied at the rate of $1x10^6$ – $1x10^8$ PFU/g.

6 Test Matrix

Salmon fillet was obtained from a local Baltimore grocery store. It was not washed or pre-treated prior to our studies.

7 EcoShield PX™ Lot and Application

- EcoShield PX™ Lot 1817K2830A88
- Titer: approx. $3x10^{10}$ PFU/mL
- EcoShield PX™ was diluted as necessary with water just prior to application
- The application rate was ca. 0.21mL EcoShield PX™ per 25g salmon (3.8mL/lb)
- EcoShield PX™ was applied using a spray bottle with pump vaporizer

8 Bacterial Strains Used to Experimentally Contaminated Salmon

The salmon test matrix was experimentally contaminated with *Escherichia coli* strain:

- Ec229: A nalidixic acid resistant mutant developed from Intralytix strain Ec133

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 µg/mL. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., Ec229). The strain was stored at -80°C, at Intralytix, in 70% LB broth/30% glycerol supplemented with 25µg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown (37±2°C, 16-24h) in LB broth supplemented with nalidixic acid (25 µg/mL). Overnight growth corresponds to ca. $2x10^8$ CFU/mL.

The salmon was experimentally contaminated with ca. $4x10^3$ CFU/g of salmon.

9 Media and Reagents

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- PBS (Phosphate buffered saline, pH 7.4) (Life Technologies, Grand Island, NY; catalog # 10010031)

- MacConkey Agar (BD, Sparks, MD; catalog # 212123)

10 General Outline of Study

1) Fifteen 25g portions of salmon were divided into five treatments A, B, C, D, E.
2) The challenge dose of bacteria was applied onto the salmon surface of treatments A, B, C, and D.
3) The samples were placed in sterile filter bags and the bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 10min.
4) Water (control) or EcoShield PX™ was applied as described in Section 7. Treatments were evenly applied to the salmon samples' surfaces as follows:
    - Group A = 0.21mL $1.2x10^{10}$ PFU/mL EcoShield PX/25g = $1x10^{8}$ PFU/g
    - Group B = 0.21mL $1.2x10^{9}$ PFU/mL EcoShield PX/25g = $1x10^{7}$ PFU/g
    - Group C = 0.21mL $1.2x10^{8}$ PFU/mL EcoShield PX/25g = $1x10^{6}$ PFU/g
    - Group D = 0.21mL water/25g (positive control)
    - Group E = 0.21mL water/25g (negative control)
5) The samples were covered and incubated at room temperature for ca. 15 minutes.
6) At 15 minutes post-treatment with water or EcoShield PX™, 225mL of sterile PBS was added. The bags were stomached for a minimum of 30 seconds at 230rpm.
7) The number of viable *E. coli* in the samples was determined by plating aliquots (0.1mL and 0.5mL) of the stomached salmon/PBS mixture onto separate MacConkey plates supplemented with nalidixic acid (25µg/mL) in duplicate. The plates were incubated (37±2°C, 24±2hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\textit{Total CFU}}{\textit{g of treated salmon}} = \frac{\textit{CFU}}{\textit{0.1 and 0.5mL plating}} \times \frac{\textit{225mL PBS}}{\textit{25g sample}}$$

Counts were used from both 0.1 and 0.5 mL platings during analysis, unless there was an uncountable (>330) number of colonies on the plates.

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study # Ec18K20JT

Group	Challenged with bacteria	Weight (g)	Treatment	~25g Samples	CFU/g
A ($1x10^8$ PFU/g)	Yes	25	$1x10^8$ PFU/g EcoShield PX	3	139.5, 99, 27
B ($1x10^7$ PFU/g)	Yes	25	$1x10^7$ PFU/g EcoShield PX	3	765, 441, 234
C ($1x10^6$ PFU/g)	Yes	25	$1x10^6$ PFU/g EcoShield PX	3	1125, 1845, 2475
D (+ Control)	Yes	25	Water	3	4725, 4635, 3645
E (- Control)	No	25	Water	3	0

11.2 Tabular presentation of results

Table 2 Reduction of *E. coli* counts on salmon treated with EcoShield PX when applied at ca. $1x10^6$ – $1x10^8$ PFU/g (0.21mL per 25g).

Group	Challenged with bacteria	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Log reduction vs. water	Significant?
A ($1x10^8$ PFU/g)	Yes	$1x10^8$ PFU/g EcoShield PX	*n* =3	88.5	98%	1.69	Yes
B ($1x10^7$ PFU/g)	Yes	$1x10^7$ PFU/g EcoShield PX	*n* = 3	480	89%	0.96	Yes
C ($1x10^6$ PFU/g)	Yes	$1x10^8$ PFU/g EcoShield PX	*n* =3	1815	58%	0.38	Yes
D (+ Control)	Yes	Water	*n* =3	4335	N/A	N/A	N/A
E (- Control)	No	Water	*n* =3	0	N/A	N/A	N/A

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

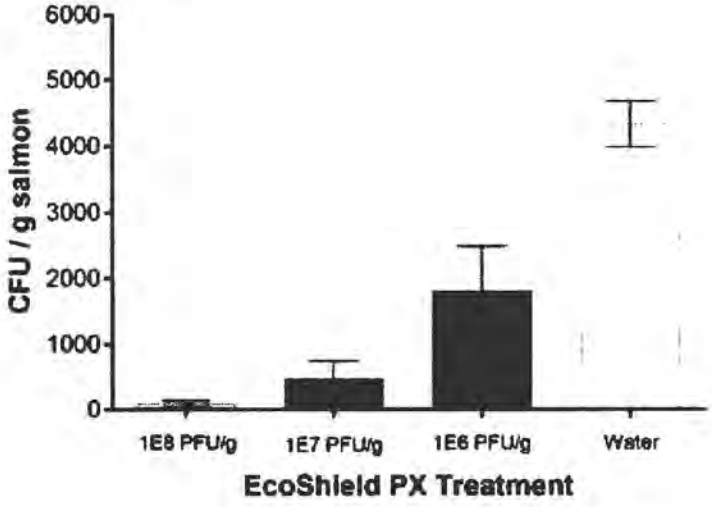
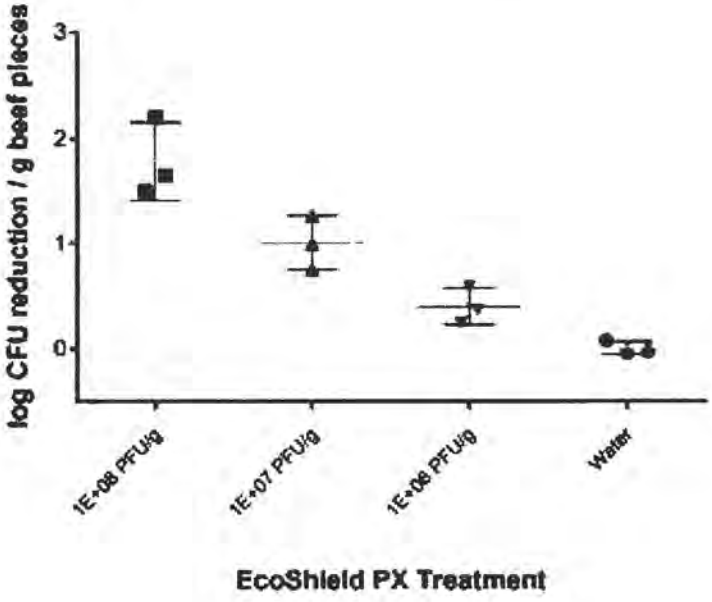


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the EcoShield PX™ treatment in reducing the number of viable *E. coli* in the experimentally contaminated salmon was evaluated by comparing the data obtained with the water-treated control samples and the EcoShield PX-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 7.04 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is <0.0001, is considered extremely significant. Variation between the means is significantly greater than expected by chance.

Comparison	Mean Difference	q value	p value
1E+08 PFU/g vs Water	-4247	15.59	**** P<0.0001
1E+07 PFU/g vs Water	-3855	14.15	**** P<0.0001
1E+06 PFU/g vs Water	-2520	9.252	*** P<0.001
1E+08 PFU/g vs 1E+07 PFU/g	-391.5	1.437	ns P>0.05
1E+08 PFU/g vs 1E+06 PFU/g	-1727	6.339	** P<0.01
1E+07 PFU/g vs 1E+06 PFU/g	-1335	4.901	* P<0.05

ns = not significant

11.5 Brief discussion of results and study's conclusions

- Applying EcoShield PX™ at ca. $1x10^8$ PFU/g salmon reduced the number of viable *E. coli* by ca. 98% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.0001).
- Applying EcoShield PX™ at ca. $1x10^7$ PFU/g salmon reduced the number of viable *E. coli* by ca. 89% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P<0.0001).
- Applying EcoShield PX™ at ca. $1x10^6$ PFU/g salmon reduced the number of viable *E. coli* by ca. 58% after 15 minutes of incubation at RT. The observed reduction was statistically significant (P>0.001).

- Reduction in *E. coli* levels achieved by using more concentrated EcoShield PX™ was higher compared to those obtained with more dilute EcoShield PX™ (98% vs. 89% vs. 58% when using ca. $1x10^8$ PFU/g, $1x10^7$ PFU/g, and $1x10^6$ PFU/g, respectively).

- The difference in *E. coli* recovered when EcoShield PX™ was applied in the two most concentrated forms (application rates $1x10^8$ PFU/g vs. $1x10^7$ PFU/g) was not statistically significant ($P>0.05$).

- The differences in *E. coli* recovered when EcoShield PX™ was applied in the two most concentrated forms vs the least concentrated (application rates $1x10^8$ PFU/g vs. $1x10^6$ PFU/g OR $1x10^7$ PFU/g vs. $1x10^6$ PFU/g) were statistically significant ($P<0.01$, $P<0.05$ respectively).

12 SUMMARY CONCLUSION OF THE STUDY

EcoShield PX™ can significantly reduce viable *E. coli* levels in experimentally contaminated salmon by ca. 58-98% after a 15 minute contact time, when applied at ca. $1x10^6$ – $1x10^8$ PFU/g.

Using the higher EcoShield PX™ application rates (ca. $1x10^8$ PFU/g or $1x10^7$ PFU/g) resulted in statistically significantly better reduction of *E. coli* levels compared to lower EcoShield PX™ application rate (ca. $1x10^6$ PFU/g).

13 SIGNATURES

[redacted]

Jeffrey Tokman
Research Scientist

[redacted]

Joelle Woolston
Director of Laboratory Operations

[redacted]

Alexander Sulakvelidze, Ph.D.
Study Director

Cournoyer, Patrick

From: Alexander Sulakvelidze <asulakvelidze@intralytix.com>
Sent: Friday, May 03, 2019 1:32 PM
To: Cournoyer, Patrick
Subject: RE: FSIS questions for GRN 834
Attachments: FDA Response re EcoShieldPX_May 2019.pdf; EcoShield_PX_SDS.pdf

Dear Dr. Cournoyer,

I am pleased to provide our responses to the questions presented in your e-mail of 4/24/2019. In the first attached document, our responses follow immediately your questions and are highlighted in BLUE font. The 2nd attached PDF file is the Safety Data Sheet (SDS) for EcoShield PX.

Please let me know if anything requires further clarification.

Sincerely,

Sandro Sulakvelidze

Alexander Sulakvelidze, Ph.D.
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Chief Scientific Officer
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From: Cournoyer, Patrick <Patrick.Cournoyer@fda.hhs.gov>
Sent: Wednesday, April 24, 2019 2:49 PM

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SAFETY BY NATURE™

May 3, 2019

Patrick Cournoyer, Ph.D.
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1019
patrick.cournoyer@fda.hhs.gov

Dear Dr. Cournoyer,

I am pleased to provide our responses to the questions presented in your e-mail of 4/24/2019. Our responses follow immediately your questions and are highlighted in BLUE font.

Please let me know if anything requires further clarification.

Sincerely,

Alexander Sulakvelidze, Ph.D.
Executive Vice President and CSO

FSIS reviewed the documentation associated with GRN834 and found that the submission is incomplete. We would appreciate your response within approximately 10 business days. The following additional information is requested:

1) Does the new technology interfere with FSIS inspection? No information was provided to support the use of Ecoshiled pxm does not interfere with FSIS inspection procedures. Descriptions and information concerning the organoleptic effects of the use of Ecoshield pmx on beef, poultry, Ready to Eat product or fish were not included.

Our understanding of your first question is whether bacteriophage application will have potential effects on the organoleptic qualities of the foods being treated and thereby interfere with FSIS inspection (another potential interfering factor was safety for FSIS inspectors which is further discussed below). As explained below, EcoShield PX™ phages will not have any effect on the organoleptic qualities of foods and are not expected to interfere with the FSIS inspection.

Phages are naturally and commonly present in all fresh foods and billions of phages are consumed daily by millions of people worldwide, with no apparent impact on organoleptic qualities of those foods[1]. Also, in controlled studies, Perera et.al., 2015[2] showed that the FSIS Directive 7120.1-listed ListShield™ did not impact the organoleptic qualities of the food when applied to deli meat. EcoShieldPX™ is technically and mechanistically equivalent to ListShield™ and several other bacteriophage products which are currently listed as GRAS and/or in FSIS Directive 7120.1 (e.g., EcoShield™, Listex™, SalmoFresh™, SalmoPro™, Salmonelex™, PhageGuard E™, and ShigaShield™)[3]. Moreover, as shown in Table 3 of our EcoShield PX™ GRAS notification, EcoShield PX™ is essentially identical in chemical composition to ListShield™ (presented in Table 3 of GRAS Notification 528), the primary components being water (99.4%), salt (0.58%), and bacteriophages (<0.1%). Bacteriophages have no taste. The only component of the EcoShield PX™ formulation that may, in theory, have impact on an organoleptic quality is salt, which could, hypothetically, affect the taste. In Section 3 of the EcoShield PX™ GRAS notification, the calculated level of salt added to foods treated with EcoShield PX™ would be equivalent to 0.0014% (42.9 mg sodium per day /3000g food eaten in a day = 0.000014) of all cumulative food items, if added at the highest acceptable level to all foods eaten in one day (assuming a 3000g diet). The recognition threshold of salt in solution is approximately 15 mmol in aqueous solution[4]. A 0.0014% salt solution would only amount to approximately 0.6 mmol (0.014 g / 22.99 g/mole Na * 1 L = 0.0006 mol), considerably lower of that threshold. Therefore, it is reasonable to believe that the salt in EcoShield PX™, as well as the EcoShield PX™ preparation overall, would not have any effect on the organoleptic qualities of foods.

2) Does the new technology cause a safety issue for FSIS inspection personnel? Information concerning, required personal protective equipment, effects of aerosols, skin contact, and other potential safety issues associated with the use of Ecoshield pmx was not included.

---

[1] Sulakvelidze, A.; Barrow, P., Phage therapy in animals and agribusiness. In *Bacteriophages: Biology and Applications*, Kutter, E.; Sulakvelidze, A., Eds. CRC Press: Boca Raton, FL, 2005, pp 335-380

[2] Perera, M. N. et.al. Bacteriophage cocktail significantly reduces or eliminates *Listeria monocytogenes* contamination on lettuce, apples, cheese, smoked salmon and frozen foods. Food Microbiology. Volume 52, December 2015, Pages 42-48

[3] EcoShield – Food Contact Substance Notification No. FCN 1018, Listex – GRAS Notice 000218, SalmoFresh – GRAS Notice 000435, SalmoPro – GRAS Notice 000752 & GRAS Notice 000603, Salmonelex – GRAS Notice 000468, PhageGuard E – GRAS Notice 000757, ShigaShield – GRAS Notice 000672

[4] Liem, D.G. et al., Reducing sodium in foods: The effect on flavor. Nutrients. Volume 3, June 2011, Pages 694-711

There are several bacteriophage products affirmed as GRAS by the FDA and/or listed in the FSIS Directive 7120.1, including EcoShield™, Listex™, SalmoFresh™, SalmoPro™, Salmonelex™, PhageGuard E™, and ShigaShield™. Similar to those bacteriophage preparations, EcoShield PX™ is safe and does not present a safety concern for the FSIS inspection personnel. Attached, please find the SDS (safety data sheet) for EcoShield PX™. The SDS is primarily based on the buffer solution of the EcoShield PX™ phage preparation, which is composed of water (ca. 99.4%) and sodium chloride (ca. 0.58%), as the lytic phages themselves are non-toxic and non-hazardous. In summary, we do not believe that EcoShield PX™ presents a safety concern for the FSIS inspectors and it is not expected to interfere with the FSIS inspection process.

3) The company request that Ecolshield pmx be considered a processing aide. The effects of a processing aide are temporary. No information concerning if the effect of Ecoshield pmx was temporary was included in the submission.

The FDA definition for processing aids (in 21 CFR 101.100(a)(3)) are "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."

In Section 3 of the EcoShield PX GRAS notification, the calculations show that EcoShield PX™ is present in the final product at insignificant levels. These calculations used a "worst case scenario," where EcoShield PX™ is applied at the highest acceptable level to all total cumulative foods (i.e., 3000 g) eaten in one day. Even with this implausible maximum use scenario, the number of phages present in a daily diet would be a mere 70.2 µg, or 23.4 ppb.

EcoShield PX™ is also not expected to have any continued technical effect after application, based on previous studies. For example, the "Notification for New Use of a Food Contact Substance" for the technically equivalent product EcoShield™ (FCN 1018; July 22, 2012) included a residual effect study. In that study, ground beef samples were artificially contaminated with *E. coli*, treated with EcoShield™, then re-contaminated with *E. coli*. The study demonstrated that the reduction in bacterial levels by EcoShield™ was a one-time occurrence and that EcoShield™ did not have an additional residual effect; i.e., it did not have any technical or functional effect in that food (Carter et. al., 2012)[5]. EcoShield™ and EcoShield PX™ are essentially equivalent (they differ in composition by just two phages, both lytic for *E. coli* O157:H7, with the rest of the composition essentially identical), therefore it can be concluded that EcoShield PX™ will also not have any continued technical effect.

FSIS has previously determined that "The use of the substance(s) is consistent with FDA's labeling definition of a processing aid." for several bacteriophage preparations, consequently EcoShield™, Listex™, SalmoFresh™, SalmoPro™, Salmonelex™, PhageGuard E™, and ShigaShield™ have no labeling requirements. EcoShield PX™ is technically equivalent to these other phage products designated as processing aids. Furthermore, with the proposed application rate for EcoShield PX™ of up to $1x10^8$ PFU per gram of food article, even in the worst case / maximum use scenario ($1x10^8$ PFU/g,) the rate is 10 times lower than the maximum $1x10^9$ PFU/g for Listex P100 cleared by the FSIS as sufficiently low enough to not require labeling (FSIS Directive 7120.1). Therefore, we believe that EcoShield PX™ also qualifies as a processing aid for its intended use.

[5] Carter, C.D.; Parks, A.; Abuladze, T.; Li, M.; Woolston, J.; Magnone, J.; Senecal, A.; Kropinski, A.M.; Sulakvelidze, A. Bacteriophage cocktail significantly reduces *Escherichia coli* O157:H7 contamination of lettuce and beef but does not protect against recontamination. Bacteriophage 2012, 2, 178-185

EcoShield PX™
Escherichia coli-specific phage preparation

Section 1: Identification

Product identifier

Product name: EcoShield PX™
Catalog #: 19EP

Recommended use of the chemical and restrictions on use

Phage preparation effective against *Escherichia coli* O157:H7

Supplier's details

Intralytix, Inc.
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21230

Emergency phone number

1-877-ITX-PHAGE
Monday–Friday 9:00 AM – 5:00 PM

Section 2: Hazard identification

Classification of substance or mixture

Not a hazardous substance or mixture

GHS label elements, including precautionary statements

Not a hazardous substance or mixture

Other hazards which do not result in classification

None

Section 3: Composition/information on ingredients

Mixture

Bacteriophages in aqueous 0.1M sodium chloride solution

Component list

Component	% composition	CAS #	Classification
Water	> 99.4	7732-18-5	Not applicable
Sodium chloride	0.58	7647-14-5	Not applicable
Escherichia coli O157:H7 – specific phages	< 0.01	Not applicable	Not applicable

Section 4: First aid measures

Description of first-aid measures

If inhaled:

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

In case of skin contact:

Wash off with soap and water.

In case of eye contact:

Flush eyes with water as a precaution.

If swallowed:

Never give anything by mouth to an unconscious person. If swallowed in excess, rinse mouth with water as a precaution.

Most important symptoms and effects, both acute and delayed:

The most important known symptoms and effects are described in the labelling (see Section 2) and/or in Section 11.

Indication of any immediate medical attention and special treatment needed:

No data available

Section 5: Fire fighting measures

Suitable extinguishing media

No restrictions

Specific hazards arising from the chemical

None

Special protective actions for fire-fighters

None

Section 6: Accidental release measures

Personal precautions, protective equipment and emergency procedures

For personal protection, see Section 8.

Environmental precautions

No special environmental precautions required

Method and materials for containment and cleaning up

Keep in suitable closed containers. Mop up or absorb with an inert dry material and place in an appropriate waste disposal container. No specific spill kit is required for this product

Section 7: Handling and storage

Precautions for safe handling

For precautions, see Section 2

Conditions for safe storage, including any incompatibilities

Keep container closed, refrigerated at 2-8°C, and protected from light.

Section 8: Exposure controls / personal protection

Control parameters

Contains no substances with occupational exposure limit values.

Appropriate engineering controls

General industrial hygiene practice

Individual protection measures, such as personal protective equipment (PPE)

Eye/face protection

When using as an aerosol, wear eye protection and provide access to eye/face flushing equipment.

Skin protection

A lab coat and/or gloves may be worn when handling this solution.

Respiratory protection

When airborne exposure limits are exceeded or ventilation is inadequate, use appropriate NIOSH approved respiratory protection equipment. Respiratory protection programs are subject to 29 CFR § 1910.134.

Section 9: Physical and chemical properties

Information on basic physical and chemical properties

Appearance	Clear/opalescent liquid
Odor	None
Odor threshold	No data available
pH	6.5 – 7.5
Melting point / freezing point	May start to solidify at –0.1°C (31.8°F) (WATER)
Initial boiling point and boiling range	The lowest known value is 99.9°C (211.8°F) (WATER).
Flash point	No data available
Evaporation rate	No data available
Flammability	No data available
Upper/lower flammability or explosive limits	No data available
Vapor pressure	No data available
Vapor density	No data available

Relative density	1.01 g/cm^3
Solubility	Soluble in water
Partition coefficient: n-octanol/water	No data available
Auto-ignition temperature	No data available
Decomposition temperature	No data available
Viscosity	No data available

Section 10: Stability and reactivity

Reactivity
No data available

Chemical stability
Stable under recommended storage conditions

Possibility of hazardous reactions
No data available

Conditions to avoid
No data available

Incompatible materials
No data available

Hazardous decomposition products
No data available

Section 11: Toxicological information

Acute toxicity
No data available

Skin corrosion/irritation
No data available

Serious eye damage/irritation
No data available

Respiratory or skin sensitization
No data available

Germ cell mutagenicity
No data available

Carcinogenicity

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

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

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

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

Reproductive toxicity

No data available

STOT-single exposure

No data available

STOT-repeated exposure

No data available

Aspiration hazard

No data available

Section 12: Ecological information

Toxicity

No data available

Persistence and degradability

No data available

Bioaccumulative potential

No data available

Mobility in soil

No data available

Other adverse effects

No data available

Section 13: Disposal considerations

Disposal methods

Product

Material does not have an EPA Waste Number and is not a listed waste, however, always contact a permitted waste disposal (TSD) to assure compliance with all current local, state, and Federal Regulations.

Packaging

Package may be recycled, if such disposal options exist.

Section 14: Transport information

UN Number

Not relevant

UN Proper Shipping Name

Not relevant

Transport hazard class

Not hazardous

Packing group

Not relevant

Environmental hazards

Not relevant

Special precautions

Keep refrigerated / cool during shipment

Section 15: Regulatory information

TSCA

Not applicable

SARA 302

Not applicable

SARA 311/312

Not applicable

SARA 313

Not applicable

CERCLA

Not applicable

California Proposition 65

This product does not contain any Proposition 65 chemicals.

US State Right-to-Know Regulations

Not applicable

Section 16: Other information

Revision date
April 30, 2019

Version
1

Further information
Notice to Reader

The statements contained herein are based upon technical data that Intralytix, Inc. believes to be reliable, are offered for information purposes only and as a guide to the appropriate precautionary and emergency handling of the material by a properly trained person having the necessary technical skills. Users should consider these data only as a supplement to other information gathered by them and must make independent determinations of suitability and completeness of information from all sources to assure proper use, storage and disposal of these materials and the safety and health of employees and customers and the protection of the environment.

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Cournoyer, Patrick

From: Alexander Sulakvelidze <asulakvelidze@intralytix.com>
Sent: Tuesday, August 27, 2019 12:20 PM
To: Cournoyer, Patrick
Subject: RE: Additional questions from FSIS re: GRN834
Attachments: EcoShield PX response to FSIS_082719.pdf

Dear Patrick,

Please find our responses to the FSIS questions in the attached letter. I hope this satisfactorily addresses all FSIS remaining concerns / questions, and that the GRAS notice would be granted so that we can start supplying the product to the industry in a timely fashion.

Thank you!

Sandro

Alexander Sulakvelidze, Ph.D.
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Chief Scientific Officer
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From: Cournoyer, Patrick <Patrick.Cournoyer@fda.hhs.gov>
Sent: Wednesday, August 21, 2019 6:56 PM
To: Alexander Sulakvelidze <asulakvelidze@intralytix.com>
Subject: Additional questions from FSIS re: GRN834

Dear Dr. Sulakvelidze

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U.S. Food and Drug Administration
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Re: Additional questions from FSIS regarding GRN 834

Dear Dr. Cournoyer,

Thank you for your letter of 8/21/19 regarding the above-referenced GRAS notification. Our understanding of your letter is that the FSIS technical team has questions regarding the (1) suitability of *Ec*229 as a surrogate for adulterant STEC and (2) use of three to eight phages for EcoShield PX™ cocktail. Please find our responses to both questions below:

1. The strain *Ec*229 is an *Escherichia coli* O157:H7 isolate. *E. coli* O157:H7 is one of the "big seven" STEC serotypes and is arguably the STEC serotype most frequently implicated in foodborne disease. Thus, *Escherichia coli* O157:H7 is a suitable surrogate for efficacy studies for STEC. If the FSIS is looking for additional information specifically about the *Ec*229 strain, we are pleased to provide the following brief summary: *Ec*229 is a nalidixic acid resistant version of *Escherichia coli* O157:H7 strain 2886-75. It has been selected, via serial passaging, for resistance to nalidixic acid, and it has been used as a surrogate for *E. coli* O157:H7 in various studies [1, 2]. Additionally, strain *Ec*229 was previously used as the *Escherichia coli* O157:H7 surrogate target strain in the efficacy studies performed in support of FCN No. 1018, in which EcoShield™ was cleared by the FDA / USDA for use in production of red meat parts and trim prior to grinding as processing aid with no labeling requirements. The original 2886-75 strain is the strain responsible for the first known U.S. case of disease caused by *E. coli* O157:H7 infection and it was identified in 1982 as a causal agent for hemorrhagic colitis [3, 4]. Since its initial identification, the strain has been well characterized phenotypically and genotypically [4, 5]. The *Ec*229 strain is the same as 2886-75 except that it is nalidixic acid resistant which ensures robustness of data when recovery of the strain is enumerated during the efficacy studies (because plating on selective media supplemented with nalidixic acid further enhances the specificity of the media and provides additional assurance that only the challenge organism – i.e., *Ec*229 – is enumerated). Therefore, *Ec*229 is an excellent surrogate for adulterant STEC.

2. EcoShield PX™ is a cocktail of lytic phages as described in the GRAS notification (GRN 834). The specific purpose of mixing three to eight monophages is to enable quick response to real-life situations when uncommon / new STEC strains or serovars may be emerging and contaminating food products. The ability to utilize various blends of up to eight lytic phages allows our phage biocontrol technology to ensure optimal efficacy which, in turn, is critical for ensuring the safety of foods. Namely, it helps in (i) warranting the broadest possible lytic

activity of the cocktail for effective control of multiple STEC strains and (ii) reducing the risk of development of bacterial resistance against EcoShield PX.

All studies presented in the current GRAS application were conducted with a formulation containing three lytic phages. Additional phages will increase the lytic range and potency of the preparation, but they also increase the cost of production. Therefore, at a minimum, Intralytix will utilize a cocktail of at least three phages, and will only add additional phages if the circumstances warrant. All phages are/will be (i) lytic in nature, (ii) free of all undesirable genes, and (iii) produced and QC-ed based on stringent criteria presented in the current GRAS notice. They will also meet all 10 (ten) criteria for inclusion in the EcoShield PX cocktail set forth in Section 2.1 of the current GRAS notice GRN 834. All of the component phages will be available through ATCC. Finally, the final concentration of phages applied onto foods – irrespective of whether the formulation contains three phages or eight – will always be up to the maximum of $1x10^8$ PFU/g for consistent efficacy, as specified in the GRAS notice GRN 834. Similar flexibility on a phage cocktail formulation has been previously granted by the FDA / USDA to another phage preparation produced by another company (GRN 724). That GRAS notice allows the use of any combination of 6 phages from the pool of 12 bacteriophages to prepare different variations of their phage cocktail. We anticipate that a similar flexibility would be granted to EcoShield PX.

I hope the above satisfactorily addresses the questions from the FSIS. We look forwarded to receiving the FDA clearance for the EcoShield PX preparation as GRAS, so that we can start supplying the product to the industry to help decrease the burden of STEC-related foodborne illness.

Sincerely,

Alexander Sulakvelidze, Ph.D.
Executive Vice President and Chief Scientific Officer

1. Abuladze, T., et al., *Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by Escherichia coli O157:H7.* Appl. Env. Microbiol., 2008. **74**(20): p. 6230-6238.
2. Carter, C., et al., *Bacteriophage cocktail significantly reduces Escherichia coli O157: H7 contamination of lettuce and beef, but does not protect against recontamination.* Bacteriophage, 2012. **2**(3): p. 178 - 185.
3. Riley, L.W., et al., *Hemorrhagic Colitis Associated with a Rare Escherichia coli Serotype.* New England Journal of Medicine, 1983. **308**(12): p. 681-685.
4. Sanjar, F., et al., *Genome sequence of Escherichia coli O157:H7 strain 2886-75, associated with the first reported case of human infection in the United States.* Genome Announcements, 2014. **2**(1): p. e01120-13.
5. Whittam, T.S., I.K. Wachsmuth, and R.A. Wilson, *Genetic Evidence of Clonal Descent of Escherichia coli O157:H7 Associated with Hemorrhagic Colitis and Hemolytic Uremic Syndrome.* The Journal of Infectious Diseases, 1988. **157**(6): p. 1124-1133.