# Broad and efficient control of major foodborne pathogenic strains of *Escherichia coli* by mixtures of plant-produced colicins

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**Enterohemorrhagic *Escherichia coli* (EHEC) is one of the leading causes of bacterial enteric infections worldwide, causing ∼100,000 illnesses, 3,000 hospitalizations, and 90 deaths annually in the United States alone. These illnesses have been linked to consumption of contaminated animal products and vegetables. Currently, other than thermal inactivation, there are no effective methods to eliminate pathogenic bacteria in food. Colicins are nonantibiotic antimicrobial proteins, produced by *E. coli* strains that kill or inhibit the growth of other *E. coli* strains. Several colicins are highly effective against key EHEC strains. Here we demonstrate very high levels of colicin expression (up to 3 g/kg of fresh biomass) in tobacco and edible plants (spinach and leafy beets) at costs that will allow commercialization. Among the colicins examined, plant-expressed colicin M had the broadest antimicrobial activity against EHEC and complemented the potency of other colicins. A mixture of colicin M and colicin E7 showed very high activity against all major EHEC strains, as defined by the US Department of Agriculture/Food and Drug Administration. Treatments with low (less than 10 mg colicins per L) concentrations reduced the pathogenic bacterial load in broth culture by 2 to over 6 logs depending on the strain. In experiments using meats spiked with *E. coli* O157:H7, colicins efficiently reduced the population of the pathogen by at least 2 logs. Plant-produced colicins could be effectively used for the broad control of pathogenic *E. coli* in both plant- and animal-based food products and, in the United States, colicins could be approved using the generally recognized as safe (GRAS) regulatory approval pathway.**

antimicrobials | colicin | EHEC | food safety | plant-made recombinant proteins

Enterohemorrhagic *Escherichia coli* (EHEC), a subset of Shiga toxin-producing *E. coli* (STEC) strains, is a leading cause of bacterial enteric infections in the United States and worldwide. EHEC causes ∼100,000 illnesses, 3,000 hospitalizations, and 90 deaths annually in the United States alone (1). Most of these illnesses have been linked to consumption of foods derived from animal products and, recently, organically grown vegetables. Nearly a quarter of all documented cases of EHEC in the United States last year were associated with fruits and vegetables, especially organically grown produce (www.cdc.gov/foodsafety/pdfs/foodborne-disease-outbreaks-annual-report-2013-508c.pdf). Although O157:H7 is currently the predominant serotype and accounts for ∼75% of EHEC infections worldwide, several non-O157 EHEC serotypes are also emerging as serious concerns for foodborne illnesses. In the United States, a group often referred to as the "Big 6" (O111, O26, O121, O103, O145, and O45) accounts for the majority of the non-O157:H7 serotypes isolated from clinical infections and, therefore, is also a focus of concern (2). One of the most serious recent cases of *E. coli* contamination outside the United States occurred in Europe in 2011, when fenugreek seeds contaminated with O104:H4 *E. coli* in an organic sprouts farm afflicted nearly 4,000 people, ultimately causing 54 deaths (3).

Currently, there are very few interventions targeted toward the inactivation of bacteria on food. Most of the available interventions involve heating or organic acids, which can adversely modify the taste and quality of the products. Currently approved bacteriophage mixtures enable narrow and specific control of O157:H7 but not of other pathogenic strains (4). Use of traditional antibiotics for the treatment of food is not appropriate and should be considered unacceptable, particularly due to the increase of antibiotic resistance seen among *E. coli* strains found in food (5). Among nonantibiotic antibacterials, several colicins have been shown to be highly effective against some EHEC strains, each individually reducing the bacterial load by up to 5 logs; some of them were found to be an effective treatment for reducing EHEC populations in both live animals and animal-derived products (6, 7).

Colicins are a group of bacteriocin-class antimicrobial proteins produced by, and effective against, *E. coli* and very closely related bacteria. Research on colicins began with their initial discovery 90 y ago (8). Colicins are classified based on their mode of bactericidal activity (either enzymatic inhibition of DNA, RNA, or cell-wall synthesis or depolarization via a pore-forming effect), their membrane receptors, and the mechanism they use for translocation through the outer membrane and across the periplasmic space of gram-negative bacteria. There are limited data on the effects of colicins on major foodborne *E. coli* strains (e.g., 7, 9, 10). We demonstrate here that we were able to express most

## Significance

**Enterohemorrhagic *Escherichia coli*-contaminated food products are among the leading causes of bacterial enteric infections in the United States and worldwide. Currently, other than thermal inactivation, there are no effective methods to control pathogenic bacteria in food. We investigated colicins, nonantibiotic antimicrobial proteins produced by certain *E. coli* strains and active against other strains of the species, as potential pathogen control agents. We demonstrate that most colicins can be expressed at high yields in plants and are fully functional. We show that mixtures of colicins applied at low concentrations are highly and broadly active against all major pathogenic *E. coli* strains of concern for foodborne illness. We propose plant-produced colicins as an inexpensive food treatment for the broad control of pathogenic *E. coli* strains.**

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colicins in plants with very high yields (up to 30% of total soluble protein, or 3 g active protein per kg of fresh green biomass) and at manufacturing costs that would allow commercial adoption of the technology. Production host plants can include tobacco and edible species such as spinach and leafy beets. Among the different colicins evaluated, colicin M was found to possess the broadest antimicrobial activity against major pathogenic *E. coli* strains. Because of their different mechanisms of action, mixtures of colicins can be used to exert complementary (additive) control of pathogens. Mixtures of colicin M and other colicins, and of colicin M and colicin E7 in particular, show very high activity against all seven pathogenic serotypes defined by the United States Department of Agriculture (USDA)/Food and Drug Administration (FDA) as major foodborne pathogens. These mixtures were also effective in controlling the emerging pathogenic serotype O104:H4. Treatments with colicin mixtures at low levels (nanomolar concentrations, or less than 10 mg total colicins per kg of treated food product) reduce the bacterial load of different pathogenic strains by 2 to >6 logs.

We propose using plant-produced colicins on food to enable broad control of pathogenic *E. coli* bacteria in animal- and plant-derived products. Because the compositions of plant-produced colicins are identical to those of native colicins produced by colicinogenic strains in many environments, including the gastrointestinal (GI) tract of humans and other animals, colicins are recognized as safe through an extensive history of human exposure, and therefore in the United States they can be approved using the GRAS (generally recognized as safe) regulatory approval pathway.

## Results

**Colicins Can Be Produced in *Nicotiana benthamiana* Using Transient Expression.** We selected 12 colicin proteins representing all four activity groups and various receptor specificities, including colicins E2, E3, E6, E7, D, N, K, 5, U, B, Ia, and M (Table S1). Respective plant codon usage-optimized sequences were subcloned into TMV-based assembled viral vectors (11), resulting in the plasmid constructs depicted in Fig. S1*A*. For colicins D, E2, E3, E6, E7, and M, coding sequences were interrupted by insertion of a plant intron to prevent cytotoxicity to the *E. coli* cells used for cloning.

In preliminary expression studies, colicins with nuclease activities (RNase and DNase) were usually highly toxic to plant tissues. The coexpression with appropriate immunity proteins reduced the toxic effect and increased the accumulation of these colicins dramatically. Immunity proteins ImmE2, ImmE6, ImmE7, and ImmD (Table S2) were thus coexpressed with colicins E2, E6, E7, and D, respectively. For coexpression, coding sequences of immunity proteins were subcloned into PVX-based assembled viral vectors, which are known to be noncompeting with TMV-based viral replicons (12). Resulting plasmid constructs are shown in Fig. S1*B*.

Expression of recombinant colicins in *Nicotiana benthamiana* plants after syringe infiltration with *Agrobacterium* cultures harboring relevant plasmid constructs resulted in reasonably high accumulation levels as revealed by SDS/PAGE analysis (Fig. 1*A*). The recombinant protein yields ranged from 0.6 to 3 mg/g fresh weight, or 7–32% of total soluble protein (TSP), depending on the particular protein (Table 1).

All plant-expressed recombinant colicins were soluble; because most of them are basic proteins (13), acidic extraction resulted in their efficient recovery with the concomitant elimination of native plant proteins (Fig. 1*B*).

Alternatively, recombinant colicins were expressed in *N. benthamiana* plants by spraying with diluted suspensions of agrobacteria supplemented with surfactant as described (14). Typically, the agrospray technique resulted in expression levels as high as those achieved with agroinfiltration (Fig. S2*A*), although for some colicins the time required for achieving maximum protein accumulation was longer, which is in conformity with ref. 14.

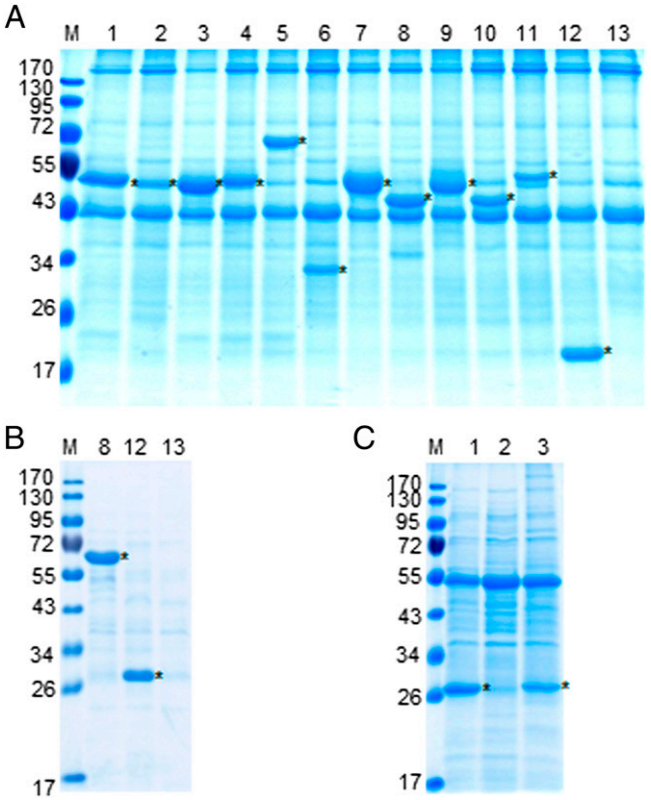


**Fig. 1.** Transient expression of colicins in plants. (*A* and *B*) Expression in *N. benthamiana* after syringe infiltration with agrobacteria carrying TMV or TMV and PVX vectors. Coomassie-stained SDS protein gels loaded with TSP extracts corresponding to (*A*) 7.5 μg protein, prepared with 50 mM Hepes (pH 7.0), 10 mM K acetate, 5 mM Mg acetate, 10% (vol/vol) glycerol, 0.05% (vol/vol) Tween 20, 300 mM NaCl or (*B*) 1.5 mg fresh weight plant material, prepared with 25 mM Na acetate (pH 4), 150 mM NaCl, 1% (vol/vol) glycerol, 0.05% (vol/vol) Tween 20 from plant material expressing colicins E2 (lane 1), E3 (lane 2), E6 (lane 3), E7 (lane 4), D (lane 5), N (lane 6), K (lane 7), 5 (lane 8), U (lane 9), B (lane 10), Ia (lane 11), M (lane 12), or nontransfected leaf tissue (lane 13). Colicins E2, E6, E7, and D were coexpressed with their respective immunity proteins. Plant material was harvested 5 dpi for colicins E3, E7, and N; 6 dpi for colicins E2, E6, Ia, and M; 7 dpi for colD; and 8 dpi for colicins K, 5, U, and B. All constructs used are described in Fig. S1. (*C*) Expression of colicins in edible plant species. *S. oleracea* cv. Frühes Riesenblatt (lanes 1 and 2) or *B. vulgaris* ssp. *maritima* (lane 3) plants were syringe-inoculated with *Agrobacterium* for colicin M expression. Coomassie-stained SDS protein gel loaded with TSP extracts corresponding to 1 mg fresh weight, prepared with 150 mM NaCl from plant material expressing colicin M at 10 dpi (lane 1), 8 dpi (lane 3), or nontransfected leaf tissue (lane 2). Asterisks mark recombinant proteins. M, molecular weight marker with weights (kDa) shown (*Left*).

**Colicins Can Be Produced Transiently in Edible Plant Species.** We tested the transient expression of recombinant colicin M in several edible crops including leafy beets, spinach, chicory, and lettuce using agroinfiltration with needleless syringe. The greatest accumulation of the protein of interest was achieved in spinach and leafy beets when TMV was used as the expression vector. Spinach (*Spinacia oleracea* L.) plants infiltrated with 1:100 dilution of agrobacterial culture developed no necrotic phenotype until the last harvesting time point of 10 d postinoculation (dpi). SDS/PAGE analysis of TSP extracts revealed abundant protein bands in Coomassie-stained gels (Fig. 1*C*). Colicin M expression was tested in sea beet (*Beta vulgaris* L. ssp. *maritima*) plants in a similar manner. Peak recombinant protein accumulation was found at 8 dpi (Fig. 1*C*). The decrease of colicin M level observed at 10 dpi correlated with developing leaf necrosis.

**Table 1. Yield of recombinant colicins expressed in *N. benthamiana* plants**

| No. | Colicin | Yield, mg/g FW | Yield, % TSP | *N* |
|---|---|---|---|---|
| 1 | E2 | 2.07 ± 0.04 | 20 ± 0 | 2 |
| 2 | E3 | 0.57 ± 0.04 | 7 ± 0 | 2 |
| 3 | E6 | 2.85 ± 0.32 | 25 ± 0 | 2 |
| 4 | E7 | 1.16 ± 0.24 | 11.83 ± 2.14 | 6 |
| 5 | D | 1.06 ± 0.06 | 10 ± 0 | 2 |
| 6 | N | 0.58 ± 0.08 | 6.75 ± 0.35 | 2 |
| 7 | K | 3.02 ± 1.11 | 32.42 ± 4.9 | 6 |
| 8 | 5 | 2.23 ± 1.6 | 20 ± 10 | 3 |
| 9 | U | 0.83 ± 0.28 | 12.25 ± 4.27 | 4 |
| 10 | B | 1.58 ± 0.24 | 19.33 ± 0.58 | 3 |
| 11 | Ia | 0.68 ± 0.09 | 9.42 ± 1.43 | 6 |
| 12 | M | 1.56 ± 0.31 | 17.92 ± 2.46 | 6 |

The yield was calculated in mg/g fresh weight (FW) of plant biomass and as a percentage of TSP [based on extraction with 50 mM Hepes, pH 7.0, 10 mM K acetate, 5 mM Mg acetate, 10% (vol/vol) glycerol, 0.05% (vol/vol) Tween 20, 300 mM NaCl] and represented as an average value and SD (AV ± SD) of several experiments. *N*, number of independent experiments.

**Colicins Can Be Produced in Transgenic *N. benthamiana* Hosts with Inducible Expression.** We generated stable transgenic *N. benthamiana* plants containing the genomic insertion of the double-inducible TMV-based viral vector pNMD18381 (Fig. S3) for ethanol-inducible colicin M expression based on the approach described in ref. 15. Selected transformants demonstrated normal growth and development with a biomass yield comparable to nontransformed control plants. Ethanol-inducible transgene expression was tested in detached leaves, and SDS/PAGE analysis revealed the accumulation of colicin M protein upon ethanol induction for the majority of selected transgenic lines (Fig. S2*B*).

**Plant-Made Recombinant Colicins Are Identical to Bacterial Counterparts.** The composition of the plant-expressed recombinant proteins was verified by two complementary mass spectrometry methods, peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF). When combined, the methods provided sufficiently high sequence coverage for verification of the amino acid sequences of the plant-made colicins [e.g., 100% coverage for colicin M (Fig. S4), 99% for colicin E7, 95% for colicin K, 89% for colicin U, and 91% for colicin B]. All plant-made colicin proteins conform to their predicted composition and match the consensus amino acid sequences of the bacterially produced native colicin proteins. Table S3 summarizes the results of amino acid sequence analyses by MALDI MS for eight representative colicins. Plant-made proteins were found to be identical to the corresponding proteins of bacterial origin. Notably, the N-terminal processing of colicins M, B, and Ia, for which information about bacterial proteins was available (16–18), was identical regardless of source host.

**Colicin Protein Purification.** We developed a simple downstream process for colicin-containing plant biomass, which includes the homogenization of plant tissue, acidic extraction, clarification, and neutralization of the plant extract followed by ultrafiltration and diafiltration steps. The product-enriched solution can then be subjected to one of two additional purification steps. If a relatively pure colicin product is desired, such as when using *Nicotiana* as the host plant, the extract is subjected to ion-exchange chromatography to remove additional host-cell proteins and plant metabolites such as alkaloids and polyphenols, resulting in a clarified, enriched product with 90% colicin protein purity (colicin isolate). Such purified product can be used in ready-to-eat food products or as a package additive. If a less-purified bulk product can suffice for certain applications such as food sprays or washes, edible plant species can be used without employment of the chromatography step. Typically this solution (colicin concentrate) contains one or a blend of colicin proteins at 40–50% purity (Fig. S5).

**Colicin M Is the Most Broadly Active Colicin for Control of *E. coli*.** We analyzed the antimicrobial activity of plant-made recombinant colicins against the bacterial strains comprising the Big 7 STEC serotypes and against the emerging pathogenic O104:H4 serotype (Table S4). In an initial screen, colicin-containing plant extracts were tested in radial diffusion assays via the spot-on-lawn method, and antimicrobial activity was evaluated semi-quantitatively based on the highest dilution of colicin-containing plant extracts causing a clearing effect. Analyzed colicin proteins significantly differed in their specificity and potency of antimicrobial activity against tested EHEC strains (Fig. 2 *A* and *B*). Surprisingly, the broadest specificity combined with relatively high potency against different strains was found for colicin M. This colicin demonstrated high antimicrobial activity against five, and moderate activity against an additional two, out of the eight pathogenic strains tested. The group of colicins including colicins E2, E6, E7, Ia, K, and 5 showed a more narrow spectrum of activity (four or five strains), with a rather similar strain specificity. In this group, colicin Ia was most broadly active, although, unlike colicin M, colicin Ia was relatively inefficient against O157:H7. Several colicins (E3, D, N, U, B) were either not active or had very narrow specificity (one or two strains). Interestingly, the strain of serotype O104:H4 was highly sensitive to almost all tested colicins, especially to colicins E2, E6, E7, and M (Fig. 2*B*).

**Colicin M Inhibits Big 7 and O104:H4 STEC Strains in Broth Culture.** For more precise quantitative evaluation of antimicrobial activity against EHEC strains, colicin-containing plant extracts were tested in broth culture. Table 2 shows the reduction of bacterial population after the application of individual colicins M or E7

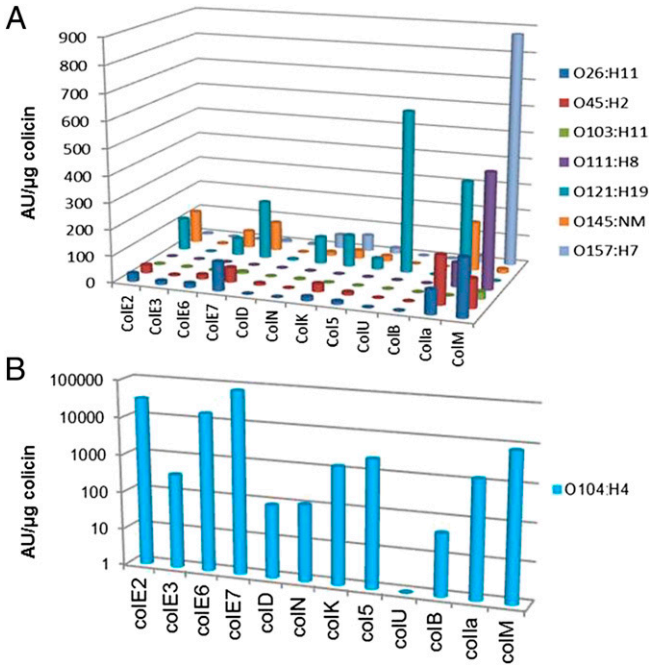


**Fig. 2.** Specific antimicrobial activity of plant extracts containing recombinant colicins. Antimicrobial activity of colicins was determined semi-quantitatively on serial dilutions of TSP extracts by soft agar overlay assay against *E. coli* strains of (*A*) Big 7 STEC and (*B*) O104:H4 serotypes. AU, arbitrary colicin activity units.

or their mixture (colicin-containing plant extract was compared with the extract from noninfected *N. benthamiana* plants). Colicin M used singly in concentrations of 1.0, 3.75, and 7.5 mg/L was able to reduce the bacterial populations by 2.5–5.0 cfu logs. Applied together, colicins M and E7 demonstrated synergistic effect for certain EHEC strains. These results suggest that using selective mixtures of two or more colicins with complementary or synergistic activities may allow for significant reductions in the amount of total colicin protein applied to food products.

**Colicin Treatment Controls *E. coli* O157:H7 Present on Meat.** Plant-produced colicins were tested for antibacterial activity on samples of meat contaminated with pathogenic *E. coli* O157:H7. Pork fillet steaks were inoculated with *E. coli* by submerging them in a bacterial solution. Steaks inoculated with *E. coli* were treated with carrier solution or colicin solution (3 mg colicin M + 1 mg colicin E7 per kg meat) by spraying. Nontreated (*E. coli* inoculation only) steaks served as an additional control. Bacterial population was analyzed after 1 h, 1 d, and 3 d of storage at 10 °C. The most significant reduction of bacterial population (2.3 and 2.7 logs) occurred as early as 1 h and 1 d of storage. Longer storage resulted in further decreases of the bacterial population (Fig. 3).

**Gastric and Intestinal Proteases Digest Plant-Produced Colicins.** Complete degradation of recombinant colicins in the human gastrointestinal tract after consuming colicin-treated foods is desirable to prevent influencing the indigenous GI tract microflora, as well as to avoid the potential development of allergic reactions. We evaluated the digestibility of colicin proteins in the human GI tract using treatments with simulated gastric and intestinal fluids (SGF and SIF, respectively). Sequential exposure studies using physiological enzyme-to-substrate ratios were conducted to mimic the natural human GI tract conditions (19–21). For these studies, the colicins were first exposed to either SGF without or with pepsin, at a pepsin-to-colicin ratio of 1:20 on a weight basis, followed by exposure of the resultant hydrolysate products to SIF supplemented with trypsin and chymotrypsin at a ratio of 1:4:400 (trypsin:chymotrypsin:colicin peptides, on a weight basis). Plant-produced individual colicins were mixed with SGF and incubated for up to 60 min, sampling every few minutes and assessing the digestion of the protein into fragments by SDS/PAGE. In parallel, aliquots of the reaction mix were evaluated for antimicrobial activity using a radial diffusion assay via the spot-on-lawn method. Fig. S6*A* shows that plant-produced colicin degrades nearly completely, producing fragments below 5 kDa already after a 10- to 20-min incubation in SGF, with a drop of antimicrobial activity to undetectable levels after 20–30 min (Fig. S6*B*). Subsequent SIF incubation results in further peptide degradation to fragments of about 2–3 kDa (Fig. S6*A*), which is below the size threshold for development of allergic reactions (22). Similar results were obtained for colicins E7, K, and U.

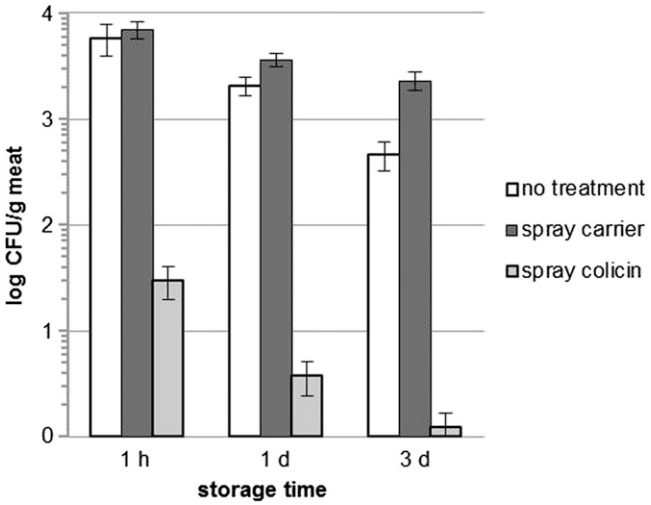


**Fig. 3.** Reduction of bacterial populations of *E. coli* O157:H7 (strain DSM19206) on fresh steak meat by colicins. Bacterial populations recovered from steak meat upon storage for various periods of time at 10 °C upon colicin treatment (colM + colE7 at 3 + 1 mg/kg meat, respectively) of contaminated meat by spray application. Error bars indicate SD of biological replicates ($n$ = 4).

**Table 2. Antibacterial activity of colicin M and colicin E7 applied individually and as mixtures to STEC strains**

| STEC serotype | Colicins, mg/mL | Reduction of bacterial population, log cfu control vs. colicin-treated |
|---|---|---|
| O121:H19 | E7 (10) | 2.7 |
| O121:H19 | M: E7 (1:1) | 2.8 |
| O145:NM | M: E7 (1:1) | 1.1 |
| O103:H11 | M (7.5) | 2.6 |
| O45:H2 | M (7.5) | 2.7 |
| O111:H8 | M (3.75) | 4.1 |
| O111:H8 | M: E7 (0.5:0.5) | 5.2 |
| O26:H11 | M (3.75) | 5.7 |
| O26:H11 | M: E7 (0.5:0.5) | 4.4 |
| O157:H7 | M (1.0) | 3.5 |
| O157:H7 | M: E7 (0.25:0.25) | 3.6 |
| O104:H4 | M: E7 (0.1:0.1) | 6.1 |

The indicated doses of colicins were incubated in LB culture at 37 °C with individual strains of different serotypes shown in Table S4 for 90 min. Reduction of bacterial cell numbers was quantified by dilution plating of cultures.

## Discussion

Colicins are nonantibiotic antibacterial proteins with multiple potential uses, including treatment of foods to eliminate pathogenic *E. coli* and as human and animal therapeutic alternatives to conventional antibiotics. Our data demonstrate that colicins can be expressed at very high levels in the plant species *N. benthamiana*, the standard manufacturing host for multiple biopharmaceuticals currently undergoing clinical trials, as well as in edible plant hosts such as spinach and beets. Uniformly, the plant-expressed proteins exhibit important properties, including proper processing and maturation, compositional identity to bacterially produced colicins, and full activity against pathogenic target strains of *E. coli*. The expression levels in many cases reached 20–30% of total soluble protein, or 2–3 g/kg of fresh leaf biomass without process optimization. Our findings show that colicins expressed alone or together with their corresponding immunity proteins, as done for colicins with nuclease activity, are not toxic to plants, and suggest that optimized industrial procedures for transfection or induction in transgenic hosts could be developed that are very inexpensive to apply on an industrial scale. Thus, plants are excellent hosts for manufacturing of not only phage lysins (23, 24) but also colicin-type bacteriocins. Technoeconomic analysis of plant-based protein manufacturing indicates that the upstream cost of goods that could be achieved in an industrial setting can be as low as $1.0–2.0 per kg of protein (25). This analysis does not take into account the costs of downstream purification or cost of containment (such as in greenhouse production) that may be necessary. Our own and our colleague's (Karen A. McDonald, University of California, Davis) conservative estimates of these additional costs indicate that the total operating costs may be on the order of $1.00 per g of purified colicin. These costs are much lower than the ceilings for colicin manufacturing prices computed from intended use rates (less than 10 mg colicin

per kg of treated foods) and intervention prices ($ per kg of treated food), and are an indication that the plant-based manufacturing of colicins is an industrially viable proposition. Manufacturing processes based on transient expression of proteins in plants and similar to those described in this report have already been brought into FDA Good Manufacturing Practice (GMP) compliance, and several biopharmaceuticals produced transiently in plants are currently undergoing clinical studies (26–28).

There is a great variety of colicin-class bacteriocins produced mostly by *E. coli* but also by some related enteric species including *Shigella* and *Yersinia*. Colicins exhibit quite diverse structures within their general three-domain (translocation, receptor, and cytotoxic) architecture. Surprisingly, practically all tested colicins or colicin–antitoxin combinations were expressed very well in plants. This may be explained by the low toxicity of colicins or colicins–antitoxins to plant cells, and by the fact that colicins are classical representatives of "intrinsically disordered proteins" (a feature essential for the ability to unfold/refold during their translocation through the bacterial cell wall and membrane), and therefore probably do not impose unusual requirements on translation or on the posttranslational machinery of the plant cell.

To the best of our knowledge, this work is the first study that shows that colicins, used singly or as mixtures, can control all (Big 7) pathogenic serotypes of *E. coli* under actual exposure modeling. We were also the first, to our knowledge, to show the identity of plant-produced colicins with their native bacterial counterparts. To our knowledge we were first to suggest that plant-based production should be scalable and can yield colicins or colicin mixtures at price points low enough to make them attractive for industrial adoption as a novel food safety strategy. The list of major serotypes (Big 7) has been defined by FDA and USDA Food Safety and Inspection Service (FSIS) based on a Centers for Disease Control and Prevention retrospective analysis of food poisonings caused by *E. coli* (29). Although about 75% of outbreaks are due to just one serotype (0157:H7), the other 25% caused by one or more of the remaining six serotypes are nevertheless significant from a public safety standpoint. Hence, any potential food safety additive intended to control *E. coli* will have market acceptance only if it provides efficient control of all seven pathogenic serotypes. In our studies, in addition to the Big 7 strains, we also tested STEC serotype 0104:H4, another emerging pathogen, because of the severity of the outbreak that it caused in Europe in 2011 (3) and its potential for recurrence.

The data presented show that, based on their ability to control major pathogenic *E. coli* strains, colicins can be divided into three subclasses. The most versatile and broadly active bacteriocin in this regard is colicin M; it exhibits high potency against five, and moderate potency against an additional two, out of the eight pathogenic strains tested. There is a group of colicins, including colicins E2, E6, E7, Ia, K, and 5, that exhibit a similar and more narrow spectrum of activity (controlling strains of four or five serotypes) but, importantly, they complement colicin M, so that the resultant mixture of colicin M and one or more colicins of this class allows for efficient control of all eight pathogenic strains of interest. Last, there are several colicins that are either not active or have very narrow specificity (strains of one or two serotypes), including colicins E3, D, N, U, and B. Based on these results, our proposed ideal mixture of colicins consists of colicin M + colicin E7, although colicin E7 can be substituted or supplemented with several colicins from among colicins E6, Ia, K, and 5. The colicin M + colicin E7 mixture has been used in studies modeling the effect of colicin treatment on meat spiked with *E. coli* strain of serotype 0157:H7. The results of our studies provide additional evidence of the potential utility of these colicins as intervention agents in food safety applications.

The use of colicins as food additives or food processing aids is especially attractive because of the magnitude of current food safety issues worldwide (30) and because these product candidates can be approved relatively quickly using the GRAS regulatory approval pathway in the United States. Approval via the GRAS review process is applicable because the human intestine is replete with colicinogenic strains of *E. coli*, and these are normal constituents of our healthy gut microflora. The compositions of plant-made colicins match those of the intestinal bacteria producing them, and plant-derived impurities from the production process would either be reduced as to not present a health risk (e.g., when *Nicotiana* is used as a host) or would be normal constituents of food crops such as spinach and beet. Further, ingestion of colicins via treated food would result in digestion of the proteins by gastric acid and gastric and duodenal proteases, as we have demonstrated in this report.

## Methods

**Bacterial Strains and Growth Conditions.** *E. coli* DH10B and STEC (Table S4) cells were cultivated at 37 °C in LB medium [lysogeny broth (31)]. *Agrobacterium tumefaciens* ICF320 (26) cells were cultivated at 28 °C in LBS medium (modified LB medium containing 1% soya peptone; Duchefa).

**Plasmid Constructs.** The methods used to construct and apply TMV- and PVX-based magnICON vectors and TMV-based vectors for EtOH-inducible expression (Figs. S1 and S3) were as described previously (11, 12, 15). Assembly of the binary construct for EtOH-inducible expression was done by modular cloning as described in ref. 32.

Coding sequences of genes of interest (colicins and colicin immunity proteins; Tables S1 and S2) were codon-optimized for *N. benthamiana*, synthesized by Entelechon, Thermo Fisher Scientific, or Eurofins Genomics and cloned into the BsaI sites of the respective destination vectors. To avoid toxicity to bacteria, for some colicin sequences an intron of *Ricinus communis cat1* gene was introduced for gene synthesis (Fig. S1).

**Plant Material and Inoculations.** *N. benthamiana*, *S. oleracea* cv. Frühes Riesenblatt, and *B. vulgaris* ssp. *maritima* plants were grown in the greenhouse (day and night temperatures of 19–23 °C and 17–20 °C, respectively, with 12 h light and 35–70% humidity). Six-week-old *N. benthamiana*, 5-week-old spinach, and 4-week-old beet plants were used for inoculations.

For plant transfection, saturated *Agrobacterium* overnight cultures were adjusted to $OD_{600}$ 1.3 (~$1.2 \times 10^9$ cfu/mL) with *Agrobacterium* inoculation solution (10 mM MES, pH 5.5, 10 mM $MgSO_4$) and diluted with same solution 1:100 for inoculation using a needleless syringe. For spray inoculation, 1:100 diluted *Agrobacterium* cultures were supplemented with 0.1% (vol/vol) Silwet L-77 (Kurt Obermeier), and inoculation was carried out using a hand sprayer (Carl Roth).

**Ethanol Induction of Transgene Expression.** Detached leaves of T0 generation transgenic *N. benthamiana* plants (primary transformants) were dipped into 4% (vol/vol) EtOH and incubated in 12 × 12-cm Petri dishes with the abaxial leaf surface on one layer of Whatman filter paper moisturized with 5 mL 4% (vol/vol) EtOH and one layer of glass fiber mesh and the petiole wrapped with tissue paper moisturized with 5 mL 4% (vol/vol) EtOH for 1 d with fluorescent light at 22 °C. Leaves were transferred to new Petri dishes composed as before but moisturized with 15 mL water and further incubated for 3 d with fluorescent light at 22 °C. Plant material was harvested 4 d postinduction.

**Plant Transformation and Regeneration.** *N. benthamiana* was transformed by leaf disk transformation and selected on kanamycin-containing medium using a slightly modified standard protocol (33). Regenerated plants (T0 generation) were transferred to the greenhouse and tested by EtOH induction of detached leaves for transgene expression.

**RT-PCR and Sequencing.** For verification of correct intron splicing, total RNA was prepared from about 100 mg of leaf tissue expressing colicins E2, E3, E6, E7, D, or M using an RNeasy Plant Mini Kit (Qiagen) and reverse-transcribed using a RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with oligo dT primers. cDNA was used as a template for PCR with gene-specific primers. PCR products purified with a GeneJET Gel Extraction Kit (Thermo Fisher Scientific) were sent for sequencing with gene-specific primers used for amplification to Seqlab.

**Protein Analysis.** Plant leaf material was ground in liquid nitrogen, and protein extracts were prepared with 5 volumes of either 2× Laemmli buffer (crude extracts) or different buffers, such as 50 mM Hepes (pH 7.0), 10 mM K acetate, 5 mM Mg acetate, 10% (vol/vol) glycerol, 0.05% (vol/vol) Tween 20, 300 mM NaCl (total soluble protein extracts). The protein concentration of TSP extracts was determined by Bradford assay using the Bio-Rad Protein Assay and BSA (Sigma-Aldrich) as a standard.

For analysis by 12% SDS/PAGE and Coomassie staining using PageBlue Protein Staining Solution (Fermentas), protein extracts were denatured at 95 °C for 5 min before loading. The estimation of the percentage of recombinant colicins of TSP was done by comparison of TSP extracts with known amounts of BSA (Sigma-Aldrich) on Coomassie-stained SDS/PAGE gels.

**Colicin M Purification.** Six days after inoculation, infected leaf-enriched material was harvested. Plant biomass was homogenized in the presence of extraction solution (10 mM HCl, 10 mM $Na_2S_2O_5$, 2.5 mM $Na_2$ EDTA) at a buffer/biomass ratio of 1:1 (vol/wt). The pH of the plant homogenate was adjusted to 4.0 and clarified by centrifugation at 20,000 × *g* for 20 min. The clarified extract was neutralized with 1 M sodium hydroxide and further clarified by centrifugation at 20,000 × *g* for 20 min and depth filtration (filter sheets: BECO KDS12; Begerow). The filtrate was concentrated fourfold by ultrafiltration with a 5-kDa hollow-fiber module. The retentate was diafiltered five times against 5 mM citrate, 50 mM NaCl (pH 5.0). The resulting concentrate was filter-sterilized using a 0.45-μm filter.

For further purification, the concentrate was loaded on a Fractogel EMD $SO_3^-$ (Merck Millipore) column equilibrated with 5 mM citric acid, 50 mM NaCl (pH 5.0). The column was washed with 25 mM sodium phosphate (pH 7.3) to reduce weakly bound proteins. The target protein was eluted with 100 mM citrate (pH 9.7). The eluate was diafiltered against 10 mM citrate, 137 mM NaCl (pH 7.3), resulting in a colicin M isolate.

The purity of the colicin concentrate was determined by using SDS/PAGE; Coomassie blue-stained protein bands were quantitated using a densitometer. The purity of the colicin M isolate was determined by capillary gel electrophoresis using a Bioanalyzer 1200 Series instrument (Agilent Technologies).

**Colicin Antimicrobial Activity Determinations.**

***Colicin assay.*** The quantitation of colicin solutions (plant TSP extracts) was carried out by serially diluting TSP with buffer 1:1 and spotting a drop (5 μL) of each dilution on an LB agar plate freshly seeded with ∼$1 \times 10^7$ cfu/mL bacterial cells (soft agar overlay assay: LB hard agar [1.5% (wt/vol)] plate overlaid with 20 mL LB soft agar [0.8% (wt/vol)] with bacterial cells in a 12 × 12-cm Petri dish). The number of colicin activity units per mg fresh weight plant material was defined as the highest dilution that gave a clear zone of growth inhibition. The colicin-specific activity was defined as the number of units of colicin activity per μg colicin.

***Quantitative bacterial enumeration.*** Antimicrobial activity was assayed in LB medium. *E. coli* strains shown in Table S4 were grown in LB medium, diluted to ∼$1 \times 10^4$ cfu/mL, and supplemented with either carrier solution (TSP extract of *N. benthamiana* WT plants) or colicin solution (TSP extract of *N. benthamiana* expressing colicins). Cultures were incubated at 37 °C and 150 rpm in an orbital shaker incubator (Multitron, Infors HT) and analyzed in triplicate for cfu numbers by plating serial dilutions of culture aliquots removed at 0, 30, 60, or 90 min of incubation on LB medium.

**Reduction of Bacterial Populations on Food.** Pork fillet steak was purchased from a local supermarket. *E. coli* O157:H7 (strain DSM19206) was grown in LB medium and diluted to $OD_{600}$ 0.005 (∼$5 \times 10^5$ cfu/mL) for contamination of the steak. Each steak of 85-g weight was inoculated by dipping in 12 mL *E. coli* contamination solution in 12 × 12-cm Petri dishes from both sides and dried for 30 min at room temperature. Subsequently, steaks were either not treated or treated by spraying (20 mL/kg) from both sides with carrier solution (TSP extract of WT *N. benthamiana* plant material) or colicin solution (mixture of TSP extracts of *N. benthamiana* plant material expressing colM or colE7) at a concentration of 3 mg/kg colM, 1 mg/kg colE7, and dried for 45 min at room temperature. Aliquots of steaks corresponding to ∼20-g meat were packed into BagFilter400P sterile bags (Interscience) and stored for 1 h, 1 d, and 3 d at 10 °C. For analysis of bacterial populations, steak aliquots were homogenized with 5 volumes of peptone water using a Bag Mixer400CC homogenizer (settings: gap 0, time 30 s, speed 4; Interscience), and colony-forming units of O157:H7 were counted on sorbitol-MacConkey medium supplemented with 0.05 μg/mL cefixime and 100 μg/mL X-gluc upon plating of serial dilutions. Samples were analyzed in quadruplicate.

**Simulated Gastroduodenal Digestion in Vitro.** The protocol followed was derived from prior reports (19, 20, 21). Lyophilized powders of enzymes and comparator proteins (α-casein from bovine milk, β-lactoglobulin from bovine milk, trypsin inhibitor from *Glycine max*) as well as reagents were purchased from Sigma-Aldrich.

***Gastric digestion in vitro: Phase I.*** Lyophilized purified colM was dissolved in Millipore water and the protein concentration was adjusted to 5 mg/mL. ColM was incubated in simulated gastric fluid (0.15 M NaCl, pH 2.5) with pepsin from porcine gastric mucosa at an approximately physiological ratio of enzyme to substrate [1:20 (wt:wt) pepsin:colM] at 37 °C with orbital shaking. Aliquots of the digestion mixture were taken at 0–60 min and quenched by raising the pH by addition of 50 mM ammonium bicarbonate for irreversible inactivation of pepsin. Control samples without enzyme addition were also analyzed.

***Duodenal digestion in vitro: Phase II.*** The pH of gastric digesta (60-min incubation) was adjusted to 6.5, and samples were supplemented with duodenal buffer containing trypsin and α-chymotrypsin from bovine pancreas for final concentrations of 4 mM sodium taurocholate, 4 mM glydeoxycholic acid, 10 mM $CaCl_2$, 25 mM Bis-Tris (pH 6.5) and approximately physiological ratio of enzymes to substrate [1:4:400 (wt:wt:wt) trypsin:chymotrypsin:colM]. Samples were incubated at 37 °C with orbital shaking, and aliquots of the digestion mixture were taken at 0–180 min and quenched by addition of the irreversible serine protease inhibitor Pefabloc SC at a final concentration of 5 mM. Control samples without enzyme addition were also analyzed.

***SDS/PAGE analysis.*** Samples taken at different time points during phase I and phase II gastroduodenal digestion were supplemented with 5× Laemmli buffer upon quenching, denatured for 10 min at 95 °C, and stored at −80 °C. Before SDS/PAGE, samples were denatured again and loaded onto precast 10–20% Mini-PROTEAN Tris-tricine gels (Bio-Rad) with loading corresponding to 2.2 μg colM per lane. Gels were run with 100 mM Tris, 100 mM tricine, 0.1% (wt/vol) SDS, fixed with 5% (vol/vol) glutardialdehyde solution, and Coomassie-stained with PageBlue Protein Staining Solution.

**Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry.** MS grade-quality reagents and enzymes were used according to the manufacturer's instructions.

***Peptide mass fingerprinting analysis.*** TSP or crude extracts of plant material were separated by SDS/PAGE, and colicin protein bands were cut from the gels and subjected to in-gel protease digestion. Before digestion, proteins were reduced and alkylated using DTT and iodoacetamide (Thermo Fisher Scientific). In-gel digests with pepsin (Protea Biosciences), trypsin, or chymotrypsin (Thermo Fisher Scientific) were conducted at 37 °C overnight, and the resulting peptides were extracted from the gel matrix in repeated extraction steps using 50% (vol/vol) acetonitrile and 1% (vol/vol) trifluoroacetic acid. Extracted peptides were spotted in triplicate onto a polished stainless steel target plate either directly (tryptic digest) or upon cleanup using PepClean C18 spin columns (Thermo Fisher Scientific) (pepsin, chymotrypsin digest). Upon drying, spotted preparations on the target plate were overlaid with 1 μL saturated alpha-cyano-4-hydroxycinnamic acid in 70% (vol/vol) acetonitrile matrix solution (HCCA; Bruker), thus allowing cocrystallization with the sample before further processing with MALDI-TOF.

Analysis was carried out using an Autoflex III Smartbeam MALDI-TOF/TOF and flexControl 3.0 software (Bruker). Ions were detected in reflector positive mode with acceleration voltage, ion-source acceleration voltage, reflection voltage, and reflector–detector voltage set to 19.0 kV, 16.6 kV, 21.0 kV, and 1.65 kV, respectively. Spectra were obtained over the *m/z* range 500–5,000, calibrated using Peptide Calibration Standard II (Bruker), and validated with a standard tryptic digest of BSA (Bruker).

The spectra were processed using flexAnalysis 3.0 software (Bruker). Peptides resulting from protease autolysis or impurities (such as keratin) were excluded for determination of sequence coverage. The assignment of peptides to the protein was done using BioTools 3.0 (Bruker) and Sequence Editor 3.0 software (Bruker). The sequence coverage for digestion of samples with single proteases was obtained by combining the assigned peptides of the triplicate measurements. The total sequence coverage resulted from combination of the individual digests with different proteases.

***Peptide fragment fingerprinting analysis.*** For confirmation of colicin N and C termini, peptides generated as described for PMF analysis were analyzed by tandem MS (MS/MS). Samples were processed using Autoflex III Smartbeam MALDI-TOF/TOF and flexControl 3.0 software at acceleration voltage, ion-source acceleration voltage, reflection voltage, and reflector–detector voltage set to 6.0 kV, 5.2 kV, 27.0 kV, and 1.65 kV, respectively. The precursor-ion selector for laser-assisted fragmentation was set to ±0.65% *m/z* of the parent ion. Fragment-ion spectra were processed using flexAnalysis 3.0 software and sequenced using BioTools 3.0 software.

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