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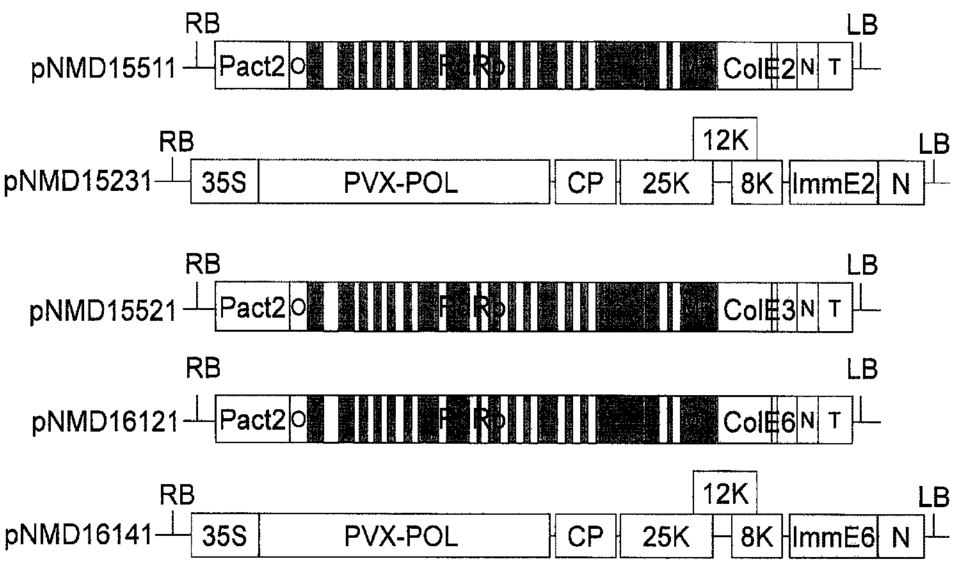

(57) Abrégé/Abstract:
The invention provides a method of preventing or reducing contamination of an object such as food with enterohaemorrhagic E. coli (EHEC), comprising contacting said object with colicin M or a derivative thereof.

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ABSTRACT

The invention provides a method of preventing or reducing contamination of an object such as food with enterohaemorrhagic *E. coli* (EHEC), comprising contacting said object with colicin M or a derivative thereof.

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Colicins for the control of EHEC

This patent application claims the priority of US provisional patent application No. 62/166,379 filed on May 26, 2015 and of European patent application No. 15 181 133.8 filed on August 14, 2015.

FIELD OF THE INVENTION

The invention provides a method of preventing or reducing contamination of food or other objects with enteropathogenic *E. coli* (EPEC) and/or enterohaemorrhagic *E. coli* (EHEC) and a use of colicin M or a derivative thereof for preventing or reducing contamination of food and other objects with EHEC. Further provided is colicin M or a derivative thereof for use in a method of treating or preventing infection with EHEC.

BACKGROUND OF THE INVENTION

Escherichia coli is a normal inhabitant of human gastro-intestinal (GI) tract, however, some *E. coli* strains are pathogenic. Enterohaemorrhagic *E. coli* (EHEC) target the small and large bowels and can cause haemorrhagic colitis and HUS (haemolytic uraemic syndrome). STEC is an abbreviation for Shiga-toxin producing *E. coli.* EHEC strains are STEC strains. Shiga-toxin (Stx) acts on the lining of the blood vessels and the vascular endothelium. The toxin penetrates into endothelial cells. When inside the cell, Stx inactivate protein synthesis leading to the death of the cell. The vascular endothelium has to continually renew itself, so this killing of cells leads to a breakdown of the lining and to hemorrhage. The first response is commonly a bloody diarrhea.

The toxin is effective against small blood vessels, such as found in the digestive tract, the kidney, and lungs, but not against large vessels such as the arteries or major veins. A specific target for the toxin appears to be the vascular endothelium of the glomerulus in the kidneys. Destroying these structures leads to kidney failure and the development of the often deadly and frequently debilitating HUS. Food poisoning with Shiga toxin often also has effects on the lungs and the nervous system.

A large number of serotypes of STEC isolated from humans is known. From studies in the USA and Canada examining human STEC infections, 50-80% were identified as being caused by E. coli O157:H7. 30 to 50% are caused by non-O157 STEC. Although E. coli O157:H7 has been most commonly identified as the cause of STEC infection, isolation of non-O157 STEC strains from clinical cases, outbreaks and environmental sources has been increasing (Posse et al., FEMS Microbiol Lett. 2008;

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282(1):124-31; Possé et al., J. Appl. Microbiol. 2008; 105(1):227-35). A study at the Center for Disease Control and Prevention showed that from 1983-2002 approximately 70% of non-O157 STEC infections in the United States were caused by strains from one of six major serogroups, namely O26, O45, O103, O111, O121 and O145 (Brooks *et al.*, 2005). Virulence factors for non-O157 STEC include, but are not limited to, production of the shiga-like toxins 1 and/or 2 (Stx1, Stx2) and intimin (*eae*). USDA-FSIS (United States Department of Agriculture (USDA) and Food Safety and Inspection Service (FSIS)) defined the so-called "Big 7" STEC panel: O157 and O26, O45, O103, O111, O121 and O145 serotypes. These are considered as most dangerous STEC serotypes ("adulterants"). EHEC serotypes are generally classified using the O antigen which is a part of the lipopolysaccharide layer, and the H antigen that is flagellin.

Prevention of EHEC or reducing contamination of food with EHEC requires, according to the WHO, control measures at all stages of the food chain, from agricultural production on the farm to processing, manufacturing and preparation of foods in both commercial establishments and household kitchens. As to industry, the WHO recommends that the number of cases of disease might be reduced by various mitigation strategies for ground beef (for example, screening the animals pre-slaughter to reduce the introduction of large numbers of pathogens in the slaughtering environment). Good hygienic slaughtering practices reduce contamination of carcasses by faeces, but do not guarantee the absence of EHEC from products. Education in hygienic handling of foods for workers at farms, abattoirs and those involved in the food production is essential to keep microbiological contamination to a minimum. So far, the only effective method of eliminating EHEC from foods is to introduce a bactericidal treatment, such as heating (e.g. cooking, pasteurization) or irradiation (see: http://www.who.int/mediacentre/factsheets/fs125/en/).

Treatment of EHEC infections in humans is difficult. A multitargeted approach is generally recommended including general supportive measures, anti-platelet and thrombolytic agents and thrombin inhibitor, selective use of antimicrobials, probiotics, toxin neutralizers and antibodies against key pathogenic pathway elements (Goldwater et al., BMC Medicine 2012, 10:12).

Most of the above mentioned methods of preventing EHEC or reducing contamination with EHEC are methods that are essentially independent from a particular pathogenic bacterium or from a particular serotype of EHEC. This has the advantage that little prior knowledge of the specific EHEC serotype in question is necessary before counter-measures are taken. However, the above mentioned methods of preventing EHEC or reducing contamination with EHEC such as heating or irradiation are not always applicable or change the treated good or food in undesirable ways. Other methods may have turned out non-effective with a particular patient. There is therefore a need for further methods of preventing or treating EHEC infections or methods for reducing or preventing contamination of objects with EHEC.

It is an object of the invention to provide methods for preventing or treating EHEC infections such as food-borne EHEC infections. It is another object to provide methods for preventing or reducing contamination of objects, notably, food with EHEC. It is a further object to provide methods for preventing or treating EHEC infections and/or methods for reducing contamination of objects with EHEC, that are effective against a wide range of EHEC serogroups such as the Big 7 or Big 6 groups of serotypes.

SUMMARY OF THE INVENTION

This problem has been solved by the following:

(1) A method of preventing or reducing contamination of an object such as food with EPEC or enterohaemorrhagic *E. coli* (EHEC), comprising contacting said object with colicin M or a derivative thereof.

(2) The method according to item 1, wherein contamination of an object such as food with EHEC serotype O157:H7 is prevented or reduced.

(3) The method according to item 1, wherein contamination of an object such as food with any one or all of the following *E. coli* serotypes is prevented or reduced: serotype O26:H11, serotype O45:H2, serotype O103:H11, serotype O111:H8, serotype O157:H7, and serotype O104:H4.

(4) The method according to item 1, wherein contamination of an object such as food with any one or all of the following *E. coli* serotypes is prevented or reduced: serotype O26:H11, serotype O45:H2, serotype O103:H11, serotype O111:H8, serotype O145:NM, serotype O157:H7, and serotype O104:H4.

(5) The method according to item 1, wherein said object is contacted with an aqueous solution of colicin M or its derivative by spraying with said aqueous solution or by dipping said object into said aqueous solution.

(6) The method according to item 1, wherein said food is immersed for at least 10 seconds, preferably for at least 1 minute, preferably for at least 5 minutes into an aqueous solution containing colicin M or its derivative.

(7) The method according to any one of items 1 to 6, wherein said colicin M or its derivative is produced by expression in a plant or in plant cells, followed by removing undesired components from said plant or said plant cells.

(8) The method according to any one of items 1 to 7, wherein said food is meat, raw fruit or raw vegetable.

(9) The method according to any one of items 1 to 8, wherein said colicin M has the amino acid sequence of SEQ ID NO: 1.

(10) The method according to any one of items 1 to 8, wherein the toxicity of the derivative of colicin M is such that the derivative and the colicin M of SEQ ID NO: 1 produce spots free of viable bacteria of sensitive *E. coli* strain DH10B of the same diameter 12 hours after spotting 5 microliters of a solution of said derivative of colicin M and the colicin M of SEQ ID NO: 1 onto a lawn of the sensitive *E. coli* strain on an agar plate and subsequent incubation of the agar plate at 37°C, wherein the concentration of the derivative of colicin M is at most 5 times that of the comparative solution of the colicin M of SEQ ID NO: 1.

(11) The method according to any one of items 1 to 10, wherein said derivative of colicin M comprises the C-terminal activity domain of residues 141 to 271 of colicin M or an activity domain having from 1 to 30, preferably from 1 to 20, amino acid substitutions, insertions and/or deletions compared to residues 141 to 271 of SEQ ID NO: 1.

(12) The method according to any one of items 1 to 11, wherein said derivative of colicin M comprises the central receptor-binding domain of residues 36 to 140 of colicin M or an activity domain having from 1 to 10 amino acid substitutions, insertions and/or deletions compared to residues 36 to 140 of SEQ ID NO: 1.

(13) The method according to any one of items 1 to 12, wherein said derivative of colicin M has amino acid residues 1 to 35 of SEQ ID NO: 1 or has from 1 to 8, preferably from 1 to 4, amino acid substitutions, insertions and/or deletions compared to residues 1 to 35 of SEQ ID NO: 1.

(14) The method according to any one of items 1 to 13, wherein said colicin M or its derivative is used in combination with one, several or all colicins selected from the group consisting of colE7, colB, colIa, colU, colK, and col5, or derivatives thereof; or said colicin M or its derivative is used in combination with colicin Ib.

(15) The method according to any one of items 1 to 13, wherein contamination with any one, several or all of the following *E. coli* serotypes is prevented or reduced: O26:H11, O45:H2, O103:H11, O111:H8, O145:NM, O157:H7, O104:H4, and O121:H19.

(16) A method of preventing or reducing contamination of an object such as food with EPEC or enterohaemorrhagic *E. coli* (EHEC), comprising contacting said object with colicin Ia or colicin Ib or a derivative thereof; preferably contamination of an object with EHEC O121:H19 is prevented or reduced.

(17) An object such as food treated with colicin M or a derivative thereof or treated with colicin Ia or colicin Ib or a derivative thereof.

(18) Use of colicin M or a derivative thereof or colicin Ib or a derivative thereof for the manufacture of a medicament for treating or preventing infection with EHEC.

(19) A composition such as an aqueous solution comprising colicin M or a derivative thereof.

(20) A composition such as an aqueous solution comprising colicin Ia or colicin Ib or derivatives thereof.

(21) The composition according to item 19 or 20, further comprising one or more further colicin selected from the group consisting of colM, colE7, colB, colIa, colU, colK, and col5, or a derivative of any of the before-mentioned colicins, preferably colM or colE7 or a derivative thereof.

(22) The composition according to item 19 or 21, further comprising one or more further colicin selected from the group consisting of colicin E5, colicin E8, colicin E9, colicin A, colicin S4, colicin 10, colicin R, colicin 28b, colicin Y, colicin Ia, colicin Ib, and cloacin DF13, or a derivative of colicin E5, colicin E8, colicin E9, colicin A, colicin S4, colicin 10, colicin R, colicin 28b, colicin Y, colicin Ib, and cloacin DF13; preferably cloacin DF13, colicin R or colicin Ib or derivatives thereof, more preferably colicin Ib or a derivative thereof.

(23) A composition such as an aqueous solution comprising any one or more colicin selected from colicin M, colicin E7, colicin B, colicin Ia, colicin U, colicin K, colicin 5, colicin E5, colicin E8, colicin E9, colicin A, colicin S4, colicin 10, colicin R, colicin 28b, colicin Y, colicin Ib, and cloacin DF13, or a derivative of colicin M, colicin E7, colicin B, colicin Ia, colicin U, colicin K, colicin 5, colicin E5, colicin E8, colicin E9, colicin A, colicin S4, colicin 10, colicin R, colicin 28b, colicin Y, colicin Ib, and cloacin DF13.

(24) A process of producing a purified colicin or a colicin-containing preparation, comprising:

(i) expressing said colicin in a plant from a nucleic acid construct encoding said colicin;

(ii) homogenizing the plant containing expressed colicin to produce a homogenate, optionally followed by removing solid or insoluble material;

(iii) acidifying the homogenate or a clarified fraction thereof to a pH of below pH5, followed by removal of insoluble material, to obtain a colicin-containing solution;

(iv) neutralizing the clarified colicin-containing solution, followed by removal of insoluble material;

(v) optionally concentrating the colicin-containing solution obtained in the previous step;

(vi) optionally freeze-drying the solutions obtained in step (iv) or (v) to obtain a freeze-dried preparation of said colicin.

(25) The process according to item 24, wherein said plant is an edible plant, such as beet, spinach, chicory or lettuce.

(26) The process according to item 24, further comprising purifying, subsequent to step (iv) or (v), said colicin by column chromatography, preferably by cation exchange chromatography.

(27) The process according to any one of items 24 to 26, wherein said colicin is colM, colE7, colB, colIa, colU, colK, col5, cloacin DF13, colicin R, colicin Ia, or colicin Ib or derivatives thereof; preferably colM, colE7, colicin Ia, or colicin Ib, or a derivative thereof, more preferably said colicin is colicin M or a derivative thereof.

(28) A method of preventing infection of a mammal with enterohaemorrhagic *E. coli*, comprising treating the mammal with colicin or a combination of colicins as described above.

(29) A use of a colicin or a combination of colicins as described above for the preparation of a medicament for preventing infection of a mammal with enterohaemorrhagic *E. coli.*

(30) A method for reducing the load of EHEC in ruminants such as cattle or sheep, comprising treating the ruminants with colicin or a combination of colicins as described above.

(31) A use of a colicin or a combination of colicins as described above for reducing the load of EHEC in ruminants such as cattle or sheep, comprising treating the ruminants with colicin or a combination of colicins as described above.

The invention also provides the use of colicin M or a derivative thereof (as defined herein) for reducing contamination of an object such as food with EHEC. The invention also provides the use of colicin Ia and/or Ib or a derivative thereof for reducing contamination of an object such as food with EHEC, notably EHEC strain O121:H19.

The inventors have surprisingly found a colicin having at the same time high activity against several EHEC serotypes and an exceptionally low specificity for particular EHEC strains or serotypes, i.e. a wide activity or toxicity against several EHEC strains. Thus, the invention allows preventing or reducing contamination of food and other objects with EHEC, generally without prior knowledge of the specific EHEC serotype having contaminated or that may contaminate the object. Further, the invention allows preventing an infection with EHEC in a patient.

The present invention as claimed relates to:

[1] a method of preventing or reducing contamination of an object with enterohaemorrhagic *E. coli* (EHEC), comprising contacting said object with colicin M or a derivative thereof, wherein
(A) said colicin M or its derivative has an N-terminal translocation domain as follows:
the N-terminal translocation domain has amino acid residues 1 to 35 of SEQ ID NO: 1,

or has from 1 to 4 amino acid substitutions, insertions, additions and/or deletions compared to amino acid residues 1 to 35 of SEQ ID NO: 1, or has an amino acid sequence identity of at least 90% to the N-terminal translocation domain of SEQ ID NO: 1, or has an amino acid sequence similarity of at least 95% to the N-terminal translocation domain SEQ ID NO: 1;

(B) said colicin M or its derivative comprises a central receptor-binding domain as follows: the receptor-binding domain has residues 36 to 140 of colicin M of SEQ ID NO: 1, or has a domain having from 1 to 10 amino acid substitutions, insertions, additions and/or deletions compared to residues 36 to 140 of SEQ ID NO: 1, or has an amino acid sequence identity of at least 90% to the receptor-binding domain of SEQ ID NO: 1, or has an amino acid sequence similarity of at least 95% to the receptor-binding domain SEQ ID NO: 1; and

(C) said colicin M or its derivative comprises a C-terminal activity domain as follows: the activity domain is of residues 141 to 271 of SEQ ID NO: 1, or the activity domain has from 1 to 30 amino acid residue substitutions, insertions, additions and/or deletions compared to amino acid residues 141 to 271 of SEQ ID NO: 1, or the activity domain has an amino acid sequence identity of at least 80% to the activity domain of SEQ ID NO: 1, or the activity domain has an amino acid sequence similarity of at least 90% to the activity domain of SEQ ID NO: 1; and

[2] a process of producing a colicin preparation, comprising: (i) expressing said colicin in a plant from a nucleic acid construct encoding said colicin; (ii) homogenizing the plant containing expressed colicin to produce a homogenate, optionally followed by removing solid or insoluble material; (iii) acidifying the homogenate or a clarified fraction thereof to a pH of below pH5, followed by removal of insoluble material, to obtain a colicin-containing solution; (iv) neutralizing the clarified colicin-containing solution, followed by removal of insoluble material; (v) optionally concentrating the colicin-containing solution obtained in the previous step; (vi) optionally freeze-drying the solutions obtained in step (iv) or (v) to obtain a freeze-dried preparation of said colicin, wherein said colicin is colicin M or a derivative thereof as defined in [1] above.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows schematically viral vectors for the expression of colicins and corresponding immunity proteins used in the Examples. Constructs for the expression of colicins are based on Tobacco mosaic virus (TMV), whereas constructs for the expression of immunity proteins are based on Potato virus X (PVX).

Colicin expression vectors include pNMD15511, pNMD15521 and pNMD16121 for the expression of colicins E2, E3 and E6, respectively (Fig. 1A), pNMD8802, pNMD10221, pNMD3680, pNMD15252 and pNMD15291 for the expression of colicins E7, M, N, K and B, respectively (Fig. 1B), and pNNMD15271, pNMD15311, pNMD19141 and pNMD19162 for the expression of colicins U, 5, Ia and D, respectively (Fig. 1C).

RB and LB stand for the right and left borders of T-DNA of binary vectors. Pact2: promoter of *Arabidopsis* actin2 gene; o: 5' end from TVCV (turnip vein clearing virus); RdRp: RNA-dependent RNA polymerase open reading frame (ORF) from cr-TMV (crucifer-infecting tobamovirus); MP: movement protein ORF from cr-TMV; ColE2: colicin E2 coding sequence; ColE3: colicin E3 coding sequence; ColE6: colicin E6 coding sequence; ColM: colicin M coding sequence; ColN: colicin N coding sequence; ColK: colicin K coding sequence; ColB: colicin B coding sequence; ColU: colicin U coding sequence; Col5: colicin 5 coding sequence; ColIa: colicin Ia coding sequence; ColD: colicin D coding sequence; N: 3'-non-translated region from cr-TMV; T: *Agrobacterium* nopaline synthase terminator; white segments interrupting grey segments in the RdRp and MP ORFs indicate introns inserted into these ORFs for increasing the likelihood of RNA replicon formation in the cytoplasm of plant cells, which is described in detail in WO2005049839. An intron was also inserted into ColE2, ColE3, ColE6, ColE7, ColM and ColD ORFs for preventing the cytotoxic effect of these proteins on *E. coli* cells used for plasmid cloning.

PVX-based vectors for the expression of immunity proteins include pNMD15231 and pNMD16141 for the expression of colicin E2 and colicin E6 immunity proteins, respectively (Fig. 1A), pNMD9060 for the expression of colicin E7 immunity protein (Fig. 1B), and pNMD15371 for the expression of colicin D immunity protein (Fig. 1C). P35S: cauliflower mosaic virus 35S promoter; PVX-pol: RNA-dependent RNA polymerase from PVX; CP: coat protein ORF; 25K, 12K and 8 together indicate the 25KDA, 12 kDa and 8 kDa triple gene block modules from PVX; N: 3'-untranslated region from PVX. ImmE2, ImmE6, ImmE7 and ImmD stand for coding sequences of colicins E2, E6, E7 and D immunity proteins, respectively.

Figure 2 shows schematically viral vectors for Colicin M expression used in the Examples. pNMD10221 and pNMD10240 constructs are based on Tobacco mosaic virus (TMV); pNMD11740 and pNMD14500 vectors are Potato virus X (PVX)-based. ColM: Colicin M coding sequence with codon usage optimized for *Nicotiana benthamiana*; CTP: chloroplast targeting peptide.

Figure 3 depicts the double-inducible viral vector pNMD18381 for ethanol-induced Colicin M expression used for stable transformation of *Nicotiana benthamiana* plants. The T-DNA region of the plasmid contains four expression cassettes: 1) neomycin phosphotransferase II coding sequence cloned under the control of nopalin synthase promoter from *Agrobacterium*; 2) coding sequence of the ethanol-sensing transcriptional activator AlcR from *Aspergillus nidulans* (GeneBank: XM_677155.1) cloned under the control of potato ST-LS1 gene promoter (GenBank: X04753.1); 3) cr-TMV replicon (with deletion, indicated by the bracket, of a movement protein coding sequence fragment and insertion of colicin M ORF) cloned under the control of the ethanol-inducible alcohol dehydrogenase (*alcA*) promoter from *Aspergillus nidulans* fused with minimal *35S* promoter sequence (Werner at al. 2011); and 4) cr-TMV movement protein coding sequence cloned under the control of *alcA* promoter.

NosT stands for nopaline synthase terminator; NPTII: neomycin phosphotransferase II for selection of transgenic plants; NosP: nopaline synthase promoter; Pstls: potato ST-LS1 gene promoter; alcR: AlcR coding sequence from *Aspergillus nidulans*; act2T: terminator of *Arabidopsis* actin2 gene; PalcA: ethanol-inducible *alcA* promoter from *Aspergillus nidulans* fused with minimal 35S promoter sequence; 35ST: cauliflower mosaic virus 35S terminator; N: 3'-non-translated region from cr-TMV; λ: the lambda insulator (999 bp long fragment between nucleotide position 31748 to 32746 of *Enterobacteria* phage lambda genome (GenBank: J02459.1); MP: movement protein ORF from cr-TMV; OcsT: terminator of octopine synthase gene from *Agrobacterium*. The position of MP deletion in TMV viral replicon is shown with brackets. Arrows indicate the direction of transcription.

Figure 4 shows comparative SDS-PAGE analysis of expression for colicins after the infiltration of *Nicotiana benthamiana* plants with agrobacteria carrying viral vectors. Plant leaf material was extracted with 5 volumes of the buffer containing 50 mM HEPES (pH7.0), 10 mM potassium acetate, 5 mM magnesium acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween-20 and 300 mM NaCl. Protein extracts were resolved in 12% polyacrylamide gels. For gel loading, aliquots containing either 8 µg of total soluble protein (TSP) (**A**) or the extract volumes corresponding to 1.5 mg fresh weight of plant tissue (**B**) were used. Before loading on the gel, aliquots of protein extracts were mixed with Laemmli buffer in the proportion 1:1 and incubated at 95°C for 5 min. Numerals above gel lanes stand for protein extracts from plant tissues expressing the following recombinant proteins: 1 - colicin E2; 2 - colicin E3; 3 - colicin E6; 4 - colicin E7; 5 - colicin D; 6 - colicin N; 7 - colicin K; 8 - colicin 5; 9 - colicin U; 10 - colicin B; 11 - colicin Ia; 12 – colicin M. Numeral 13 corresponds to the extract from uninfected leaf tissue used as a negative control. L – PageRulerTM Prestained Protein Ladder (Fermentas, #SM0671). Arrows indicate specific protein bands corresponding to expressed recombinant colicins.

Figure 5 shows the semi-quantitative evaluation of specific antimicrobial activity of colicin-containing plant extracts against Big 7 EHEC strains. The antimicrobial activity was tested using radial diffusion assay via a spot-on-lawn method and calculated in arbitrary units (AU) per mg fresh weight of plant biomass expressing recombinant colicins. Thereby, it reflects the yield of specific active agent per unit of biomass; *i. e.* the specific production capacity of the host is being evaluated. Arbitrary units are calculated as a dilution factor for the highest dilution of protein extract causing the detectable clearing effect in the radial diffusion assay. Tested recombinant colicins and EHEC strains are indicated.

Figure 6 shows the semi-quantitative evaluation of specific antimicrobial activity of colicin-containing plant extracts against Big 7 EHEC strains. The antimicrobial activity was tested using radial diffusion assay via spot-on-lawn-method and calculated in arbitrary units (AU) per µg of recombinant colicin, which reflects the specific activity of colicins against particular strains; *i. e.* the specific antimicrobial potency of colicins is being evaluated. Tested colicins and EHEC strains are indicated.

Figure 7 shows the semi-quantitative evaluation of specific antimicrobial activity of plant extracts containing recombinant colicins against O104:H4 strain of EHEC. The antimicrobial activity is expressed in either arbitrary units (AU) per mg fresh weight of plant biomass (**A**) or arbitrary units (AU) per µg of colicin (**B**).

Figure 8 shows SDS-PAGE analysis of Colicin M expression after the infiltration of *N. benthamiana* plants with agrobacteria carrying TMV and PVX viral vectors. 15 µl aliquots of total soluble extracts at different harvesting time points were resolved in 12% polyacrylamide gels. *Nicotiana benthamiana* plants were inoculated with GV3101 strain of *Agrobacterium tumefaciens* carrying either TMV-based vector pNMD10220 (lane 1) or PVX-based vector pNMD11740 (lane 2).

Figure 9 shows SDS-PAGE analysis of Colicin M expression in edible plants. Spinach *Spinacea oleracea* (**A**) and sea beets *Beta vulgaris* ssp *maritima* (**B**) plants were infiltrated with suspension of *Agrobacteria* carrying TMV-based viral vector pNMD10220. 15 µl aliquots of total soluble extracts at different harvesting time points were resolved in 12% polyacrylamide gels. Gels were stained with Coomassie blue. Plants were inoculated with either ICF320 (lane 1) or GV3101 (lane 2) strains of *Agrobacterium tumefaciens*.

Figure 10 shows the analysis of stable transgenic *Nicotiana benthamiana* plants for ethanol-inducible Colicin M expression. 7.5 µl aliquots of Laemmli buffer extracts of non-induced and induced (4 days post induction) plant material were analyzed by SDS-PAGE (12% gel) with Coomassie staining. NbWT: non-transgenic wild type *Nicotiana benthamiana* plants; Nb18381T0#41, Nb18381T0#42, Nb18381T0#43, Nb18381T0#44 and Nb18381T0#45: independent lines of primary transformants (T0 generation) obtained using pNMD18381 construct; pNMD18381 transient: transient delivery of pNMD18381 construct using agroinfiltration; "-": no induction; "+": with ethanol induction.

Figure 11 shows the reduction of *E. coli* O157:H7 (DSM19206) cell population in contaminated steak meat pieces by treatment with two-component colicin mixture comprising colicin M and colicin E7.

Figure 12 shows the reduction of E. coli O157:H7 (DSM19206) cell population in contaminated fresh-cut RTE cantaloupe melon pieces by treatment with two-component colicin mixture comprising colicin M and colicin E7.

Figure 13 shows the reduction of *E. coli* O157:H7 (DSM19206) cell population on contaminated fresh-cut RTE apple pieces by treatment with a two-component colicin mixture containing colicin M and colicin E7 and with a five-component colicin mixture comprising colicins M, E7, K, B and 5.

Figure 14 shows the reduction of *E. coli* O157:H7 (DSM19206) cell population on contaminated fresh arugula leaves by treatment with a two-component colicin mixture containing colicin M and colicin E7.

Figure 15 shows the reduction of *E. coli* O157:H7 (DSM19206) cell population on contaminated beef steak meat by treatment with a four-component colicin mixture containing colicin M, E7, Ia and K.

Figure 16 shows the reduction of *E. coli* O157:H7 (DSM19206) cell population in ground beef meat by treatment with a four-component colicin mixture containing colicin M, E7, Ia and K.

Figure 17 Summarized process (flow) diagram for colicin M production in plants.

Figure 18 Summary of purification of colicin M from *N. benthamiana* plants. The SDS-PAGE gel shows molecular weight marker in lane M. The initial green homogenate is shown in lane S1; the clarified acidic extract is shown in lane S2 and the neutralized, filtered extract in lane S3; lane S4 shows the UF concentrate; lane S5 and S6 show the retentate and permeate of diafiltration. S7 is the clarified Fractogel SO3- load, S8 corresponds to column flow through, S9 is the column eluate and E2 corresponds to the tailing part of elution peak. Lane S10 and S11 are permeate and retentate of the final formulation in 10 mM Citrate, 137 mM NaCl pH 7.3. The final retentate final retentate (S11) corresponds to colicin isolate.

Figure 19 shows schematically viral vectors for the expression of colicins E5, E8, E9, A, S4, 10, R, 28b, Y, Ib, and cloacin DF13, and immunity proteins for colicins E5, E8, E9, and cloacin DF13 used in the examples. Constructs for the expression of colicins are based on Tobacco mosaic virus (TMV), whereas constructs for the expression of immunity proteins are based on Potato virus X (PVX).

Constructs for the expression of colicins are shown in Fig. 14A. ColX stands for colicin coding sequence. All other designations as described in the legend to Fig. 1. Coding sequences of colicins E5, E8, E9, Y, and cloacin DF13 contain the intron, which was inserted to prevent the cytotoxic effect of these proteins on *E. coli* cells used for plasmid cloning. The positions of intron insertion are represented in the table.

Constructs for the expression of colicin immunity proteins are shown Fig. 14B. ImmP stands for the coding sequence of colicin immunity protein. All other designations as described in the legend to Fig. 1.

transcription.

Figure 20 shows comparative SDS-PAGE analysis of expression for colicins E5, E8, E9, A, S4, 10, R, 28b, Y, Ib, and cloacin DF13 after the infiltration of *N. benthamiana* plants with agrobacteria carrying viral vectors.

Plant leaf material was extracted with 5 volumes of 2xLaemmli buffer containing 125 mM Tris-HCl (pH6.8), 4% SDS, 20% (v/v) glycerol, 10% 2-mercaptoethanol, and 0,002% bromophenol blue (Fig. 15A), or with 5 volumes of the buffer containing 50 mM HEPES (pH7.0), 10 mM potassium acetate, 5 mM magnesium acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween-20 and 300 mM NaCl. Protein extracts were resolved in 12% polyacrylamide gels. For gel loading, aliquots containing extract volumes corresponding to 1.5 mg fresh weight of plant tissue were used. Plant extracts were loaded in the next order: 1 - colicin E8; 2 - colicin E8 + Immunity Protein E8; 3 - colicin E9; 4 - colicin E9 + Immunity Protein E9; 5 - colicin A; 6 - colicin S4; 7 - colicin 10; 8 - colicin R; 9 - colicin 28b; 10 - colicin Y; 11 - colicin Ib; and 12 – uninfected leaf tissue used as a negative control. L – Protein Mass Ladder. Arrows show specific protein bands corresponding to expressed recombinant colicins. Expected protein molecular masses are: colicin E8 – 61 kDa, colicin E9 – 62 kDa, colicin A – 63 kDa; colicin S4 – 54 kDa; colicin 10 – 53 kDa; colicin R – 68 kDa; colicin 28b – 48 kDa; colicin Y – 67 kDa; colicin Ib – 70 kDa.

Figure 21 shows the semi-quantitative evaluation of specific antimicrobial activity of colicin-containing plant extracts against Big 7 EHEC strains. The antimicrobial activity for colicins E5, E8, E9, A, S4, 10, R, 28b, Y, Ib, and cloacin DF13 was tested using radial diffusion assay via spot-on-lawn-method and calculated in arbitrary units (AU) per mg fresh weight of plant biomass expressing recombinant colicins. Arbitrary units are calculated as a dilution factor for the highest dilution of protein extract causing the detectable clearing effect in the radial diffusion assay. Tested recombinant colicins and EHEC strains are indicated.

Figure 22 shows the semi-quantitative evaluation of specific antimicrobial activity of plant extracts containing recombinant colicins E5, E8, E9, A, S4, 10, R, 28b, Y, Ib, and cloacin DF13 against O104:H4 strain of EHEC. The antimicrobial activity is expressed in arbitrary units (AU) per mg fresh weight of plant biomass.

DETAILED DESCRIPTION OF THE INVENTION

Colicins are plasmid-encoded cytotoxins synthesized by *Escherichia coli*, which are secreted into the medium and kill sensitive strains of *E. coli*. A colicin is sometimes abbreviated "Col" herein. Four cytotoxic classes of colicin have thus far been identified according to the mechanism by which they kill sensitive strains of *E. coli*:

- pore-forming colicins such as ColA, ColE1, ColN, ColK, Colla, Collb, and ColD, which kill cells by causing membrane depolarization
- RNase colicins, such as ColE3, ColE4, ColE6, and cloacin DF13, which specifically cleave 16S ribosomal, or ColD and ColE5, which cleave the anticodon loops of distinct tRNA
- DNase colicins such as ColE2, ColE7, ColE8, and ColE9, which are nonspecific endonucleases
- inhibitors of cell wall synthesis such as ColM.

Colicins from the above-mentioned groups have been characterized to some extent, and suggestions for practical applications of some colicins have been made. However, colicins are generally highly specific for their target *E. coli* strain or serotype, which is presumably due to the fact that colicins need specific receptor binding for entering the compartment of the target cells where they can exert their function. Thus, even where their mode of action would, in principle, allow broad antibacterial activity, the mechanism of internalization into target cells or compartments thereof generally prevents activity against diverse strains or serotypes of potential target *E. coli* cells. Thus, for practical application in antibacterial measures, prior knowledge of the target EHEC serotype would be necessary for selecting an appropriate colicin, which is time-consuming, laborious and requires specially trained personnel. This is probably a reason as to why colicins have, apparently, not been used in practice for antibacterial treatment so far.

The inventors have found that, among many colicins, colicin M has a surprisingly broad (or low) target cell specificity. Consequently, colicin M and derivatives thereof can be used, even without prior determination of an EHEC serotype to be attacked, for reducing the contamination with EHEC or for reducing viable EHEC cell density on objects such as food. For analogous reasons, colicin M can be used for preventing EHEC infection in patients. Moreover, colicin M may be used for reducing the load of EHEC in the digestive tract of farm animals such as cattle, sheep, and goats.

Colicin M is a naturally occurring *E. coli*-produced protein (CAS 39386-24-8; Swiss-Prot Entry PO5820; SID 135305941, deposit date 2012-03-21). Colicin M has a molecular weight of about 29.45 kDa and consists of a single polypeptide chain of 271 amino acid residues. The amino acid sequence was filed with the GenBank database (AAA23589.1; Köck 1987) and is also shown in SEQ ID NO:1.

Colicin M is a peptidoglycanase that specifically cleaves the bond between the lipid moiety and the pyrophosphoryl group of the peptidoglycan lipid I and lipid II intermediates, located at the periplasmic side of the inner membrane (Gross and Braun, Mol. Gen. Genet. 251 (1996) 388-396; Barreteau et al., Microbial Drug Resistance 18 (2012), 222-229). The released C55-polyisoprenol no longer translocates MurNAc-pentapeptide-GlcNAc across the cytoplasmic membrane. Although the major part of colicin M produced remains inside cells and is not released into the culture medium, it does not kill the producer cells. Instead, it kills sensitive strains after it has been taken up across the outer membrane into the periplasm. Colicinogenic strains are protected against the toxin they produce by co-expression of a specific immunity protein.

The mode of action of colicin M involves the steps of adsorption to the FhuA outer membrane receptor, energy-dependent translocation through the outer cell membrane into the periplasm by the TonB import machinery (TonB, ExbB and ExbD), and catalytic action of its substrate. Each of these steps is performed by a specific protein domain. Accordingly, colicins share a three-domain structural organization and a narrow antibacterial spectrum. Barreteau et al., Microbial Drug Resistance 18 (2012) 222-229 reviews recent knowledge on the biology of colicin M. The three domains of colicin M are referred to as translocation domain, receptor-binding domain, and activity domain from the N- to the C-terminus. The amino acid sequence stretch of the N-terminal translocation domain is usually defined as ranging from amino acid position 1 to (and including) position 35 in SEQ ID NO: 1. The amino acid sequence stretch of the central receptor-binding domain is usually defined as ranging from amino acid position 36 to (and including) position 140 in SEQ ID NO: 1. The amino acid sequence stretch of the C-terminal activity domain is generally defined as ranging from position 141 to 271 in SEQ ID NO: 1.

In the methods of the invention, colicin M of SEQ ID NO:1 may be used or a derivative thereof. Colicin M and its derivative as defined in the following are also referred to herein collectively as "protein of interest". The derivative preferably has a peptidoglycanase activity of at least 20 % of the peptidoglycanase activity of colicin M of SEQ ID NO:1 in the standard assay with lipid I as substrate for colicin M activity described by El Ghachi cited below.

With regard to the activity domain, the amino acid sequence is not particularly limited provided the derivative has at least 20 % of the peptidoglycanase activity of colicin M of SEQ ID NO:1. In preferred embodiments, the peptidoglycanase activity is at least 40 %, more preferably at least 60 %, and most preferably at least 80 %, of the peptidoglycanase activity of colicin M of SEQ ID NO:1. The activity is determined according to the standard assay for colicin M activity described by El Ghachi *et al.*, J. Biol. Chem. 281 (2006) 22761-22772 using lipid I as the substrate.

The activity domain may, alternatively or additionally, have from 1 to 30, preferably from 1 to 20, more preferably from 1 to 15, even more preferably from 1 to 10, and most preferably from 1 to 5, amino acid residue substitutions, insertions, additions and/or deletions compared to amino acid residues 141 to 271 of SEQ ID NO: 1. The activity domain may preferably have from 1 to 30, preferably from 1 to 20, more preferably from 1 to 15, even more preferably from 1 to 10, and most preferably from 1 to 5, amino acid residue substitutions and/or terminal deletions compared to amino acid residues 141 to 271 of SEQ ID NO: 1. Herein, substitutions, insertions, additions and deletions may be combined; the number of substitutions, insertions, additions and/or (terminal) deletions given herein refers to the sum of substitutions, insertions, additions and deletions made compared to the applicable sequence stretch of SEQ ID NO: 1.

In another embodiment, the activity domain has an amino acid sequence identity of at least 80 %, preferably of at least 85%, more preferably of at least 90%, even more preferably at least 95%, and most preferably of at most 97 %, to the activity domain of SEQ ID NO: 1. Alternatively, the activity domain of the derivative has an amino acid sequence similarity of at least 90 %, preferably at least 95%, to the activity domain SEQ ID NO: 1. The conditions above based on peptidoglycanase activity and structural similarity to the activity domain of SEQ ID NO: 1 may be combined. Thus, the derivative of colicin M may have a peptidoglycanase activity as listed above and may have from 1 to 30, preferably from 1 to 20, more preferably from 1 to 15, more preferably from 1 to 10, and most preferably from 1 to 5 insertions and/or additions and/or deletions and/or substitutions compared to residues 141 to 271 of SEQ ID NO: 1. In another embodiment, the derivative of colicin M may have a peptidoglycanase activity as listed above and may have an amino acid sequence identity of at least 80 %, preferably of at least 85%, more preferably of at least 90%, more preferably at least 95% and most preferably of at most 97 %, to the activity domain of SEQ ID NO: 1.

In the derivatives of the activity domain, one or more of the following amino acid residues influence the catalytic activity and are therefore preferably those of SEQ ID NO: 1, i.e. are not altered in the derivative: P176, D226, Y228, D229, H235 and R236, preferably all of these amino acid residues are those of SEQ ID NO: 1. More preferably, any one or all of the following amino acid residues are those of SEQ ID NO: 1: P176, D226, Y228, D229, H235, R236, R222, N231, E241 and T244.

In a preferred embodiment, the activity domain of the derivative has from 1 to 20, more preferably from 1 to 15, more preferably from 1 to 10, and most preferably from 1 to 5 insertions and/or additions and/or deletions and/or substitutions (preferably substitutions and/or terminal deletions) compared to residues 141 to 271 of SEQ ID NO: 1 and all of the following amino acid residues are those of SEQ ID NO: 1: P176, D226, Y228, D229, H235, R236, R222, N231, E241 and T244.

The protein of interest has a central receptor-binding domain of residues 36 to 140 of colicin M or a receptor-binding domain having from 1 to 15, preferably from 1 to 10, more preferably from 1 to 6, and most preferably from 1 to 3 amino acid substitutions, insertions, additions, and/or deletions compared to amino acid residues 36 to 140 of SEQ ID NO: 1. The protein of interest may have a central receptor-binding domain of residues 36 to 140 of colicin M or a receptor-binding domain having from 1 to 15, preferably from 1 to 10, more preferably from 1 to 6, and most preferably from 1 to 3 amino acid substitutions and/or terminal deletions compared to amino acid residues 36 to 140 of SEQ ID NO: 1. In another embodiment, the receptor-binding domain has an amino acid sequence identity of at least 90 %, preferably of at least 95%, and most preferably of at most 97 %, to the receptor-binding domain of SEQ ID NO: 1. Alternatively, the receptor-binding domain of the derivative has an amino acid sequence similarity of at least 95 %, preferably at least 97%, to the receptor-binding domain SEQ ID NO: 1. In addition to the amino acid residues given above from the activity domain that are preferably not changed compared to SEQ ID NO: 1, P107 and P129 from the central domain are, in one embodiment, present in the colicin M derivative.

The protein of interest has an N-terminal translocation domain of amino acid residues 1 to 35 of colicin M or an N-terminal translocation domain having from 1 to 8, preferably from 1 to 4, more preferably from 1 to 2, more preferably of one amino acid substitutions, insertions, additions, and/or deletions (preferably substitutions and/or terminal deletions) compared to residues 1 to 35 of SEQ ID NO: 1. The term "terminal deletions" refers to deletions at the termini of the sequence referred to, such as SEQ ID NO: 1. In another embodiment, the N-terminal translocation domain has an amino acid sequence identity of at least 90 %, preferably of at least 95%, and most preferably of at most 97 % to the N-terminal translocation domain of SEQ ID NO: 1. Alternatively, the N-terminal translocation domain of the derivative has an amino acid sequence similarity of at least 95 %, preferably at least 97%, to the N-terminal translocation domain SEQ ID NO: 1. In one embodiment, the derivative as defined above comprises, in the N-terminal translocation domain, the TonB box of residues 2 to 7 of SEQ ID NO: 1. In the same or another embodiment, the derivative has no N-terminal amino acid residue addition compared to SEQ ID NO: 1.

A derivative of colicin M may comprise an additional C-terminal amino acid sequence stretch such as purification tags, e.g. as a His-tag of 6 or more contiguous histidine residues; the derivative has, preferably, no N-terminal amino acid residue addition.

For the purpose of determining similarity between amino acid sequences in the present invention, the amino acid residues belonging to each of the following groups are considered similar (in the standard one-letter code):

- F, Y, W
- V, I, L
- R, K, H
- D, E
- N, Q
- A, T, S

The derivative of colicin M has minimum toxicity against a colicin M-sensitive *E. coli* strain compared to colicin M of SEQ ID NO:1. The toxicity of the derivative of colicin M should be such that the derivative and the colicin M of SEQ ID NO: 1 produce spots free of viable bacteria of the sensitive *E. coli* strain of the same diameter 12 hours after spotting 5 microliters of a solution of said derivative of colicin M and the colicin M of SEQ ID NO: 1 onto a lawn of the sensitive *E. coli* strain on agar plates and subsequent incubation of the agar plates at 37°C, wherein the concentration of the derivative of colicin M is at most 5 times that of the comparative solution of the colicin M of SEQ ID NO: 1. Preferably, the concentration of the derivative of colicin M is at most 3 times, preferably at most twice that of the comparative solution of the colicin M of SEQ ID NO: 1. The colicin M-sensitive *E. coli* strain may be any sensitive *E. coli* strain. For convenience, *E. coli* strain DH10B may be used for testing the toxicity of colicin M or its derivative. Otherwise, the assay described in Example 3 may be employed. Thus, the radial diffusion assays via spot-on-lawn-method may be used.

The agar plates for the assay may be overlaid with soft agar containing cells of tested *E. coli* strain. 10x10 cm quadratic petri dishes may be poured with 15-20 ml LB agar medium (1.5% w/v agar). LB soft agar medium (0.8% (w/v) agar) is melted, 20 ml aliquots are transferred into 50 ml plastic tubes and their temperature is adapted to 50-55°C. *E. coli* overnight cultures of the test bacteria adjusted to OD600=1.0 with LB medium are added to the soft agar medium in a ratio of 200 µl bacterial culture per 20 ml medium resulting in the final OD600=0.01 or approximately $1x10^7$ cells/ml. Raw colicin preparations such as plant leaf material containing expressed colicin M or its derivative may be extracted as described in Example 2. A 1:1 dilution series of plant extracts starting with undiluted samples by using extraction buffer may be prepared. 5 µl aliquots of total soluble protein (TSP) dilution series may be applied to the agar plates that are then incubated at 37°C overnight. Antimicrobial activity (toxicity) of the colicin M or its derivative can be evaluated visually based on clearing zones and the diameter of spots may be measured.

The above definitions of the colicin M and its derivatives of the three domains of colicin M may be combined. An embodiment of the protein of interest is as follows:

a colicin M or a derivative of colicin M comprising:

an N-terminal translocation domain having up to 4 amino acid residue substitutions, insertions, additions, and/or deletions compared to residues 1 to 35 of SEQ ID NO: 1, comprises residues 2 to 7 of SEQ ID NO: 1 and has no N-terminal addition compared to SEQ ID NO: 1;

a central receptor-binding domain having up to 10, preferably up to 6, and most preferably up to 3 amino acid residue substitutions, insertions, additions, and/or deletions compared to the amino acid sequence segment of residues 36 to 140 of SEQ ID NO: 1 and comprises P107 and P129 of SEQ ID NO: 1; and

an activity domain having up to 20, preferably up to 10 amino acid residue substitutions, insertions, additions and/or deletions compared to residues 141 to 271 of SEQ ID NO: 1 and having amino acid residues P176, D226, Y228, D229, H235 and R236 of SEQ ID NO: 1, preferably amino acid residues P176, D226, Y228, D229, H235, R236, R222, N231, E241 and T244 of SEQ ID NO: 1.

The colicin M or its derivatives to be used according to the invention may be produced by known methods of protein expression in a standard expression system. For producing the colicin M or its derivative, a nucleotide sequence encoding the colicin M or its derivative may be expressed in a suitable host organism. Methods for producing and purifying colicin M have been described in the prior art and any such methods may be used. An expression method employing an *E. coli* expression system was described by Zeth et al., J. Biol. Chem. 283 (2008) 25324-25331. If eukaryotic expression systems are used, one or more introns may be inserted in the coding sequence of the colicin for preventing toxic effects on bacteria used for cloning.

Particularly efficient expression methods are plant expression systems that are known in the prior art. Plant expression systems for expressing colicin M or a derivative thereof and for expressing other colicins or derivatives thereof are described in the Examples. A possible way of achieving expression of a nucleotide sequence of interest in plants is the use of self-replicating (viral) replicons containing the nucleotide sequence encoding the colicin M or its derivative, or encoding another colicin or its derivative. Plant viral expression systems have been described in many publications, such as in WO2008028661, WO2006003018, WO2005071090, WO2005049839, WO2006012906, WO02101006, WO2007137788 or WO02068664 and many more publications are cited in these documents. Various methods for introducing a nucleic acid molecule, such as a DNA molecule, into a plant or plant part for transient expression are known. Agrobacteria may be used for transfecting plants with the nucleic acid molecule (vector) or nucleic acid construct e.g. by agroinfiltration or spraying with agrobacterial suspensions. For references see WO 2012/019660, WO 2014/187571, or WO 2013/149726.

In embodiments wherein strong expression of the protein of interest (or other colicin) is desired, a nucleic acid construct containing the nucleotide sequence encoding the colicin M or its derivative (or encoding another colicin) may encode a viral vector that can replicate in plant cells to form replicons of the viral vector. In order to be replicating, the viral vector and the replicons contain an origin of replication that can be recognized by a nucleic acid polymerase present in plant cells, such as by the viral polymerase expressed from the replicon. In case of RNA viral vectors (referred to as "RNA replicons"), the replicons may be formed by transcription under the control of a promoter active in plant cells, from the DNA construct after the latter has been introduced into plant cell nuclei. In case of DNA replicons, the replicons may be formed by recombination between two recombination sites flanking the sequence encoding the viral replicon in the DNA construct, e.g. as described in WO00/17365 and WO 99/22003. If the replicon is encoded by the DNA construct, RNA replicons are preferred. Use of DNA and RNA viral vectors (DNA or RNA replicons) has been extensively described in the literature over the years. Some examples are the following patent publications: WO2008028661, WO2007137788, WO 2006003018, WO2005071090, WO2005049839, WO02097080, WO02088369, WO02068664. An example of DNA viral vectors are those based on geminiviruses. For the present invention, viral vectors or replicons based on plant RNA viruses, notably those based on plus-sense single-stranded RNA viruses may be used. Accordingly, the viral replicon may be a plus-sense single-stranded RNA replicon. Examples of such viral vectors are those based on tobacco mosaic virus (TMV) and potexvirus X (PVX). "Based on" means that the viral vector uses the replication system such as the replicase and/or other proteins involved in replication of these viruses. Potexvirus-based viral vectors and expression systems are described in EP2061890 or WO2008/028661.

The colicin such as colicin M or its derivative may be expressed in a multi-cellular plant or a part thereof, notably a higher plant or parts thereof. Both monocot and dicot (crop) plants can be used. Common plants usable for expressing the protein of interest include Nicotiana benthamiana, Nicotiana tabacum, spinach, Brassica campestris, B. juncea, beets (Beta vulgaris), cress, arugula, mustard, Strawberry, Chenopodium capitatum, lettuce, sunflower, cucumber, Chinese cabbage, cabbage, carrot, green onion, onion, radish, lettuce, field peas, cauliflower, broccoli, burdock, turnip, tomato, eggplant, squash, watermelon, prince melon, and melon. Expression in edible plants may be used for preventing contamination of the plants or food made therefrom with EHEC. Expression in edible plants may also be used for preparing a colicin-containing composition that may, in turn, be used for contacting an object such as food for preventing or reducing contamination of the object with EHEC. Any remaining components in the composition from the edible plant will not be harmful for consumers if the composition is used for treating food. Examples of edible plants are those listed above, except *Nicotiana benthamiana* and *Nicotiana tabacum*. In another embodiment, plants are used that do not normally enter the food chain such as *Nicotiana* species such as *N. tabacum* and *N. benthamiana* may be used.

Generally, the protein of interest (or other colicin) is expressed in the cytosol of cells of the plants or plant parts. In this case, no signal peptide directing the protein of interest into a particular compartment is added to the enzyme. Alternatively, the protein of interest can be expressed in or targeted into chloroplasts of the plants; in the latter case, an N-terminal pre-sequence, generally referred to as plastid transit peptide or chloroplast targeting peptide, is added to the N-terminal or C-terminal end, preferably the C-terminal end, of the protein of interest.

Expressed colicin such as colicin M or its derivative may be purified from the cells or plants having expressed it. The method of Zeth et al., J. Biol. Chem. 283 (2008) 25324-25331 employing affinity purification via a His-tag at the N-terminus of colicin M may be employed. If the colicin or its derivative such as colicin M or the derivative thereof is expressed in plants, the plants or tissue thereof having expressed protein may be harvested, the tissue may be homogenized, and insoluble material may be removed by centrifugation or filtration. If relatively pure colicin (such as colicin M or its derivative) is desired, the solution that may have been further purified by acid extraction and subsequent neutralization and removal of insoluble material, may be subjected to cation-exchange chromatography which can remove other host-cell proteins and plant metabolites such as polyphenols. Purified colicin solutions such as colicin M solutions may be concentrated and/or freeze-dried.

Accordingly, the invention provides processes of producing a colicin such as colicin M or a derivative thereof, or a colicin-containing preparation. In a first general process, the invention provides a process of producing a colicin such as colicin M or a derivative thereof, or a colicin-containing preparation, comprising:

(i) expressing said colicin in a plant from a nucleic acid construct encoding said colicin;

(ii) harvesting the plant and homogenizing the plant or material of the plant containing expressed colicin to produce a homogenate, optionally followed by removing solid or insoluble material;

(iii) acidifying the homogenate or a clarified fraction thereof to a pH of below pH5, followed by removal of insoluble material, to obtain a colicin-containing solution;

(iv) neutralizing the clarified colicin-containing solution, followed by removal of insoluble material;

(v) optionally concentrating the colicin-containing solution obtained in the previous step; and

(vi) optionally freeze-drying the solutions obtained in step (iv) or (v) to obtain a freeze-dried preparation of said colicin.

If the colicin is expressed in edible plants, crude protein extracts from the edible plants or semi-purified concentrates may be used for preventing or reducing contamination of an object such as food with EHEC. Therefore, in the first general process, the plant in which the colicin (or its derivative) is expressed is preferably an edible plant. It is understood that, in the context of the production processes, references to the colicins includes the derivatives of the colicins.

In a second general process, the invention provides a process of producing a colicin such as colicin M or a derivative thereof, or a colicin-containing preparation, comprising:

(i) expressing said colicin in a plant from a nucleic acid construct encoding said colicin;

(ii) harvesting the plant and homogenizing the plant or material of the plant containing expressed colicin to produce a homogenate, optionally followed by removing solid or insoluble material;

(iii) acidifying the homogenate or a clarified fraction thereof to a pH of below pH5, followed by removal of insoluble material, to obtain a colicin-containing solution;

(iv) neutralizing the clarified colicin-containing solution, followed by removal of insoluble material;

(v) optionally concentrating the colicin-containing solution obtained in the previous step;

(vi) purifying said colicin by column chromatography, preferably by cation exchange chromatography; and

(vii) optionally freeze-drying the solutions obtained in step (iv) or (v) to obtain a freeze-dried preparation of said colicin.

The second general process leads to high purity colicin. Preferably, the plant in the second general process is *N. tabacum* or *N. benthamiana*.

In the above production processes, the colicin may be colicin M, colicin E7, colicin B, colicin Ia, colicin U, colicin K, colicin 5, colicin E5, colicin E8, colicin E9, colicin A, colicin S4, colicin 10, colicin R, colicin 28b, colicin Y, colicin Ia, colicin Ib, or cloacin DF13, or a derivative of any of these colicins. A preferred colicin is colicin M or its derivative.

Colicin M or its derivative may be used in the invention for preventing or reducing contamination of an object such as food with EHEC. Contamination of an object with EHEC means adhesion of viable EHEC cells to the object. Reducing contamination with EHEC means reducing the number of viable EHEC cells adhering to the object. Determining contamination of objects with EHEC is part of the general knowledge. For example, dilution plating of solutions or dispersions of homogenized food as done in Examples 8 and 9 or dilution plating of rinsing solution of other objects may be used, followed by counting bacterial cells.

Colicins generally have a very narrow toxicity range against EHEC serotypes. The inventors have found that colicin M not only has toxicity against an exceptionally broad range of target EHEC, but also higher toxicity against several EHEC serotypes including *E. coli* O157:H7, O111:H8 and O26:H11 than many other colicins. Thus, the invention can be used for preventing or reducing contamination of an object such as food with any one or all of *E. coli* O157:H7, O111:H8 and O26:H11, preferably serotype O157:H7. The invention can further be used for preventing or reducing contamination of an object such as food with any one or all of the following *E. coli* serotypes: O26:H11, O45:H2, O103:H11, O111:H8, O157:H7, and O104:H4. The invention can further be used for preventing or reducing contamination of an object such as food with any one or all of the following *E. coli* serotypes: O26:H11, O45:H2, O103:H11, O111:H8, O145:NM, O157:H7, and O104:H4. Colicin M or a derivative thereof can further be used in a method of preventing infection of a mammal with enterohaemorrhagic *E. coli*.

For preventing or reducing contamination of an object with EPEC or EHEC, the surface of the object may be wetted with a composition such as a solution containing colicin M or its derivative. For example, the object such as food may be dipped into or sprayed with a solution of the colicin M or its derivative. The solution is preferably aqueous and may contain a buffer. The buffer may be an inorganic or organic acid or salts thereof. An example of an inorganic acid is phosphoric acid or salts thereof. Examples of the organic acid are HEPES, acetic acid, succinic acid, tartaric acid, malic acid, benzoic acid, cinnamic acid, glycolic acid, lactic acid, citric acid, and ascorbic acid. Preferred organic acids are malic acid, lactic acid, citric acid, and ascorbic acid. The pH of the solution may be from 4 to 8, preferably from 5 to 8, more preferably from 6 to 7.5. Further, the solution may contain isotonic agents such as glycerol or sodium chloride. The concentration of the colicin M or its derivative in the solution may be from 1 to 100 000 μg/l, preferably from 10 to 50 000 μg/l, more preferably from 100 to 10 000 μg/l, and even more preferably from 500 to 5 000 μg/l.

For broadening the range of target cells to be treated, colicin M or its derivative may be used in combination with any one or more other colicins such as with colE7, colB, colla, collb, colU, colK, col5, or derivatives thereof. A preferred colicin to be combined with colicin M or its derivative is colE7 or a derivative thereof. Other preferred colicins to be combined with colicin M or its derivative are colicin la (colla) or colicin lb (collb) or a derivative thereof. Thus, the composition or solution containing colicin M or its derivative may contain any one or more of these further colicins or their derivatives. For each of the other colicin to be combined with colicin M or its derivative, the same concentrations as given above for colicin M or its derivative may be used. Alternatively, the total concentration of all colicins combined (including any colicin derivatives) may be as those given above for colicin M.

When colicin M or its derivative is used in combination with one or more other colicins, the spectrum of EHEC serotypes, contamination with which can be reduced or prevented, increases. In one embodiment, colicin M or its derivative is combined with any one or more or all of colE7, colB, colla, collb, colU, colK, col5 (or derivatives thereof), and contamination with any one or all of the following *E. coli* serotypes is prevented or reduced: serotypes O26:H11, O45:H2, O103:H11, O111:H8, O145:NM, O157:H7, O104:H4, and O121:H19. In a preferred embodiment, colicin M or its derivative is combined with colicin E7 or a derivative thereof for preventing or reducing contamination of an object such as food with any one or all O26:H11, O45:H2, O103:H11, O111:H8, O145:NM, O157:H7, O104:H4, and O121:H19.

After wetting, the wetted object may be further processed or may be left to dry. In the case of food, the wetted food may be further processed such as by slicing or grinding and/or may be packed for shipping to customers or prepared for consumption.

In other embodiments, colicin M or its derivative may be used in combination with any one or more other colicins selected from the group consisting of colicin E5, colicin E8, colicin E9, colicin A, colicin S4, colicin 10, colicin R, colicin 28b, colicin Y, colicin Ia, colicin Ib, and cloacin DF13 or a derivative of E5, colicin E8, colicin E9, colicin A, colicin S4, colicin 10, colicin R, colicin 28b, colicin Y, colicin Ia, colicin Ib, and cloacin DF13.

The invention also provides a composition containing a colicin or its derivative. The colicin may be selected from colicin M, colicin E7, colicin B, colicin Ia, colicin U, colicin K, colicin 5, colicin E5, colicin E8, colicin E9, colicin A, colicin S4, colicin 10, colicin R, colicin 28b, colicin Y, colicin Ib, and cloacin DF13, or a derivative of colicin M, colicin E7, colicin B, colicin Ia, colicin U, colicin K, colicin 5, colicin E5, colicin E8, colicin E9, colicin A, colicin S4, colicin 10, colicin R, colicin 28b, colicin Y, colicin Ib, and cloacin DF13. Colicin M or its derivative is preferred. Alternatively, the colicin may be any one or a combination of two or more of these colicins or derivatives of these colicins. In a preferred embodiment, the composition contains colicin M or its derivative and one or more further colicin selected from the group of colE7, colB, colIa, colU, colK, and/or col5, or a derivative of any of these colicins. More preferred embodiments are as follows:

- a composition containing colicin M or its derivative and colicin E7 or a derivative thereof;
- a composition containing colicin M or its derivative and colicin Ia or a derivative thereof;
- a composition containing colicin M or its derivative and colicin Ib or a derivative thereof;
- a composition containing colicin M or its derivative, colicin E7 or its derivative, colicin Ia or its derivative, and colicin K or its derivative;
- a composition containing colicin M or its derivative, colicin E7 or its derivative, colicin K or its derivative, colicin B or its derivative, and colicin 5 or its derivative.

If the composition contains more than one colicin or its derivative, the multiple colicins or their derivatives may be separately expressed in plants and purified to the desired degree. Thereafter, the colicins or their derivatives, or preparations containing the colicins may be combined. Binary colicin compositions, i.e. compositions containing two different colicins (or derivatives thereof) may be mixed from 1:10 to 10:1 by weight, preferably from 1:5 to 5:1 by weight. Further colicins (or their derivatives) may be added in comparable amounts. In the case of a combination of colicin M (or its derivative) and colicin E7 (or its derivative), they may be mixed in a weight ratio of from 1:3 to 5:1, preferably from 1:1 to 4:1. Where the composition is a solution, the total concentration of all colicins and any derivatives thereof in the solution may be from 1 to 100 000 µg/l, preferably from 10 to 50 000 µg/l, more preferably from 100 to 10 000 µg/l, and even more preferably from 500 to 5 000 µg/l.

The composition of the invention may further contain a solvent that dissolves the colicin(s) or its/their derivatives. The solvent is preferably water. Thus, the composition may be an aqueous solution of the colicins. For storage, such compositions may be cooled or frozen. In another embodiment, the composition is a freeze-dried solid obtained by lyophilization of an aqueous solution of the colicin(s) or its derivatives. The composition may further contain additives such as one or more preservatives, buffers, stabilizers and the like. As preservatives or stabilizers, those generally known to be compatible with food such as benzoic acid, glycerol, ascorbic acid, ethanol and the like may be mentioned. As buffers, substances mentioned above in the context of preventing or reducing contamination of an object with EPEC or EHEC may be mentioned.

Any of the above-mentioned colicins other than colicin M may be produced in plants as described above for colicin M. Introns may be inserted in the coding sequences to be expressed for ease of cloning. Further, coding sequences of the colicins may be codon optimized for expression in plants or in a particular plant. Accession numbers such as from the Uniprot database of the colicins are given in the Examples. In the following, the colicin amino acid sequences given in the database entries are referred to herein as "parent colicin" as opposed to the derivatives of the colicins.

The derivative of the colicins mentioned above may be a protein

(A) comprising the known amino acid sequence of the parent colicin, and may have additional N- and/or C-terminal amino acid sequence stretches, such a purification tags;

(B) comprising an amino acid sequence having an amino acid sequence identity of at least 80 %, preferably of at least 85%, more preferably of at least 90%, even more preferably at least 95%, and most preferably of at least 97 %, to the amino acid sequence of the parent colicin;

(C) comprising an amino acid sequence similarity of at least 90 %, preferably at least 95%, to the amino acid sequence of the parent colicin; similar amino acid residues are as defined above; and/or

(D) comprising an amino acid sequence having from 1 to 30, preferably from 1 to 20, more preferably from 1 to 15, even more preferably from 1 to 10, and most preferably from 1 to 5, amino acid residue substitutions, insertions, additions and/or deletions compared to the amino acid sequence of the parent colicin; these number refer to the total of substitutions, insertions, additions and deletions;

(E) comprising an amino acid sequence having from 1 to 30, preferably from 1 to 20, more preferably from 1 to 15, even more preferably from 1 to 10, and most preferably from 1 to 5, amino acid residue substitutions and/or terminal deletions compared to the amino acid sequence of the parent colicin. These numbers refer to the total of substitutions and terminal deletions.

The (parent) amino acid sequences of colicin E7 is given in SEQ ID NO: 6. The (parent) amino acid sequences of colicin Ia is given in SEQ ID NO: 7. The (parent) amino acid sequences of colicin Ib is given in SEQ ID NO: 8.

The derivatives of the above colicins (other than of colicin M), preferably have a minimum toxicity against an *E. coli* strain sensitive to the parent colicin compared to the parent colicin. The toxicity of the derivative of the colicin should be such that the derivative and the parent colicin produce spots free of viable bacteria of the sensitive *E. coli* strain of the same diameter 12 hours after spotting 5 microliters of a solution of said derivative of the colicin and the parent colicin onto a lawn of the sensitive *E. coli* strain on agar plates and subsequent incubation of the agar plates at 37°C, wherein the concentration of the derivative of the colicin is at most 5 times that of the comparative solution of the parent colicin. Preferably, the concentration of the derivative is at most 3 times, preferably at most twice that of the comparative solution of the parent colicin, and more preferably the same as that of the parent colicin. The colicin-sensitive *E. coli* strain may be any sensitive *E. coli* strain. For convenience, *E. coli* strain DH10B may be used for testing the toxicity of the colicin and its derivative. Otherwise, the assay described in Example 3 may be employed analogously. Thus, the radial diffusion assays via spot-on-lawn-method may be used. Similarly as decribed above for colicin M, the agar plates for the assay may be overlaid with soft agar containing cells of tested *E. coli* strain. 10x10 cm quadratic petri dishes may be poured with 15-20 ml LB agar medium (1.5% w/v agar). LB soft agar medium (0.8% (w/v) agar) is melted, 20 ml aliquots are transferred into 50 ml plastic tubes and their temperature is adapted to 50-55°C. *E. coli* overnight cultures of the test bacteria adjusted to OD600=1.0 with LB medium are added to the soft agar medium in a ratio of 200 µl bacterial culture per 20 ml medium resulting in the final OD600=0.01 or approximately $1x10^7$ cells/ml. Raw colicin preparations such as plant leaf material containing expressed colicin or its derivative may be extracted as described in Example 2. A 1:1 dilution series of plant extracts starting with undiluted samples by using extraction buffer may be prepared. 5 µl aliquots of total soluble protein (TSP) dilution series may be applied to the agar plates that are then incubated at 37°C overnight. Antimicrobial activity (toxicity) of the colicin or its derivative can be evaluated visually based on clearing zones and the diameter of spots may be measured.

If a colicin as described above such as colicin M or a derivative thereof is used for preventing infection of a mammal with enterohaemorrhagic *E. coli*, the colicin may be administered to the mammal. The mammal is preferably a human. Further, colicin M or a derivative thereof, or combinations of colicin M or its derivative with other colicins (or their derivatives) as described above may be used for reducing the load of EHEC in ruminants such as cattle (e.g. cows) or sheep. Generally, a liquid or solid pharmaceutical composition containing the colicin such as the colicin M or a derivative thereof, or combinations with other colicins or their derivatives, is prepared for administration to the mammal. Liquid compositions may be aqueous solutions. Solid compositions may be tablets containing the colicin such as the colicin M or its derivative, or combinations of colicin M or its derivative with other colicins (or their derivatives), e.g. in freeze-dried form. Administration may be oral. In this case, the pharmaceutical preparation is one that allows passage through the stomach without being attacked by the acid medium in the stomach. The colicins or their derivatives should then be released from the pharmaceutical preparation in the intestine. Such pharmaceutical preparations are known in the art. Examples are tablets and capsules resistant to the acid medium in the stomach. It is further possible to administer orally a biological material such as *E. coli* or plant material containing expressed colicin such as colicin M or a derivative thereof to a patient. The colicin such as the colicin M or its derivative may be administered to an adult in amounts of 1 mg to 1000 mg per day, preferably of from 10 mg to 250 mg per day to a human patient.

Similarly as described above for the method of reducing or preventing contamination with EHEC, or the method or reducing the load of EHEC in ruminants, colicin M or its derivative can be combined with any one or more other colicins such as with colE7, colB, colIa, colU, colK, col5, col Ia or col Ib for preventing infection of a patient with EHEC such as any one or all of the following *E. coli* serotypes: O26:H11, O45:H2, O103:H11, O111:H8, O145:NM, O157:H7, O104:H4, and O121:H19.

In a probiotic approach, a patient may be treated by administering to the patient a genetically-modified microorganism expressing a colicin such as colicin M or its derivative. The genetically-modified microorganism may be a genetically-modified non-pathogenic E. coli or a lactic acid-producing microorganism as commonly employed in fermentation of milk products. Examples of lactic acid-producing microorganism are bacteria from the genera *Lactobacillus* such *Lactobacillus lactis* and *Bifidobacterium* such as *Bifidobacterium bifidium or Bifidobacterium breve.*

Another route of administration is by injection into the blood stream of a patient for preventing infection with EHEC or EPEC. For this purpose the colicin such as the colicin M or a derivative, or combinations of colicin M or its derivative with other colicins (or their derivatives), may be dissolved in a physiological saline and the solution be sterilized.

EXAMPLES

Example 1: Plasmid constructs

Twelve colicins representing all four activity groups and various receptor specificities were selected (Table 1).

Table 1. List of colicins used in Examples

No.	Colicin	Receptor	Activity	Accession No.
1	colE2	BtuB	DNase	AAA23068.1
2	colE3	BtuB	RNase	AAA88416.1
3	colE6	BtuB	RNase	AAA23080.1
4	colE7	BtuB	DNase	AAA98054.1
5	colD	FepA	tRNase	P17998.1
6	colN	OmpF, LPS	pore-forming	P08083.1
7	colK	Tsx	pore-forming	Q47502.1
8	col5	Tsx	pore-forming	CAA61102.1
9	colU	OmpA	pore-forming	CAA72509.1
10	colB	FepA	pore-forming	P05819.3
11	colla	Cir	pore-forming	WP_001283344.1
12	colM	FhuA	inhibition of cell wall synthesis	AAA23589.1

The list comprises colicins E2, E3, E6, E7, D, N, K, 5, U, B, Ia and M. Respective amino acid sequences were retrieved from GenBank; corresponding nucleotide sequences with codon usage optimized for *Nicotiana benthamiana* were synthesized by Life Technologies GmbH (Darmstadt, Germany). In case of colicins E2, E3, E6, E7 and M, the coding sequence was interrupted by insertion of the *cat 1* intron (the first intron from *Ricinus communis* cat1 gene for catalase CAT1 (GenBank: D21161.1, nucleotide positions between 679 and 867)) to prevent the cytotoxicity in *Escherichia coli* cells used for cloning. Colicin coding sequences were inserted into TMV-based assembled viral vector pNMD035 (described in detail in WO2012/019660) resulting in plasmid constructs depicted in Fig.1 A-C.

In preliminary expression studies, it was found that colicins with nuclease (RNase and DNase) activities are usually highly toxic for plant tissues where they are expressed. Their expression resulted in tissue necrosis and poor accumulation of recombinant protein. However, co-expression with appropriate immunity proteins reduced the toxic effect and increased the accumulation of these colicins dramatically. Colicin immunity proteins used in our studies are listed in the Table 2.

Table 2. List of immunity proteins used in examples

No.	Immunity protein	Specificity	Accession No.
1	ImmE2	colE2 (DNase)	AAA23069.1
3	ImmE6	colE6 (RNase)	AAA23081.1
4	ImmE7	colE7 (DNase)	AAA23071.1
5	ImmD	colD (tRNase)	P11899.2

Immunity proteins ImmE2, ImmE6, ImmE7 and ImmD are specific for colicins E2, E6, E7 and D, respectively. Amino acid sequences of immunity proteins were retrieved from GenBank; corresponding nucleotide sequences with codon usage optimized for *Nicotiana benthamiana* were synthesized by Life Technologies and subcloned into PVX-based assembled viral vector pNMD670 as described in WO2012/019660. Resulting plasmid constructs are shown in Fig. 1 A-C.

For colicin M, several other constructs were created. They include TMV-based vector pMD10240 for chloroplast targeting of colicin M protein expressed as a translational fusion with chloroplast targeting pre-sequence (Marillonnet et al. 2004). Two other constructs are PVX-based viral vectors for cytosolic accumulation (pNMD11740) and chloroplast targeting (pNMD14500) of colicin M.

pNMD18381, the double-inducible viral vector for ethanol-induced Colicin M expression was created using the Golden Gate Modular Cloning approach (Engler et al. 2009; Weber et al. 2011; WO 2011/154147). This vector was further used for stable transformation of *Nicotiana benthamiana* plants.

Basically, level 0 vectors were generated by PCR amplification of modules with flanking BsaI endonuclease restriction sites bearing specific nucleotide sequences in sticky end regions and cloning of BsaI-restricted PCR fragments into BpiI-restricted level 1 entry cloning vectors with matching sticky end regions. Five different types of modules were used: 1) promoter module (P; BsaI 5´GGAG/BsaI 3´TACT); 2) 5´NTR module (5´non-translated region; BsaI 5´ TACT/BsaI 3´AATG), 3) ORF module (open reading frame; BsaI 5´AATG/BsaI 3´GCTT), 4) 3´NTR module (3´non-translated region; BsaI 5´ GCTT/BsaI 3´GGTA) and 5) terminator module (T; BsaI 5´GCTT or Bsa 5´GGTA/BsaI 3´CGCT). Nucleotide sequences were verified by sequencing.

Four different expression cassettes were assembled by single pot BsaI cloning of level 0 modules into BsaI sites of level 1 destination vectors which are flanked by BpiI sites with specific nucleotide sequences in sticky end regions for position and orientation of expression cassettes as: position 1, reverse (BpiI 5´TGCC/BpiI 3´GCAA); position 2, forward (BpiI 5´GCAA/BpiI 3´ACTA); position 3, reverse (BpiI 5´ ACTA/BpiI 3´TTAC); position 4, forward (BpiI 5´TTAC/BpiI 3´CAGA). Generated level 1 expression cassette vectors were: pNMD3420 (position 1, reverse; in pICH41344 (level 1 destination vector); pNMD3320 (level 0 promoter module vector, nos P (*Agrobacterium tumefaciens* nopaline synthase promoter)); pICH41403 (level 0 5´NTR module vector, Ω translational enhancer from TMV); pNMD3410 (level 0 ORF module vector, NPTII (neomycine phosphotransferase II); and pNMD3330 (level 0 terminator module vector, nos T (*Agrobacterium tumefaciens* nopaline synthase terminator)); pNMD13981 (position 2, forward; pICH47742 (level 1 destination vector), pICH41551 (level 0 promoter module vector, pSTLS (potato ST-LS1 gene promoter, GenBank: X04753.1)), pICH41571 (level 0 5´NTR-ORF module vector, alcR (Werner at al. 2011)), pICH53411 (level 0 3´NTR module vector, *U1* 3´NTR (3´non-translated region of Tobacco mosaic virus U1 isolate)), pICH53461 (level 0 terminator module vector, act2-t (*Arabidopsis actin 2* terminator)); pNMD14002 (position 3, reverse; pICH47822 (level 1 destination vector), pICH41561 (level 0 promoter module vector, pAlcA (*alcA* promoter from *Aspergillus nidulans* fused with minimal *35S* promoter sequence (Werner at al. 2011)), pICH52122 (level 0 5´NTR module vector, RdRp *TVCV* viral RNA-dependant RNA polymerase with 9 introns), pICH58391 (level 0 ORF module vector, *LacZ*), pICH45567 (level 0 terminator module vector, 3´NTR and 35S-t (*TVCV* viral 3´-nontranslated region and *CaMV 35S* terminator)); pNMD4590 (position 4, forward; pICH47761 (level 1 destination vector), pNMD4571 (level 0 promoter module vector, lambda insulator (999 bp long fragment between nucleotide position 31748 to 32746 of *Enterobacteria* phage lambda genome, GenBank: J02459.1) fused to *alcA* promoter), pICH41581 (level 0 5´NTR-ORF module vector, MP (viral movement protein) and 5´NTR), pICH53411 (level 0 3´NTR module vector, U1 3´NTR), pICH53444 (level 0 terminator module vector, ocs-t (*Agrobacterium tumefaciens* octopine synthase promoter)).

Level 2 binary expression vector with kanamycin resistance selection marker on the vector backbone and a T-DNA consisting of 4 expression cassettes in different orientations flanked by left and right border was generated by single pot BpiI cloning using pICH45066 (level 2 destination vector (BpiI 5´TGCC/BpiI 3´GGGA)), pNMD3420 (level 1 vector, position 1, reverse; kanamycin plant selection marker expression cassette), pNMD13981 (level 1 vector, position 2, forward; *alcR* transcriptional regulator expression cassette), pNMD14002 (level 1 vector, position 3, reverse; viral expression cassette for gene of interest containing *lacZ* for counterselection) pNMD4590 (level 1 vector, position 4, forward; expression cassette for viral MP for cell-to-cell movement), pICH41780 (end-linker to bridge expression cassette 4 and vector backbone).

Colicin M sequence from *E. coli* (AAA23589.1) was codon-optimized for *N. benthamiana*; *cat 1* intron was inserted at position corresponding to codon G81 and the corresponding DNA was synthesized by Life Technologies GmbH (Darmstadt, Germany). Level 0 ORF module vector containing PCR-cloned colM, pNMD18371, was used to replace LacZ in position 3 expression cassette of level 2 binary destination vector pNMD14021 by BsaI cloning to generate pNMD18381.

Example 2: Colicin expression screening

6 weeks old *Nicotiana benthamiana* plants were infiltrated using needleless syringe with diluted *Agrobacterium tumefaciens* cultures carrying TMV-based assembled vectors for cytosolic colicin expression. In case of colicins E2, E6, E7 and D, *Agrobacterium* cultures carrying TMV-based vector for colicin expression were mixed in equal proportions with other cultures carrying PVX-based vectors for the expression of corresponding immunity proteins. Individual overnight cultures were adjusted to OD600=1.5 and further diluted 1:100 with infiltration buffer containing 10mM MES, pH 5.5 and 10mM MgSO4. Plasmid constructs used in this experiment are summarized in Table 3. One week post infiltration, plant material was harvested and used for protein extraction. Total soluble protein (TSP) concentration was determined using the Bradford assay, and TSP extracts were analyzed using SDS-PAGE with Coomasssie staining. In our experiment, all tested colicins were expressed on reasonably high levels varying between 0.4 and 6.3 mg recombinant colicin/g FW or 6 and 50% of TSP (Table 4) as determined by comparison with bovine serum albumin (BSA) protein.

Table 3. Summary of colicin expression screen

No.	Colicin	Construct	Construct (feature)
1	colE2/ImmE2	pNMD15511/pNMD15231	TMV/PVX
2	colE3	pNMD15521	TMV
3	colE6/ImmE6	pNMD16121/pNMD16141	TMV
4	colE7/ImmE7	pNMD8802/pNMD9060	TMV/PVX
5	colD/ImmD	pNMD19162/pNMD19183	TMV/PVX
6	colN	pNMD3680	TMV
7	colK	pNMD15252	TMV
8	col5	pNMD15311	TMV
9	colU	pNMD15271	TMV
10	colB	pNMD15291	TMV
11	colla	pNMD19141	TMV

Table 4. Yield of recombinant colicins expressed in *Nicotiana benthamiana* plants. FW stands for fresh weight, TSP for total soluble protein.

No.	Colicin	Yield (mg/g FW)	Yield (% TSP)
1	Colicin E2	2.1	20
2	Colicin E3	0.6	7
3	Colicin E6	3.99	38
4	Colicin E7	1.17	13
5	Colicin D	1.1	10
6	Colicin N	0.63	7
7	Colicin K	2.13	25
8	Colicin 5	6.25	50
9	Colicin U	0.42	6
10	Colicin B	1.8	19
11	Colicin Ia	0.7	10
12	Colicin M	1.17	13

Example 3: Colicin activity screen

We analyzed the antimicrobial activity of plant-made recombinant colicins against the Big 7 STEC strains and against emerging O104:H4 EHEC strain. Details of standard quality control strains of Big 7 (#5219, Microbiologics Inc., St. Cloud, MN, USA) and O104:H4 (#01104, Microbiologics Inc.) used in our experiments are given in Table 5.

Table 5. EHEC strains used for antimicrobial activity screen

No.	Strain	Characteristics
1	*E. coli*, serotype O26:H11 (CDC03-3014)	Big 7 STEC QC Set (#5219, Microbiologics) (positive for presence of virulence genes *stx1* and/or *stx2* and *eae*)
2	*E. coli*, serotype O45:H2 (CDC00-3039)	
3	*E. coli*, serotype O103:H11 (CDC06-3008)	
4	*E. coli*, serotype O111:H8 (CDC2010C-3114)	
5	*E. coli*, serotype O121:H19 (CDC02-3211)	
6	*E. coli*, serotype O145:NM (CDC99-3311)	
7	*E. coli*, serotype O157:H7 (ATCC 35150)	
8	*E. coli*, serotype O104:H4 (ATCC BAA-2326)	QC control strain (#01104, Microbiologics) emerging EHEC

Antimicrobial activity of recombinant colicin-containing plant extracts was tested in radial diffusion assays via spot-on-lawn-method. For this purpose, we prepared agar plates overlaid with soft agar containing cells of tested *E. coli* strains. 10x10 cm quadratic petri dishes were poured with 15-20 ml LB agar medium (1.5% w/v agar). LB soft agar medium (0.8% (w/v) agar) was melted, 20 ml aliquots were transferred into 50 ml plastic tubes and their temperature was adapted to 50-55°C. *E. coli* overnight cultures adjusted to OD600=1.0 with LB medium were added to the soft agar medium with a ratio of 200 µl bacterial culture per 20 ml medium resulting in the final OD600=0.01 or approximately $1x10^7$ cells/ml. Plates for each strain were prepared in duplicate.

Plant leaf material was extracted as described in Example 2. We prepared 1:1 dilution series of plant extracts starting with undiluted samples by using same extraction buffer. 5 µl aliquots of TSP dilution series were applied to agar plates; plates were incubated at 37°C overnight. Antimicrobial activity was evaluated based on clearing zones. Colicins significantly differed in their specificity and potency of antimicrobial activity against different EHEC strains. The majority of tested colicins demonstrated rather narrow strain specificity with reasonably high activity against 1-3 strains. Surprisingly, the broadest specificity combined with relatively high potency against different strains was found for colicin M.

For semi-quantitative comparison, we represented relative antimicrobial activity of recombinant colicins in arbitrary units (AU), calculated as a dilution factor for the highest dilution of protein extract causing the detectable clearing effect in the radial diffusion assay. Colicin antimicrobial activity against Big 7 STEC strains calculated in AU per mg FW of the plant tissue is shown in Fig. 5. Fig. 6 demonstrates the same activity calculated in AU per µg of colicin protein. Both figures show the superiority of colicin M over other colicins concerning the spectrum of antimicrobial activity (Fig. 6) and the yield of active antimicrobial agent in plant tissue (Fig. 5). Fig. 7 shows the activity of tested recombinant colicin proteins against O104:H4 strain represented in either AU/mg FW (**A**) or AU/µg colicin (**B**). This strain is quite sensitive to the majority of tested colicins, however, colicins E2, E6, E7 and M are most active against this strain.

To summarize, colicin M shows the broadest antimicrobial activity against tested EHEC strains. Thus, it can be used as a main ingredient of colicin cocktails for the control of EHEC.

Example 4: Transient expression of Colicin M in *Nicotiana benthamiana* with different viral vectors

6 weeks old *Nicotiana benthamiana* plants were infiltrated using needleless syringe with diluted suspension of *Agrobacterium tumefaciens* GV3101 cells carrying TMV-based assembled vector pNMD10221 or PVX-based assembled vector pNMD11740 (Fig. 2). For infiltration, OD600 of overnight cultures was adjusted to 1.5 and further diluted 1:100 with infiltration buffer containing 10mM MES (pH 5.5) and 10mM MgSO4. Plant phenotype was analyzed and leaf samples were harvested after 4, 5, 6, 7 and 9 days post infiltration (dpi). Total soluble extracts were prepared at different harvesting time points by grinding the plant tissue in liquid nitrogen and adding of 5 volumes of extraction buffer followed by incubation for 30min on ice. The extraction buffer contained 20 mM acetate; 250 mM sodium chloride; 15 mM sodium ascorbate, 10 mM sodium metabisulfite (pH 4.0).

The analysis of the plant phenotype revealed no necrosis till 9 dpi when plants were infiltrated with the PVX based construct, whereas some necrosis at 9 dpi appeared when leaves were infiltrated with the TMV based construct. SDS-PAGE analysis revealed a high expression of ColM after the inoculation with both vectors (Fig. 8). The TMV based expression, however, resulted in higher accumulation of the protein of interest. The optimal harvesting time found for both vectors was 6 or 7 days post infiltration.

Example 5: Expression of Colicin M in edible plants

We successfully expressed Colicin M in the spinach and the beet plants.

7.5 weeks old plants of spinach *Spinacea oleracea* were infiltrated using syringe without needle with diluted suspension of *Agrobacterium tumefaciens* cells (ICF320 or GV3101 strains) carrying TMV-based assembled vector pNMD10220. Plant phenotypes were analyzed and plant material was harvested at 6, 8 and 10 dpi. For infiltration, OD600 of overnight cultures was adjusted to 1.5 and further diluted 1:100 with infiltration buffer containing 10mM MES (pH 5.5) and 10mM MgSO4. No necrotic phenotype was observed until last harvesting time point of 10 dpi. SDS-PAGE analysis of TSP extracts revealed abundant protein bands in Coomassie-stained gels without significant difference between the two *Agrobacterium* strains (Fig. 9A).

Beta vulgaris ssp. *maritima* (the sea beet) plants were infiltrated by syringe with 1:100 dilutions of *Agrobacterium* cultures of ICF320 and GV3101 strains for expression of cytosolic Colicin M using an assembled TMV based vector pNMD10220. Plant phenotypes were analyzed and plant material was harvested at 6, 8 and 10 dpi. Some necrosis was observed in the infiltrated areas at 10 dpi which seemed to be stronger for GV3101 strain compared to ICF320. SDS-PAGE analysis revealed detectable protein bands in Coomassie stained gels with a peak of recombinant protein accumulation at 8 dpi (Fig. 9B). The decrease of Colicin M level observed at 10 dpi correlated with leaf necrosis.

Example 6: Ethanol-inducible expression of Colicin M in stable transgenic *Nicotiana benthamiana* plants

For ethanol-inducible Colicin M expression, we generated stable transgenic *Nicotiana benthamiana* plants containing the genomic insertion of a double-inducible TMV-based viral vector (the approach is described in Werner et al. 2011).

The pNMD18381 construct (Fig. 3) created for this purpose was first tested in transient assay. Leaves of 5.5 weeks old *Nicotiana benthamiana* plants were infiltrated with a suspension of *Agrobacterium tumefaciens* cells of OD600=1.3 diluted 1:100 with a buffer for infiltration (10 mM MES, pH5.5; 10 mM MgSO4) using the syringe without needle. 2 days post infiltration, plants were sprayed with 4% (v/v) ethanol and drenched each plant with 40 ml of 4% (v/v) ethanol, incubated under plastic box with 500 ml of 4% (v/v) ethanol for 24h (for 4 plants). Leaf material was harvested 4 days post induction. For SDS-PAGE analysis, leaf samples were extracted with 5 volumes of Laemmli buffer, resolved in 12% polyacrylamide gel and stained with Coomassie. SDS-PAGE analysis detected the specific protein bands in ethanol-induced tissue (Fig. 10).

Construct pNMD18381 was transformed into *Nicotiana benthamiana* with *Agrobacterium*-mediated leaf disc transformation and selection on kanamycin-containing medium using a slightly modified standard protocol (Horsch et al. 1985; Werner et al. 2011). Regenerated plants were transferred to the greenhouse and tested for Colicin M expression upon ethanol induction.

Ethanol-inducible transgene expression was tested in detached leaves. Each leaf (one per plant) was incubated in 12.5 cm petri dish containing one layer of Whatman filter paper (10 cm in diameter) moisturized with 5 ml of 4% (v/v) ethanol and one layer of glass fibre mesh (10 cm in diameter) (Fiberglasgewebe für Licht- und Kellerschächte, Schellenberg, Germany). After 2 days incubation (fluorescent light, 22°C), leaves were transferred into new petri dishes as described above but moisturized with water (15 ml in total) and incubated 3 additional days at same conditions. 4 days post induction 100 mg samples of plant material were harvested and frozen in liquid nitrogen.

SDS-PAGE analysis was performed as described above for the transient assay. The accumulation of Colicin M protein upon ethanol induction was shown for the majority of selected transgenic lines (Fig. 10).

Example 7: Colicin M inhibition of 'Big Seven' STEC and EAHEC O104:H4 strains in broth culture

E. coli cultures were grown overnight in liquid LB medium, diluted with a fresh LB medium to OD_{600}=0.05 and further grown to OD_{600}=0.3. After that, we prepared 100 ml cultures via dilution with fresh LB medium to approx. $1x10^4$ cfu/ml (predilution to OD_{600}=0.3, dilute 1:3000). Each culture was aliquoted into 6 flasks (each containing 14 ml). Aliquot of the culture was taken for dilution plating (T=0 min). After addition of 1 ml of analyzed plant TSP extract with known colicin concentration, cultures were incubated at 37°C with 150 rpm agitation. Aliquots for dilution plating were taken at 30, 60 and 90 min of incubation. 100 µl aliquots of bacterial cultures were plated on LB agar medium; plates were incubated at 37°C overnight and used for colony counting next day. The plating was done in triplicate; bacterial population in the tested liquid cultures was evaluated as a number of cfu (colony forming units) per ml.

Table 6 shows the reduction of bacterial population after the application of colicin M (colicin containing plant extract was compared with the extract from uninfected *Nicotiana benthamiana* plants). Significant reduction of bacterial population was demonstrated for individual strains as well as for the mixture of all Big 7 strains.

Table 7 shows the result of application of colicin M mixed with colicin E7. These two colicins demonstrated synergistic effect for certain EHEC strains. Using a mixture of two or more colicins may be used for decreasing the amount of applied proteins.

Table 6. Antibacterial activity of colicin M applied individually to STEC strains

E. coli strain	Colicin M (mg/l)	*E. coli* cells (cfu/ml)	
		Reduction log	Initial cell number
O103:H11	7.5	2.6	0.9×10^4
O45:H2	7.5	2.7	1.3×10^4
O111:H8	3.75	4.1	1.5×10^4
O26:H11	3.75	5.0	1.1×10^4
O157:H7	1	3.5	1.2×10^5
Mix of Big 7 strains	1	0.8	1.0×10^4
O104:H4	1	5.0	1.0×10^4

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

E. coli strain	Colicin M+colicin E7 (mg/l)	*E. coli* cells (cfu/ml)	
		Reduction log	Initial cell number
O121:H19	1+1	2.8	0.7×10^4
O145:NM	1+1	0.8	2.3×10^4
O103:H11	1+1	0.9	1.8×10^4
O45:H2	1+1	0.9	1.3×10^4
O111:H8	0.5+0.5	5.2	0.8×10^4
O26:H11	0.5+0.5	4.4	0.7×10^4
O157:H7	0.25+0.25	3.6	1.1×10^5
Mix of Big 7 strains	1+1	0.9	1.4×10^4
O104:H4	0.1+0.1	>6.1	1.4×10^4

Example 8: Treatment of *E. coli* contaminated steak meat pieces with two-component colicin mixture consisting of colicin M and colicin E7

Plant-produced colicins were tested for antibacterial activity on samples of meat steak contaminated with pathogenic *E. coli*.

Pork fillet steaks were purchased in the local supermarket. Steaks were trimmed to a final weight of each 85 g using a sterile scalpel and put into 12x12 cm sterile petri dishes. *E. coli* O157:H7 inoculum, strain DSM19206, was prepared by dilution of a saturated LB overnight culture to OD_{600}=0.05 and a freshly grown LB culture at end of exponential phase (OD_{600}≈0.3) was diluted to OD_{600}=0.005 (approx. $5x10^5$ cfu/ml) by dilution with LB medium. Each steak was inoculated with *E. coli* by dipping from both sides into 12 ml of this bacterial solution in 12x12 cm sterile petri dishes. Upon inoculation, steaks were dried for 30 min at room temperature and turned around upon 15 min. The solutions for carrier or colicin treatment were prepared by extraction of *N. benthamiana* leaf material expressing colicin M or colicin E7 or *N. benthamiana* uninfected wild type leaf material stored at -80°C and ground to fine powder in liquid nitrogen with 5 volumes prechilled buffer (50 mM HEPES (pH7.0), 10 mM potassium acetate, 5 mM magnesium acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween®20 (Sigma-Aldrich, St. Louis, MO, USA) and 300 mM sodium chloride), respectively. Steaks inoculated with *E. coli* were treated with carrier solution or colicin solution (3 mg colM + 1 mg colE7/kg meat) by spraying in total 1.6 ml on both sides of a 85 g steak, non-treated (*E. coli* inoculation only) steaks served as control. Steaks were dried again at room temperature for 45 min and turned around upon 20 min. Afterwards, aliquots of steaks of about 20 g were packed for microbial analysis and storage into lateral filter bags BagFilter®P (Interscience, St Nom la Bretêche, France). After a total incubation time of 1.5 hours at room temperature upon colicin treatment, samples were incubated for 1 hour, 1 day or 3 days at 10°C before microbial analysis. Samples were inspected in quadruplicates for microbes by homogenization of steaks with 5 volumes of peptone water for 30 s using BagMixer®400CC (Interscience, St Nom la Bretêche, France) and analysis of homogenized material from filtered part of the bag by dilution plating on sorbitol-MacConkey medium supplemented with 0.05 µg/ml cefixime and 100 µg/ml X-Gluc for O157:H7 cfu numbers.

The results of bacteria count is shown in Fig. 11. Most significant reduction of bacterial population (2.3 logs) occurred already after 1 hour storage. More prolonged storage resulted in further decrease of bacterial population.

Example 9: Treatment of *E. coli* contaminated fresh-cut RTE pieces of melon with two-component colicin mixture consisting of colicin M and colicin E7

Ready-to-eat (RTE) cut segments of melon were infected with E. coli O157:H7 as an indicator pathogen and then sprayed with a two-component (colicins M + E7 formulations) and the results were compared to washing alone and spraying a control carrier solution containing plant extract without colicins.

Cantaloupe melon was purchased in the local supermarket and surface-sterilized by incubation of one fruit in 2 liters of 200 ppm sodium hypochlorite solution prepared with sterile tap water for 5 min with subsequent washing in 2 liters sterile tap water. Fruit pieces were prepared by cutting melon into flat pieces (thickness of approx. 1 cm) of suitable size using knifes and chopping board cleaned with Bacillol, only fruit pulp after removal of kernels and rind was used. 10 pieces corresponding to approx. 65 g were aliquoted into 12x12 cm sterile petri dishes.

E. coli O157:H7 inoculum, strain DSM19206, was prepared by dilution of a saturated LB overnight culture to OD_{600}=0.05 and a freshly grown LB culture at the end of exponential phase (OD_{600}≈0.3) was diluted to OD_{600}=0.001 (approx. $1x10^5$ cfu/ml) by dilution with sterile tap water. Each 10 fruit pieces were inoculated with *E. coli* by dipping from both sides into 12 ml of this bacterial solution in 12x12 cm sterile petri dishes. Upon inoculation, fruit pieces were dried for 30 min at room temperature and turned around upon 15 min. The solution for carrier or colicin treatment were prepared by extraction of *N. benthamiana* leaf material expressing colicin M or colicin E7 or *N. benthamiana* wild type leaf material stored at -80°C and ground to fine powder in liquid nitrogen with 5 volumes prechilled buffer (50 mM HEPES (pH7.0), 10 mM potassium acetate, 5 mM magnesium acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween®20, 300 mM sodium chloride), respectively. Melon pieces inoculated with *E. coli* were treated with carrier solution or colicin solution (3 mg colM + 1 mg colE7/kg fruit) by spraying in total 1.8 ml on both sides of 65 g fruit pieces. Non-treated fruit pieces (*E. coli* inoculation only) served as control. Fruit pieces were dried again at RT for 30 min and turned around upon 15 min. Afterwards, aliquots of fruit pieces of about 20 g were packed for microbial analysis and storage into lateral filter bags BagFilter®P. Upon a total incubation time of 1 hour at room temperature upon colicin treatment, samples were incubated for 1 hour, 1 day or 3 days at 4°C before microbial analysis. Samples were inspected in quadruplicates for microbes by homogenization of fruit pieces with 5 volumes peptone water for 30 s using BagMixer®400CC and analysis of homogenized material from filtered part of the bag by dilution plating on sorbitol-MacConkey medium supplemented with 0.05 µg/ml cefixime and 100 µg/ml X-Gluc for O157:H7 cfu numbers.

Results of colicin spray treatment of RTE melon segments are summarized in Fig. 12. Colicin treatment resulted in approx. 1 log reduction of bacterial population if compared with a treatment with spray carrier only.

Example 10: Treatment of *E. coli* contaminated fresh-cut RTE pieces of apple with two-component colicin mixture (colicin M and colicin E7) and five-component colicin mixture (colicin M, colicin E7, colicin K, colicin B, colicin 5).

A similar exposure study to the one described in Example 9 with RTE melon was performed on Golden Delicious apples. Apple fruits purchased in the local supermarket were surface sterilized in the bleach solution and cut into flat pieces (thickness of ~1cm). These ready-to-eat (RTE) segments of apple were infected with E. coli O157:H7 as an indicator pathogen and then sprayed with either a two-component (colicins M + E7) or a five-component (colicins M + E7 + K + B + 5) formulations and the results were compared to spraying a control carrier solution containing plant extract without colicins.

For this series, the density of the O157:H7 (strain DSM19206) inoculum was set to OD_{600}=0.005 (about 5 x 10^5 cfu/ml) resulting in a measured actual load of about 1 x 10^4 CFU/g of fruit, and the pooling of 4 apple pieces per sample was done.

Significant differences between control treatment and colicin treatments were observed already upon 1h storage at 4°C (Fig. 13). These differences increased further with time. After 3 days storage at 4°C, more than one log reduction of bacterial load was observed for both colicin formulations if compared with colicin-free carrier control.

Example 11: Treatment of *E. coli* contaminated fresh arugula leaves with a two-component colicin mixture (colicin M and colicin E7).

Plant-made colicins were tested for antimicrobial activity against *E. coli* O157:H7 in fresh leaves of arugula (rocket salad, *Eruca sativa* Mill.). Arugula leaves were contaminated with *E. coli* O157:H7 as an indicator pathogen and then washed in the two-component colicin solution (colicins M + E7) (wash colicin). The results were compared to *E. coli* contaminated leaves without any washing (no treatment) and contaminated leaves washed with a carrier solution containing plant extract without colicins (wash carrier).

Arugula leaves have been purchased in the local supermarket. Leaves were inoculated with *E. coli* O157:H7 (strain DSM19206) by dipping in the sterile water containing bacterial cells. The inoculum was set to 1x10^5 cfu/ml (OD_{600}=0.001) resulting in a measured actual load of ~1 x 10^4 CFU/g of arugula leaves. After 5 minutes incubation, the bacterial solution was decanted, and the excess liquid was removed from leaves using a salad spinner. The leaves were left to dry at room temperature for 30 minutes.

For colicin treatment, the leaves were dipped into 5 volumes of colicin solution and incubated for 10 minutes with an agitation. Because of the high leaf surface to mass ratio, the colicin dosage was increased compared to previous examples. We applied 3 mg colM + 1 mg colE7 per liter of wash solution (corresponds to 15 mg colM + 5 mg colE7 per kg of food). After removing the liquid, leaves were incubated at 10°C. Leaf samples were analyzed for microbial contamination after 1 hour and 1 day storage.

Interestingly, simple wash with a carrier solution efficiently removed bacterial cells with nearly one log CFU reduction after 1 hour storage compared to untreated samples (difference between colicin washed and untreated samples was 1.5 logs). However, after one day storage, there was practically no difference between untreated and carrier washed samples, and nearly one log CFU reduction for colicin treated samples compared to untreated or carrier washed ones (Fig. 14).

Example 12: Treatment of *E. coli* contaminated beef steak meat with a two-component colicin mixture (colicin M, colicin E7, colicin Ia and colicin K).

A similar exposure study to the one described in Example 8 with pork fillet steaks was performed on beef steak meat. Beef meat pieces of about 1 kg weight purchased in the local supermarket were trimmed to steaks of about 85 g weight and contaminated with *E. coli* O157:H7 (strain DSM19206) by dipping into bacterial solution. *E. coli* contamination resulted in a measured actual load of ~$1x10^4$ CFU/g of meat. After 30 minutes drying at room temperature, steaks were sprayed with the four-component colicin solution (diluted plant extracts containing colicins M + E7 + Ia + K). The colicin dosage was 3 mg/kg meat for colicin M and 1 mg/kg meat for each other colicin. Control samples were sprayed with a carrier solution only.

For analysis of efficacy, meat samples were stored at 4°C for up to 3 days and analyzed for microbial counts at 1 hour, 24 hours, and 72 hours storage. One log CFU reduction of *E. coli* O157:H7 by colicin in comparison to carrier application was detected already at 1 hour post treatment; for two later time points, the CFU reduction was approximately two logs (Fig. 15).

Example 13: Treatment of *E. coli* contaminated beef steak meat with four-component colicin mixture (colicin M, colicin E7, colicin Ia and colicin K) prior grinding.

Beef meat pieces of about 1 kg weight were trimmed to cubes of about 100 g weight and inoculated by addition of 10 ml/kg bacterial suspension of *E. coli* O157:H7 (strain DSM19206) of ~$1x10^6$ cfu/ml. The bacterial culture was equally distributed on beef cubes by tumbling.

Colicin (the blend of 3+1+1+1 mg/kg colM+colE7+colla+colK) or carrier treatment was carried out by spraying and equal distribution of carrier/colicin solution on meat cubes by tumbling. Upon 30 minutes incubation at room temperature with colicin or carrier solution, meat was ground using a ø 6 mm and a ø 3 mm die subsequently with ProfiCook® PC-FW 1003 meat grinder (ProfiCook-Clatronic International GmbH, Kempen, Germany) and 25 g samples of ground meat were packed in sampling bags and stored at 4°C. In total, meat was incubated for 2h at room temperature upon colicin application and stored at 4°C. Microbial counts were performed after 1 hour, 24 hours, and 72 hours storage at 4°C.

Nearly two logs reduction of *E. coli* population upon colicin treatment was observed already after 1 hour storage (Fig. 16). The difference was even higher (2.5 logs) after 24 hours. It was not possible to quantify the colicin effect after 72 hours storage, as no *E. coli* bacteria could be detected in colicin treated samples at this time point.

Furthermore, a very high reduction (nearly two logs) of detected O157:H7 CFUs in carrier samples from 1h to 72 h storage at 4°C was observed. One possible explanation for this finding could be that grinding of contaminated meat poses the stress to bacteria and reduces their viability.

Example 14: General scheme of colicin production

Fig. 17 shows a flow diagram of steps of two expression and purifcation processes. A flow diagram summarizing key steps in a production process of producing colicin proteins is shown. Key process steps are described in the following (step numbers correspond to the steps indicated in Fig. 18. The induction of gene expression can be accomplished by one of two alternative methods (described below), which share common downstream purification unit operations.

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

The *Agrobacterium tumefaciens* bacterial vector containing a TMV transcript with the gene insert for a colicin of interest is grown in defined medium under aseptic conditions following strict quality SOPs; this bacterial suspension constitutes the inoculum. An *Agrobacterium* strain harboring a colicin expression vector, such as colicin M expression vector pNMD 10220, is grown in medium containing de-mineralized water, yeast extract, peptones, minerals, kanamycin and rifampicin. The removal of residual antibiotics and fermentation chemicals is achieved by high dilution of the bacterial solution before inoculation of plants and the ultra- and dia-filtration procedures during plant biomass extraction and processing. All raw materials and processing aids are food grade. A multivial Master Vector Bank of the vector is prepared and stored at -80°C, from which aliquots are removed as Working Vector Banks of the inoculum for each manufacturing batch.

Each Working Bank of *Agrobacterium* strain pNMD 10220 is handled in a way to reduce the risk of contamination by foreign microorganisms. This includes use of sterile materials for bacterial cultivation, quality control checks to ensure axenic culture, and confirmation of strain identity before plant inoculation. Samples not meeting criteria are rejected and disposed, and new aliquots are drawn from the Master Bank. If a problem is identified at the Master Bank level, a new Master Bank is generated and subjected to quality control procedures before further use.

Step 1b. Ethanol induction of transgenic plants

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

Step 2. Host plant preparation

For agroinduction, normal seeds of *Spinacia oleracea* (spinach), *Beta vulgaris* (beet) or other suitable host plants are obtained from qualified seed producers. For ethanol induction, transgenic seeds developed by Notifier are used, which contain the gene insert for the desired colicin driven by an ethanol-inducible promoter. With either method of induction, plants are propagated in trays using a soil based substrate, fertilizer and water. For seeding, plant propagation, target expression and plant harvest, the principles of Good Agriculture and Collection Practices (GACP) are applied. All used materials underlie a quality management system ensuring a predefined quality.

Step 3a. Inoculation of host plants with agrobacterial vector

The *A. tumefaciens* inoculum carrying the selected colicin replicon is applied to greenhouse-grown and quality tested host plants through the stomata (pores) in the leaves. The plant hence takes the place of a conventional "fermenter" in the production of the product. The *Agrobacterium* inoculum and the host plants are cultured under predefined and controlled conditions. At a specified time point after seeding the plants are treated with a defined concentration of *Agrobacterium* in dilution buffer.

Inoculation of plants is accomplished by either vacuum-mediated infiltration after dipping the plant leaves in a suspension of the inoculum, or via a procedure wherein the inoculum is sprayed onto plant leaves mixed with a surfactant (Gleba 2014; Tusé 2014). Via either method, the agrobacteria are efficiently internalized into the plant and gain systemic distribution.

The agrobacteria infect the plant cells and insert the T-DNA plasmid into the nucleus, which initiates synthesis of colicin-encoding RNA transcripts. Amplification of the transcript and translation of the colicin RNA message into colicin occurs in the cytoplasm of each plant cell.

Step 3b. Ethanol induction

In this variation of the method, a simple treatment of the transgenic plants carrying the colicin gene with dilute ethanol (2.5% v/v) releases the replicon leading to RNA amplification and high-level colicin production. To achieve tight control of replicon activation and spread in the non-induced state, the viral vector has been deconstructed, and its two components, the replicon and the cell-to-cell movement protein, have each been placed separately under the control of an inducible promoter (Werner 2011). Throughout the induction period, colicin protein accumulates in the tissues of the host plant. The inducer (ethyl alcohol) is diluted during plant growth and any traces remaining are removed during downstream purification.

Step 4. Incubation

After agro-inoculation or ethanol induction, the plants are incubated for 5-10 days under controlled temperature, humidity, and light condition to allow for accumulation of the desired protein. During this incubation period, there is rapid systemic replication of the vector and expression and accumulation of the induced product.

Step 5. Harvest

Plants producing colicin protein are harvested typically 8-9 days post inoculation/induction. Samples of plant biomass are taken for analyses of colicin protein content, general health and other process QC procedures prior to large-scale extraction. Plants in trays are transported to the cutting operation. The plants' aerial biomass (i.e. leaves and part of the stems) are mechanically cut and harvested into bins, which are transported to the extraction room.

Step 6. Homogenization of plant tissue

Cut plant biomass is disintegrated by homogenization in a grinder using an extraction buffer; the course plant material and fibers are removed, and the protein-containing soluble stream is further purified through a series of pH-assisted precipitations and filtration steps.

Step 7. Acidic extraction

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

Step 8. First clarification

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

Step 9. Neutralization

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

Step 10. Second clarification

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

Step 11 and Step 12. Ultrafiltration / diafiltration

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

Step 13. Chromatography

At this stage, the product-enriched solution can be subjected to one of two additional purification steps. If a relatively pure colicin product is desired, the solution is subjected to cation-exchange chromatography, which removes additional host-cell proteins and plant metabolites such as polyphenols, resulting in a clarified, enriched product. One or more colicin proteins prepared by this method and to meet this level of purity can be blended into a final solution that will be further processed into **COLICIN Isolate**. If a less purified bulk product will suffice for certain applications, the chromatography step is eliminated, and this solution (containing one or a blend of colicin proteins) will be further processed into **COLICIN Concentrate**.

Steps 14 – 17. Formulation, fill and finish

The final **COLICIN Concentrate** or **COLICIN Isolate** precursor solution is stabilized and standardized by the addition of water, sodium citrate/citric acid and sodium chloride. Finally, the solution is filter-sterilized through a 0.22 µm membrane filtration unit and filled as a bulk liquid concentrate, or freeze dried to produce a dry, off-white to light tan powdered product. Prior to release, the bulk products are tested to ensure compliance with the respective final product specification for COLICIN Concentrate or COLICIN Isolate.

In-Process controls and quality assurance

Notifier applies rigorous in-process controls to manage the quality of process intermediates and final products throughout the manufacturing process. Materials not meeting pre-determined specifications are rejected. Product release is done after each batch passes rigorous identity and potency tests. A Quality Management system is in place to ensure conformance with industry standards and federal and local regulatory guidelines.

A.1 Possible Specifications

Specifications for each grade of COLICIN produced by this process are shown in Table 8 (COLICIN Concentrate) and Table 9 (COLICIN Isolate).

Table 8. Specification for COLICIN Concentrate Product

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

Table 9. Specification for COLICIN Isolate Product

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

Example 15: Colicin protein purification

We developed a simple downstream process for colicin-containing plant biomass, which includes homogenization of plant tissue, acidic extraction, clarification and neutralization of the plant extract followed by ultrafiltration and diafiltration steps. The colicin-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 suffices 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. 18).

Six days after inoculation, infected leaf enriched material was harvested. Plant biomass was homogenized in the presence of extraction solution (10mM HCl, 10mM

$Na_2S_2O_5$, 2.5mM 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 x g for 20 minutes. The clarified extract was neutralized with 1 M sodium hydroxide and further clarified by centrifugation and depth filtration (Filter sheets: BECO® KDS12 -Begerow). The filtrate was 4-fold concentrated by ultrafiltration with a 5 kDa hollow-fiber module. The retentate was diafiltered 5 times against 5 mM citrate, 50mM 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, 50mM NaCl pH 5.0. The column was washed with 25mM 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, 137mM NaCl pH 7.3 resulting in colicin M isolate.

The purity of the colicin concentrate and isolate was determined by using SDS-PAGE (Fig. 18); 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, Böblingen, Germany).

Example 16: Plasmid constructs for the expression of colicins E5, E8, E9, A, S4, 10, R, 28b, Y, Ib, and cloacin DF13

Aiming to find other potential colicin candidates with desired antimicrobial activity, we selected eleven additional colicin genes for the expression in plants. They represented three activity groups and various receptor specificities (Table 10). Thus, we were able to express 23 colicin genes covering nearly all *E. coli* colicins described up to now.

The second set comprises colicins colicins E5, E8, E9, A, S4, 10, R, 28b, Y, Ib, and cloacin DF13. Respective amino acid sequences were retrieved from GenBank; corresponding nucleotide sequences with codon usage optimized for *Nicotiana benthamiana* were synthesized by Life Technologies GmbH (Darmstadt, Germany). In case of colicins E5, E8, E9, Y and cloacin DF13 the coding sequence was interrupted by by insertion of the *cat 1* intron (the first intron from *Ricinus communis* cat1 gene for catalase CAT1 (GenBank: D21161.1, nucleotide positions between 679 and 867)) to prevent the cytotoxicity in *Escherichia coli* cells used for cloning. Colicin coding sequences were inserted into TMV-based assembled viral vector pNMD035 (described in detail in WO2012/019660) resulting plasmid constructs depicted in Fig. 19A.

Table 10. List of colicins used in examples (the second set).

No.	Colicin	Receptor	Activity	Accession No.
1	colE5	BtuB	tRNase	AHK10569.1
2	colE8	BtuB	DNase	ACS71682.1
3	colE9	BtuB	DNase	ACM07430.1
4	cloacin DF13	lutA	16S rRNase	NP_052372.1
5	colA	BtuB	pore-forming	P04480.1
6	colS4	OmpF	pore-forming	CAB46008.1
7	col10	Tsx	pore-forming	CAA57998.1
8	colR	OmpA	pore-forming	AGV40809.1
9	col28b	OmpA	pore-forming	CAA44310.1
10	colY	FepA	pore-forming	AAF82683.1
11	colIb	Cir	pore-forming	AAA23188.1

Colicins with nuclease activities were co-expressed with corresponding immunity proteins to reduce the toxic effect and increase the accumulation of these colicins. Colicin immunity proteins used in this example are listed in the Table 11.

Table 11. List of immunity proteins used in examples (the second set).

No.	Immunity protein	Specificity	Accession No.
1	ImmE5	colE5 (tRNAse)	AHK10570.1
3	ImmE8	colE8 (DNAse)	ACS71683.1
4	ImmE9	colE9 (DNAse)	ACM07431.1
5	ImmDF13	colDF13 (rRNAse)	NP_052371.1

Immunity proteins ImmE5, ImmE8, ImmE9 and ImmDF13 are specific for colicins E5, E8, E9 and cloacin DF13, respectively. Amino acid sequences of immunity proteins were retrieved from GenBank; corresponding nucleotide sequences with codon usage optimized for *Nicotiana benthamiana* were synthesized by Life Technologies and subcloned into PVX-based assembled viral vector pNMD670 as described in WO2012/019660. An overview over the resulting plasmid constructs is shown in Fig. 19B.

Example 17: Expression screen for colicins E5, E8, E9, A, S4, 10, R, 28b, Y, Ib, and cloacin DF13

6 weeks old *Nicotiana benthamiana* plants were infiltrated using needleless syringe with diluted *Agrobacterium tumefaciens* cultures carrying TMV-based assembled vectors for colicin expression. In case of colicins E5, E8, E9 and cloacin DF13, *Agrobacterium* cultures carrying TMV-based vector for colicin expression were mixed inn equal proportions with other cultures carrying PVX-based vectors for the expression of corresponding immunity proteins. Overnight cultures were adjusted to OD600=1.5 and further diluted 1:100 with infiltration buffer containing 10mM MES, pH 5.5 and 10mM $MgSO_4$. 5-6 days post infiltration, plant material was harvested and used for the protein extraction. Protein were extracted either with Laemmli buffer to recover all protein forms or with HEPES buffer to recover total soluble protein (TSP) fraction only. Total soluble protein concentration was determined using Bradford assay, and protein extracts were analyzed using SDS-PAGE with Coomasssie staining (Fig. 20). In our experiment, all tested colicins were expressed on reasonably high levels varying between 6 and 50% of TSP as determined by comparison with Bovine Serum Albumin (BSA) protein.

Example 18: Antimicrobial activity screen for colicins E5, E8, E9, A, S4, 10, R, 28b, Y, Ib, and cloacin DF13

We analyzed antimicrobial activity of plant-made recombinant colicins against Big 7 STEC strains and against emerging O104:H4 EHEC as described in Example 3. For semi-quantitative comparison, relative antimicrobial activity of recombinant colicins was represented in arbitrary units (AU) per mg of fresh weight of plant tissue. The results of colicin activity screen were represented in Fig. 21.

The majority of tested colicins (7 out of 11) had no or very low activity against tested strains. Four other tested colicins demonstrated rather narrow strain specificity with reasonably high activity against 1-3 strains.

Overview of nucleic acid and amino acid sequences

SEQ ID NO: 1 Amino acid sequence of colicin M

METLTVHAPSPSTNLPSYGNGAFSLSAPHVPGAGPLLVQVVYSFFQSPNM

CLQALTQLEDYIKKHGASNPLTLQIISTNI GYFCNADRNL VLHPGISVYD AYHFAKPAPS

QYDYRSMNMKQMSGNVTTPIVALAHYLWGNGAERSVNIAN IGLKISPMKI NQIKDIIKSG

VVGTFPVSTK FTHATGDYNVITGAYLGNITLKTEGTLTISANGSWTYNGVVRSYDDKYDF

NASTHRGIIGESLTRLGAMFSGKEYQILLPGEIHIKESGKR

SEQ ID NO: 2 pNMD035: empty TMV-based vector for cloning

SEQ ID NO: 3 pNMD10221: TMV-based vector with ColM insertion

SEQ ID NO: 4 pNMD670: an empty PVX-based vector for cloning

SEQ ID NO: 5 pNMD11740: PVX-based vector with ColM insertion

SEQ ID NO: 6 amino acid sequence of colicin E7

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

SEQ ID NO: 7 amino acid sequence of colicin Ia

MSDPVRITNPGAESLGYDSDGHEIMAVDIYVNPPRVDVFHGTPPAWSSFGNKTIWGGNEW
VDDSPTRSDIEKRDKEITAYKNTLSAQQKENENKRTEAGKRLSAAIAAREKDENTLKTLR
AGNADAADITRQEFRLLQAELREYGFRTEIAGYDALRLHTESRMLFADADSLRISPREAR
SLIEQAEKRQKDAQNADKKAADMLAEYERRKGILDTRLSELEKNGGAALAVLDAQQARLL
GQQTRNDRAISEARNKLSSVTESLNTARNALTRAEQQLTQQKNTPDGKTIVSPEKFPGRS
STNHSIVVSGDPRFAGTIKITTSAVIDNRANLNYLLSHSGLDYKRNILNDRNPVVTEDVE
GDKKIYNAEVAEWDKLRQRLLDARNKITSAESAVNSARNNLSARTNEQKHANDALNALLK
EKENIRNQLSGINQKIAEEKRKQDELKATKDAINFTTEFLKSVSEKYGAKAEQLAREMAG
QAKGKKIRNVEEALKTYEKYRADINKKINAKDRAAIAAALESVKLSDISSNLNRFSRGLG
YAGKFTSLADWITEFGKAVRTENWRPLFVKTETIIAGNAATALVALVFSILTGSALGIIG
YGLLMAVTGALIDESLVEKANKFWGI

SEQ ID NO: 8 amino acid sequence of colicin Ib

MSDPVRITNPGAESLGYDSDGHEIMAVDIYVNPPRVDVFHGTPPAWSSFGNKTIWGGNEW
VDDSPTRSDIEKRDKEITAYKNTLSAQQKENENKRTEAGKRLSAAIAAREKDENTLKTLR
AGNADAADITRQEFRLLQAELREYGFRTEIAGYDALRLHTESRMLFADADSLRISPREAR
SLIEQAEKRQKDAQNADKKAADMLAEYERRKGILDTRLSELEKNGGAALAVLDAQQARLL
GQQTRNDRAISEARNKLSSVTESLKTARNALTRAEQQLTQQKNTPDGKTIVSPEKFPGRS
STNHSIVVSGDPRFAGTIKITTSAVIDNRANLNYLLTHSGLDYKRNILNDRNPVVTEDVE
GDKKIYNAEVAEWDKLRQRLLDARNKITSAESAINSARNNVSARTNEQKHANDALNALLK
EKENIRSQLADINQKIAEEKRKRDEINMVKDAIKLTSDFYRTIYDEFGKQASELAKELAS
VSQGKQIKSVDDALNAFDKFRNNLNKKYNIQDRMAISKALEAINQVHMAENFKLFSKAFG
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FAIIMAAVSALVNDKFIEQVNKLIGI

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Horsch RB, Fraley RT, Rogers SG, Sanders PR, Lloyd A (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231.

Marillonnet S, Giritch A, Gils M, Kandzia R, Klimyuk V, Gleba Y (2004) *In planta* engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium*. Proc Natl Acad Sci U S A 101(18): 6852-7.

Weber E, Engler C, Gruetzner R, Werner S, Marillonnet S (2011) A modular cloning system for standardized assembly of multigene constructs. PLoS ONE 6(2): e16765.

Werner S, Breus O, Symonenko Y, Marillonnet S, Gleba Y (2011) High-level recombinant protein expression in transgenic plants by using a double-inducible viral vector. PNAS 108(34): 14061-14066.

Sequence Listing In Electronic Form

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 76766-102 SEQ 03-05-2016 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

CLAIMS:

1. A method of preventing or reducing contamination of an object with enterohaemorrhagic *E. coli* (EHEC), comprising contacting said object with colicin M or a derivative thereof, wherein

(A) said colicin M or its derivative has an N-terminal translocation domain as follows:

the N-terminal translocation domain has amino acid residues 1 to 35 of SEQ ID NO: 1, or

has from 1 to 4 amino acid substitutions, insertions, additions and/or deletions compared to amino acid residues 1 to 35 of SEQ ID NO: 1, or

has an amino acid sequence identity of at least 90 % to the N-terminal translocation domain of SEQ ID NO: 1, or

has an amino acid sequence similarity of at least 95 % to the N-terminal translocation domain SEQ ID NO: 1;

(B) said colicin M or its derivative comprises a central receptor-binding domain as follows:

the receptor-binding domain has residues 36 to 140 of colicin M of SEQ ID NO: 1, or

has a domain having from 1 to 10 amino acid substitutions, insertions, additions and/or deletions compared to residues 36 to 140 of SEQ ID NO: 1, or

has an amino acid sequence identity of at least 90 % to the receptor-binding domain of SEQ ID NO: 1, or

has an amino acid sequence similarity of at least 95 % to the receptor-binding domain SEQ ID NO: 1; and

(C) said colicin M or its derivative comprises a C-terminal activity domain as follows:

the activity domain is of residues 141 to 271 of SEQ ID NO: 1, or

the activity domain has from 1 to 30 amino acid residue substitutions, insertions, additions and/or deletions compared to amino acid residues 141 to 271 of SEQ ID NO: 1, or

the activity domain has an amino acid sequence identity of at least 80% to the activity domain of SEQ ID NO: 1, or

the activity domain has an amino acid sequence similarity of at least 90% to the activity domain of SEQ ID NO: 1.

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2. The method according to claim 1, wherein contamination of an object with EHEC serotype O157:H7 is prevented or reduced.

3. The method according to claim 1, wherein contamination of an object with any one or all of the following *E. coli* serotypes is prevented or reduced: serotype O26:H11, serotype O45:H2, serotype O103:H11, serotype O111:H8, serotype O157:H7, and serotype O104:H4.

4. The method according to claim 1, wherein contamination of an object with any one or all of the following *E. coli* serotypes is prevented or reduced: serotype O26:H11, serotype O45:H2, serotype O103:H11, serotype O111:H8, serotype O145:NM, O157:H7, and O104:H4.

5. The method according to claim 1, wherein said object is contacted with an aqueous solution containing colicin M or its derivative by spraying with said aqueous solution or by dipping said object into said aqueous solution.

6. The method according to any one of claims 1 to 4, wherein said object is food.

7. The method according to claim 6, wherein said food is immersed for at least 10 seconds into an aqueous solution of colicin M or its derivative.

8. The method according to claim 6, wherein said food is immersed for at least 1 minute into an aqueous solution of colicin M or its derivative.

9. The method according to claim 1, wherein said colicin M or its derivative is produced by expression in a plant or in plant cells, followed by removing undesired components from said plant or said plant cells.

10. The method according to claim 1, wherein said object is food selected from meat, raw fruit and raw vegetable.

11. The method according to claim 1, wherein said colicin M has the amino acid sequence of SEQ ID NO: 1.

12. The method according to claim 1, wherein toxicity of the derivative of colicin M is such that the derivative and the colicin M of SEQ ID NO: 1 produce spots free of viable bacteria of sensitive *E. coli* strain DH10B of the same diameter 12 hours after spotting 5 microliters of a solution of said derivative of colicin M and the colicin M of SEQ ID NO: 1 onto a lawn of the sensitive *E. coli* strain on an agar plate and subsequent incubation of the agar plate at 37°C, wherein the concentration of the derivative of colicin M is at most 5 times that of the comparative solution of the colicin M of SEQ ID NO: 1.

13. The method according to claim 1, wherein said colicin M or its derivative is used in combination with one or more colicins selected from the group consisting of colicin E7 or a derivative thereof, colicin B or a derivative thereof, colicin Ia or a derivative thereof, colicin U or a derivative thereof, colicin K or a derivative thereof, and colicin 5 or a derivative thereof.

14. The method according to claim 1, wherein said colicin M or its derivative is used in combination with one or more colicins selected from the group consisting of colicin E5 or a derivative thereof, colicin E8 or a derivative thereof, colicin E9 or a derivative thereof, colicin A or a derivative thereof, colicin S4 or a derivative thereof, colicin 10 or a derivative thereof, colicin R or a derivative thereof, colicin 28b or a derivative thereof, colicin Y or a derivative thereof, colicin Ib or a derivative thereof, and cloacin DF13 or a derivative thereof.

15. The method according to claim 1, wherein said colicin M or its derivative is used in combination with colicin E7 or a derivative thereof.

16. The method according to claim 15, wherein contamination with any one or all of the following *E. coli* serotypes is prevented or reduced: serotypes O26:H11, O45:H2, O103:H11, O111:H8, O145:NM, O157:H7, O104:H4, and O121:H19.

17. A process of producing a colicin preparation, comprising:

(i) expressing colicin in a plant from a nucleic acid construct encoding said colicin;

(ii) homogenizing the plant containing expressed colicin to produce a homogenate, optionally followed by removing solid or insoluble material;

(iii) acidifying the homogenate or a clarified fraction thereof to a pH of below pH5, followed by removal of insoluble material, to obtain a colicin-containing solution;

(iv) neutralizing the clarified colicin-containing solution, followed by removal of insoluble material;

(v) optionally concentrating the colicin-containing solution obtained in the previous step;

(vi) optionally freeze-drying the solutions obtained in step (iv) or (v) to obtain a freeze-dried preparation of said colicin,

wherein said colicin is colicin M or a derivative thereof as defined in claim 1.

18. The process according to claim 17, wherein said plant is an edible plant.

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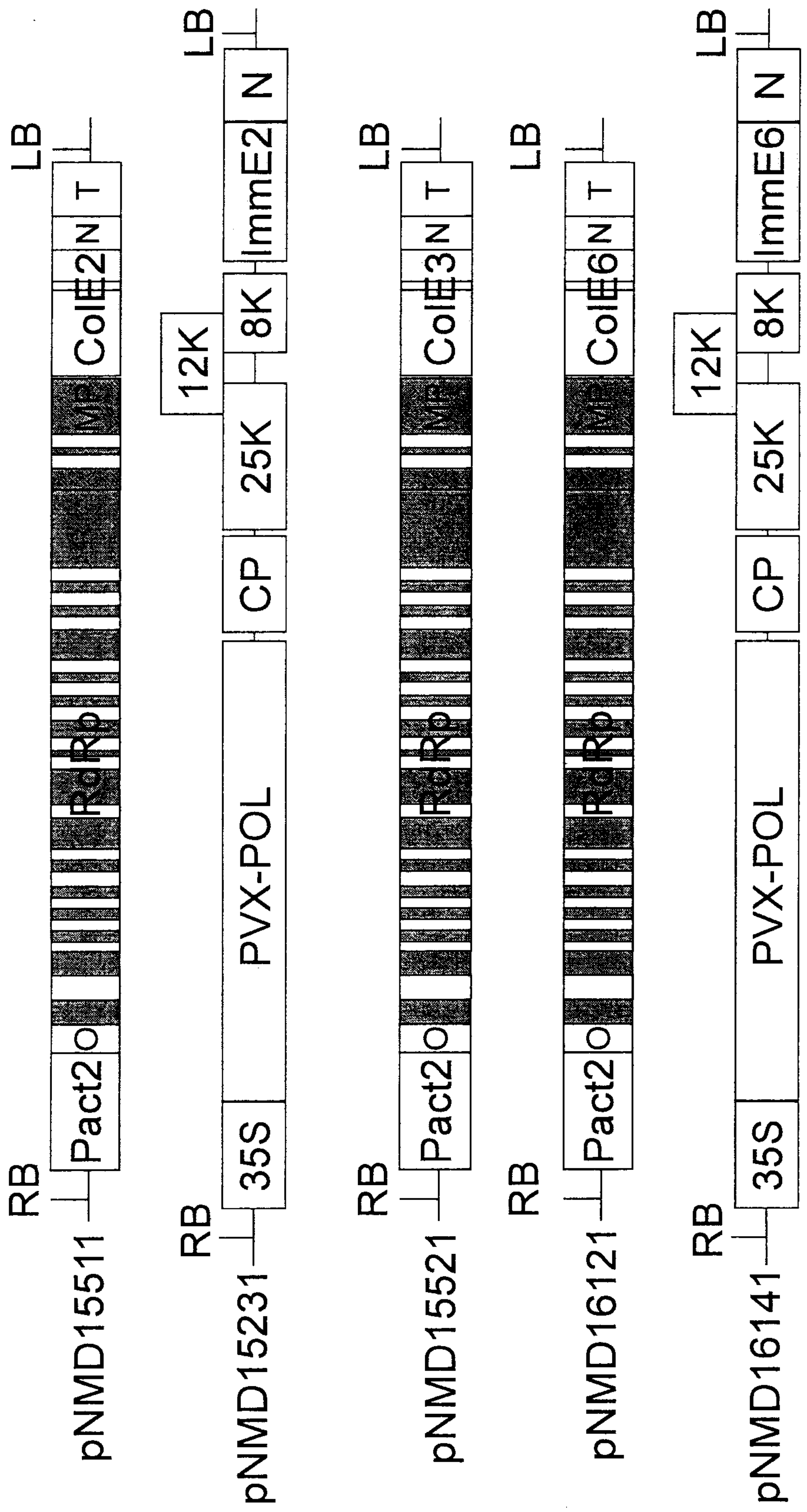
19. The process according to claim 17, further comprising purifying, subsequent to step (iv) or (v) and before step (vi), said colicin by cation exchange chromatography.



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O
RdRp
MP
ColE2
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T
LB
pNMD15231
RB
35S
PVX-POL
CP
25K
12K
8K
ImmE2
N
LB
pNMD15521
RB
Pact2
O
RdRp
MP
ColE3
N
T
LB
pNMD16121
RB
Pact2
O
RdRp
MP
ColE6
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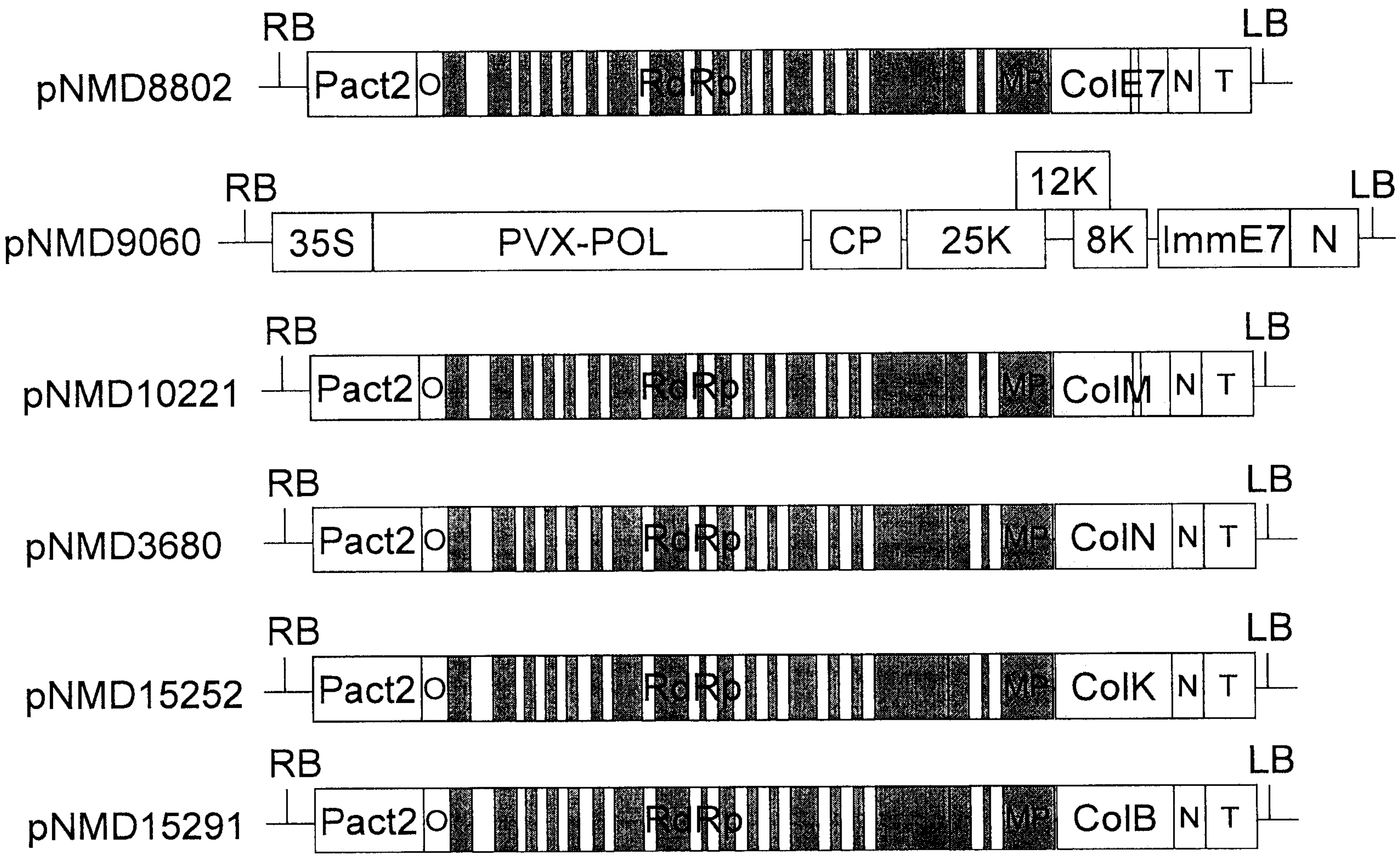
Fig. 1A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8K
ImmE7
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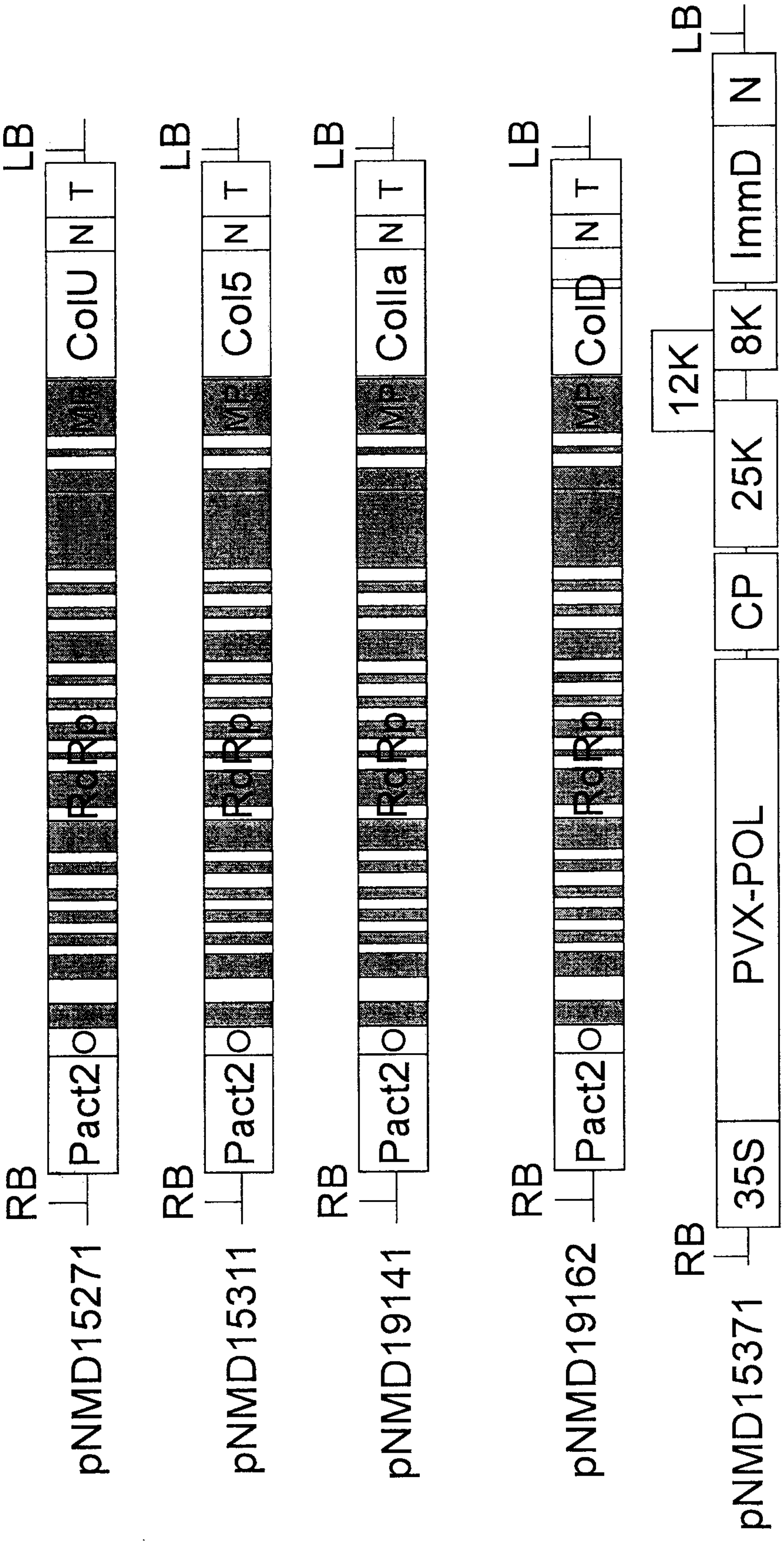
ColB

Fig. 1B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T
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MP
Col5
N
T
LB
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RB
Pact2
O
RdRp
MP
Colla
N
T
LB
pNMD19162
RB
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RdRp
MP
ColD
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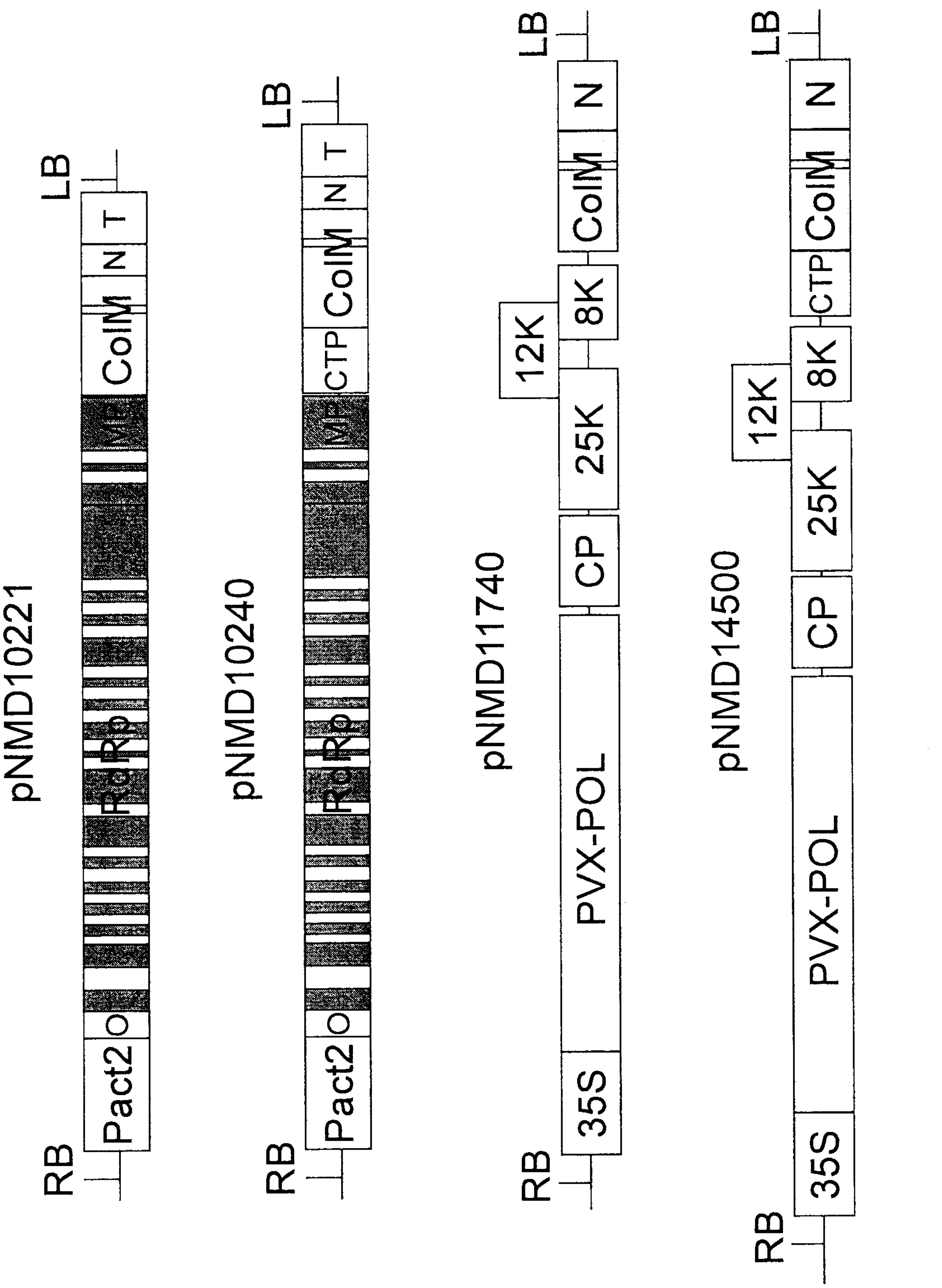
ImmD
N
LB

Fig. 1C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CP
25K
12K
8K
ColM
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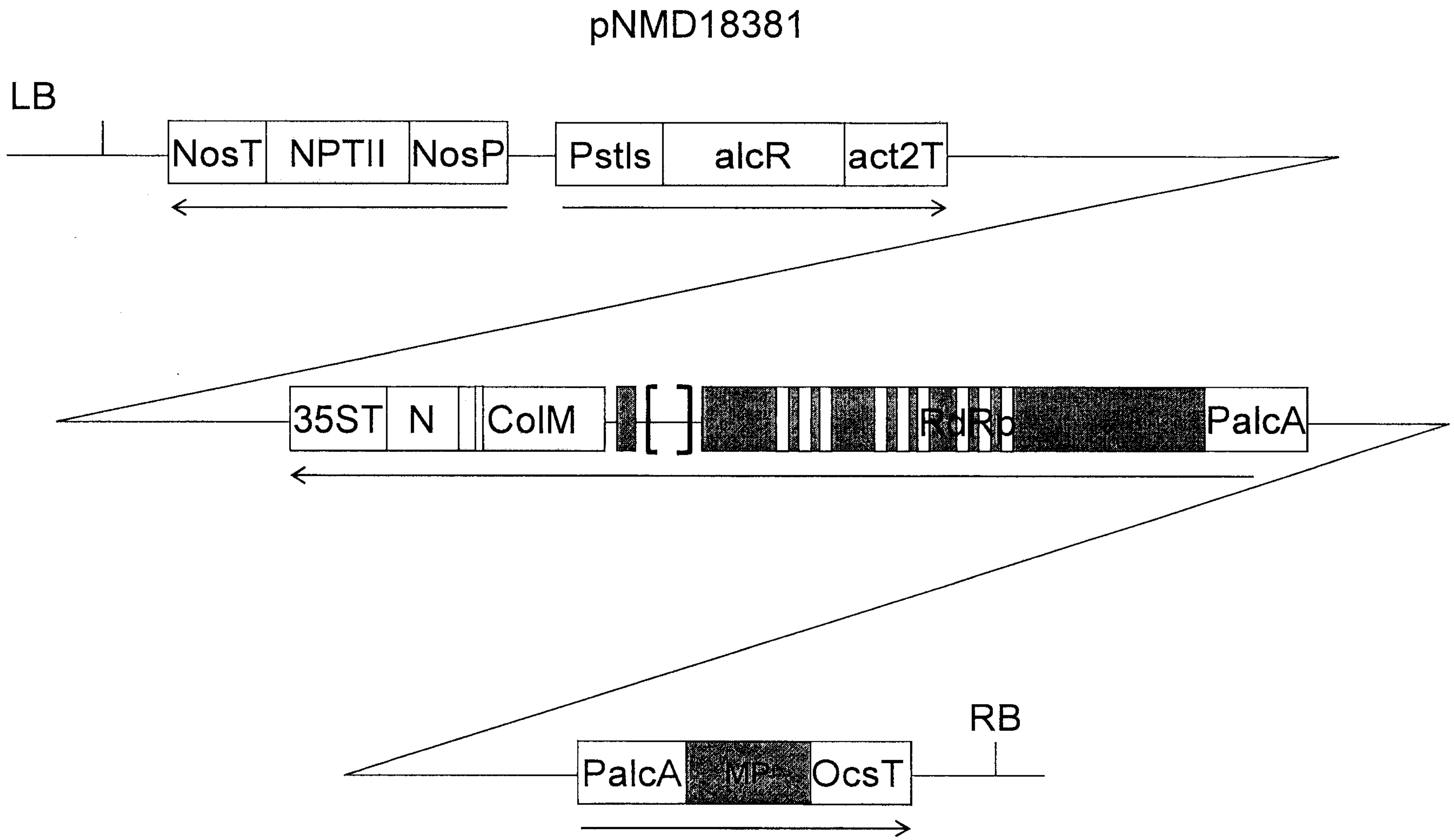
ColM
N
LB

Fig. 2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RB

Fig. 3

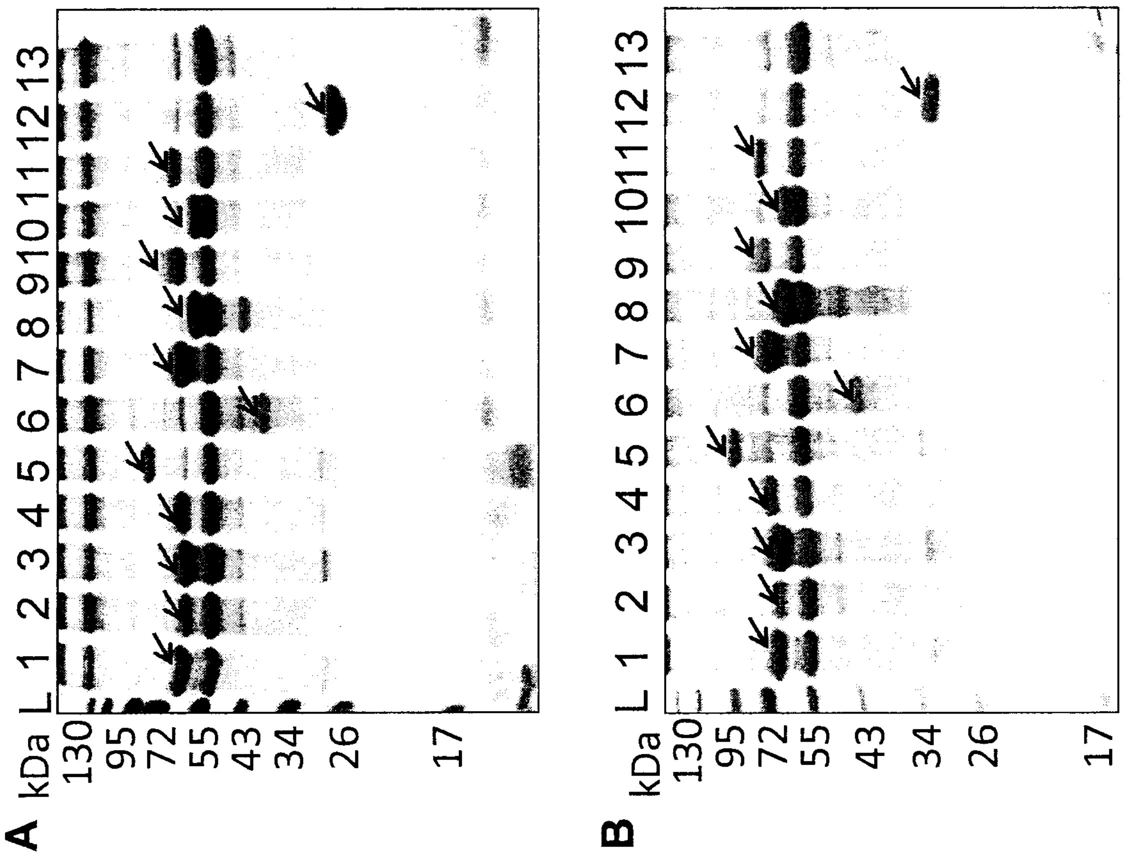
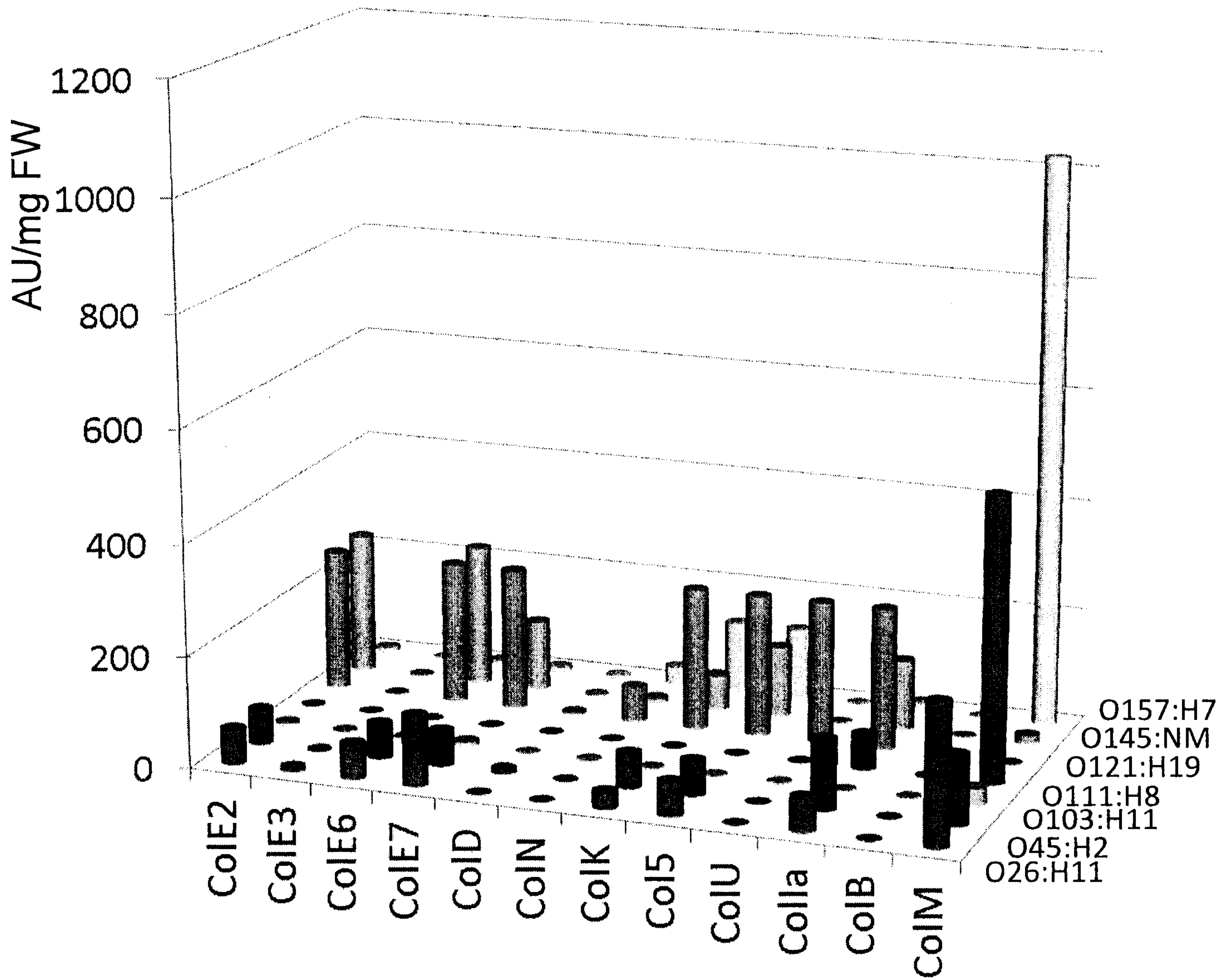


Fig. 4


AU/mg FW
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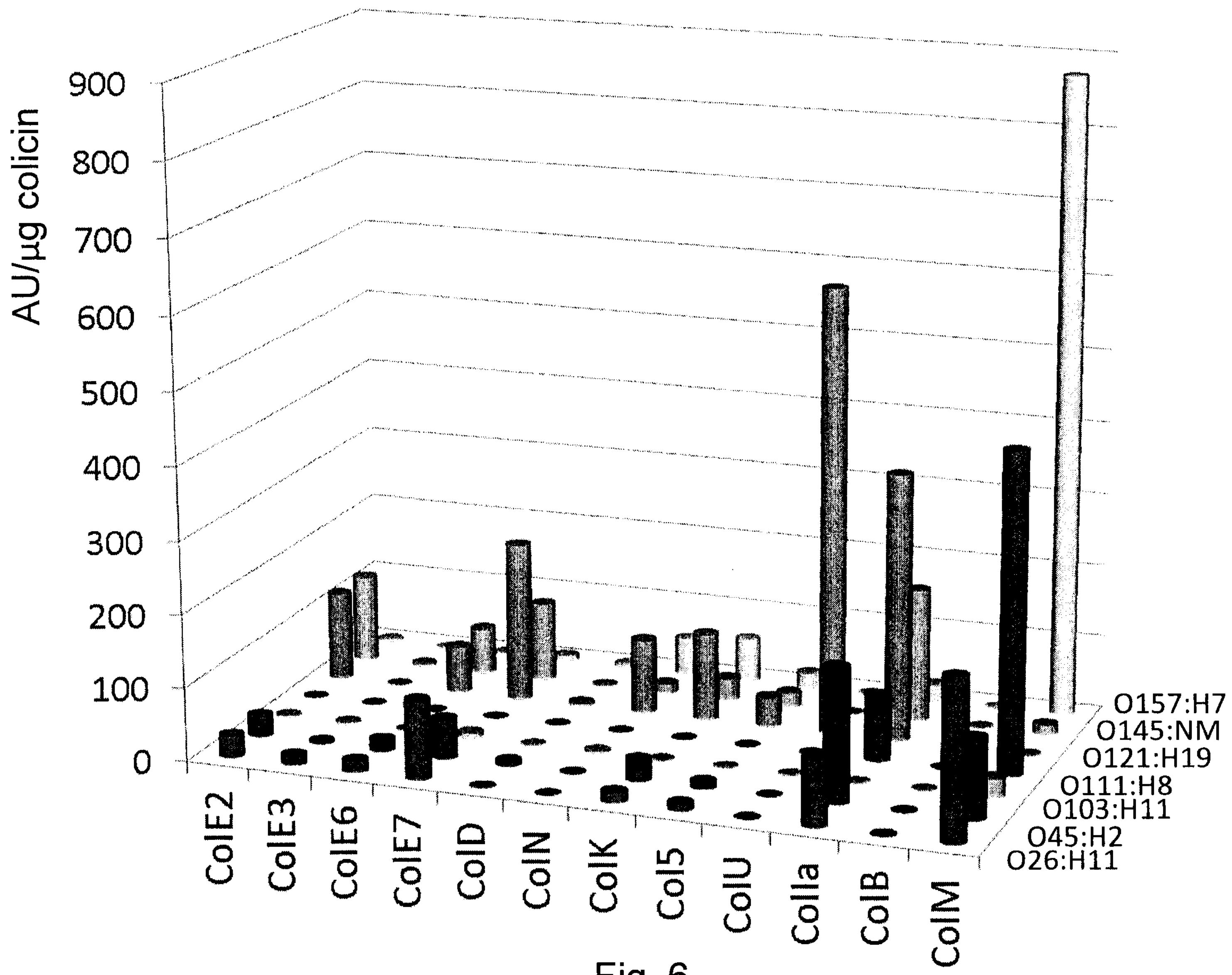
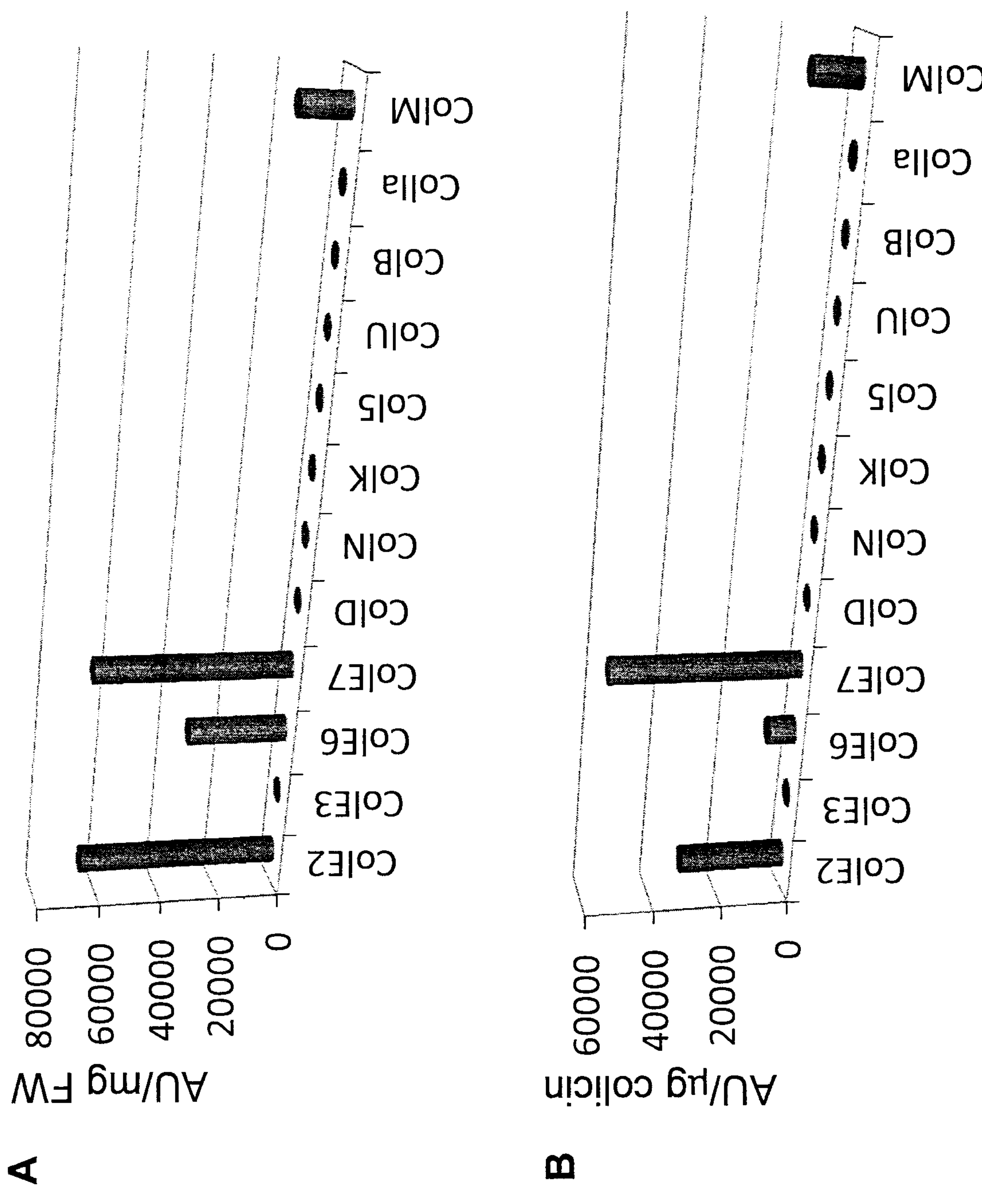
1000
800
600
400
200
0
ColE2
ColE3
ColE6
ColE7
ColD
ColN
ColK
Col5
ColU
ColIa
ColB
ColM
O157:H7
O145:NM
O121:H19
O111:H8
O103:H11
O45:H2
O26:H11

Fig. 5



AU/µg colicin
900
800
700
600
500
400
300
200
100
0
ColE2
ColE3
ColE6
ColE7
ColD
ColN
ColK
Col5
ColU
ColIa
ColB
ColM
O157:H7
O145:NM
O121:H19
O111:H8
O103:H11
O45:H2
O26:H11

Fig. 6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AU/mg FW
80000
60000
40000
20000
0
ColE2
ColE3
ColE6
ColE7
ColD
ColN
ColK
Col5
ColU
ColB
ColIa
ColM
B
AU/µg colicin
60000
40000
20000
0
ColE2
ColE3
ColE6
ColE7
ColD
ColN
ColK
Col5
ColU
ColB
ColIa
ColM

Fig. 7

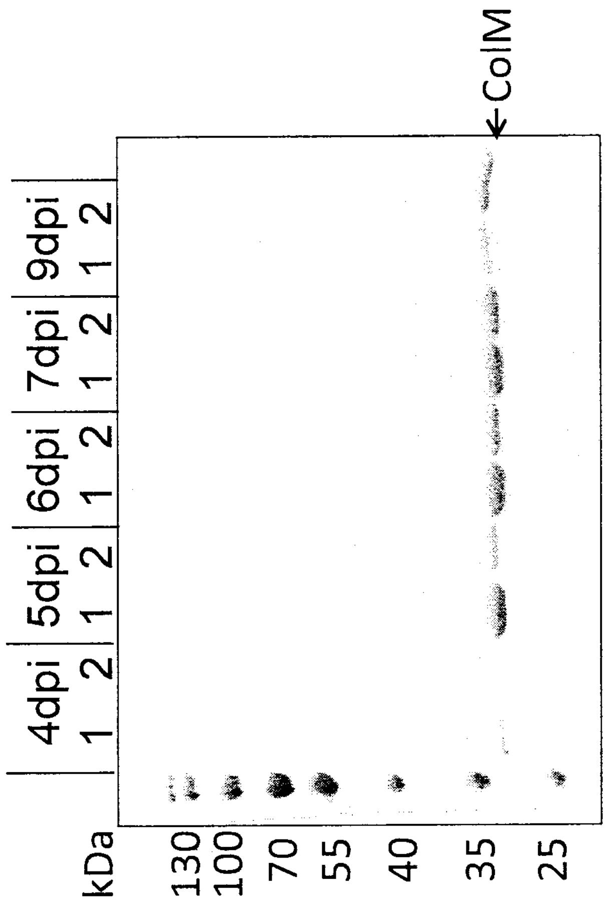

4dpi
5dpi
6dpi
7dpi
9dpi
1 2 1 2 1 2 1 2 1 2
kDa
130
100
70
55
40
35
25
ColM

Fig. 8

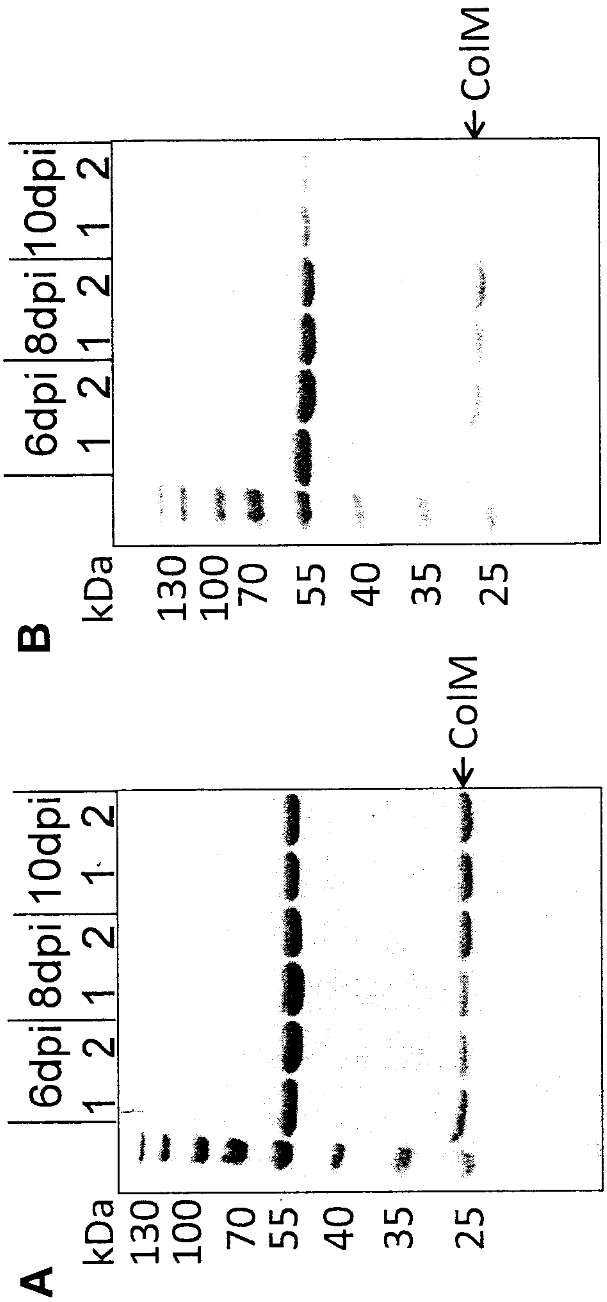
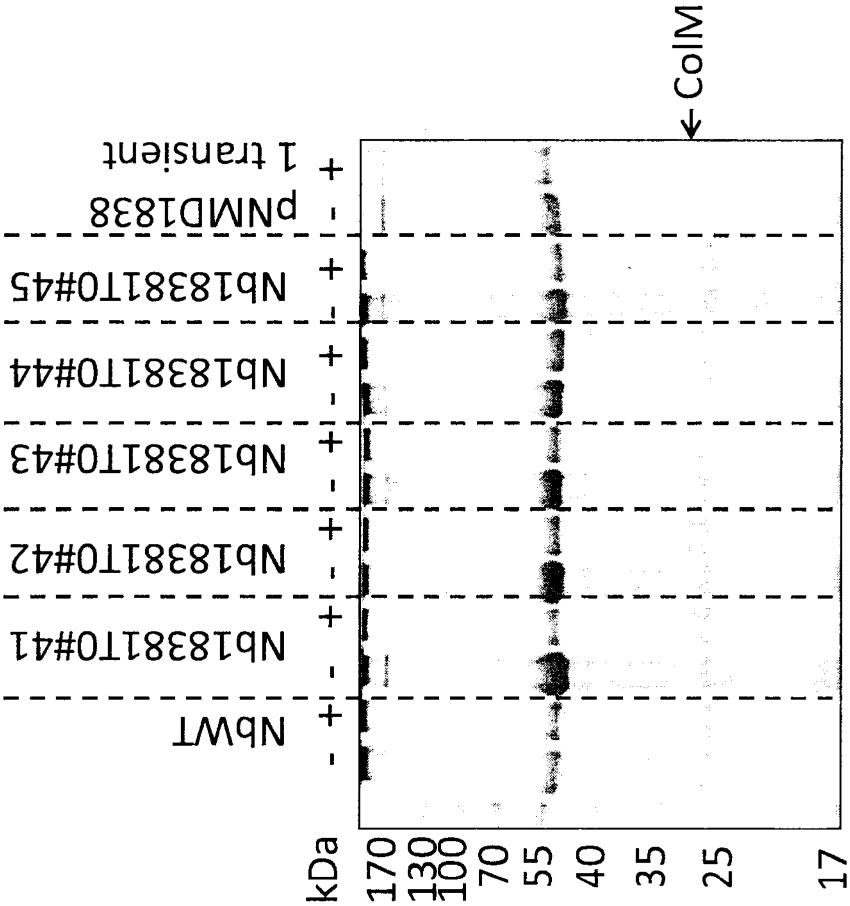


Fig. 9


NbWT
Nb1838T0#41
Nb1838T0#42
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Nb1838T0#44
Nb1838T0#45
pNMD1838
1 transient
ColM
kDa
170
130
100
70
55
40
35
25
17

Fig. 10

Effect of colicin treatment on bacterial populations of *E. coli* O157:H7 (DSM19206) on fresh steak meat

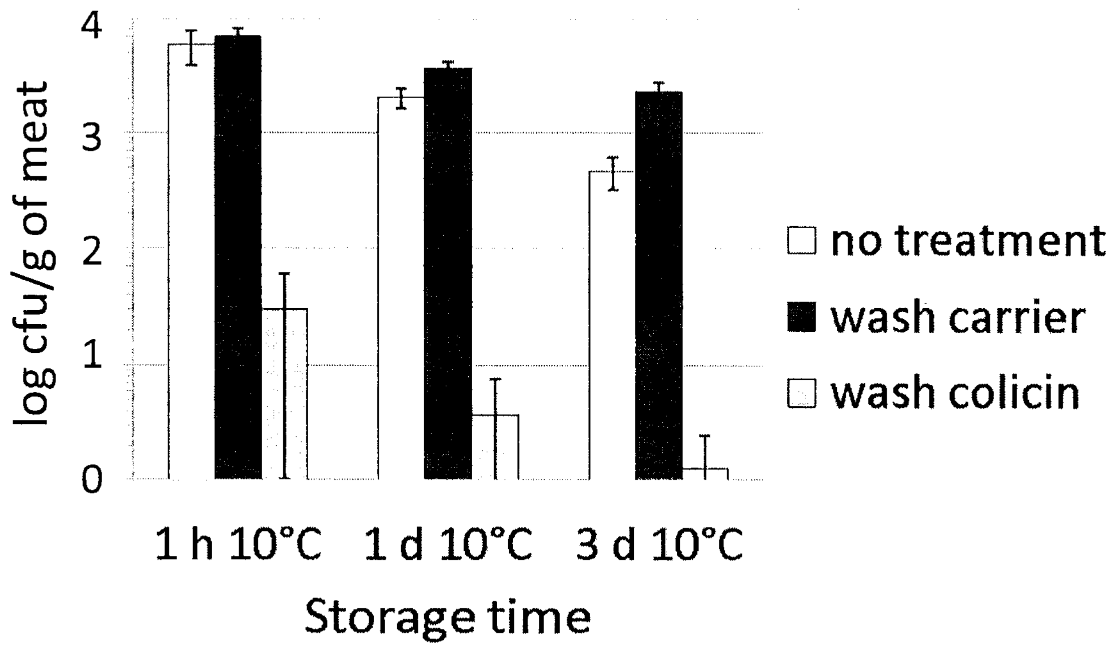


Fig. 11

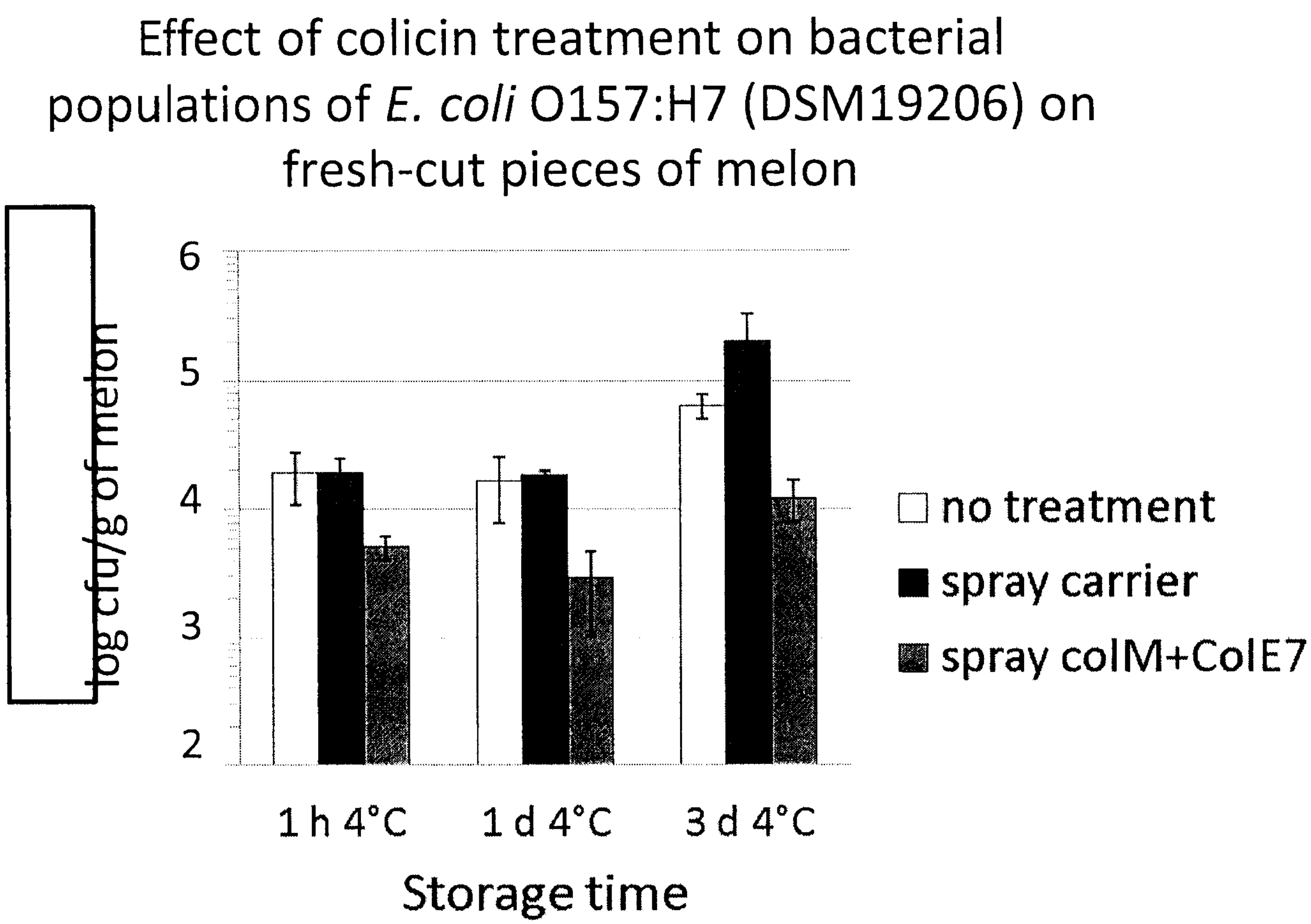

Effect of colicin treatment on bacterial populations of E. coli O157:H7 (DSM19206) on fresh-cut pieces of melon
log cfu/g of melon
6
5
4
3
2
1 h 4°C
1 d 4°C
3 d 4°C
Storage time
no treatment
spray carrier
spray colM+ColE7

Fig. 12

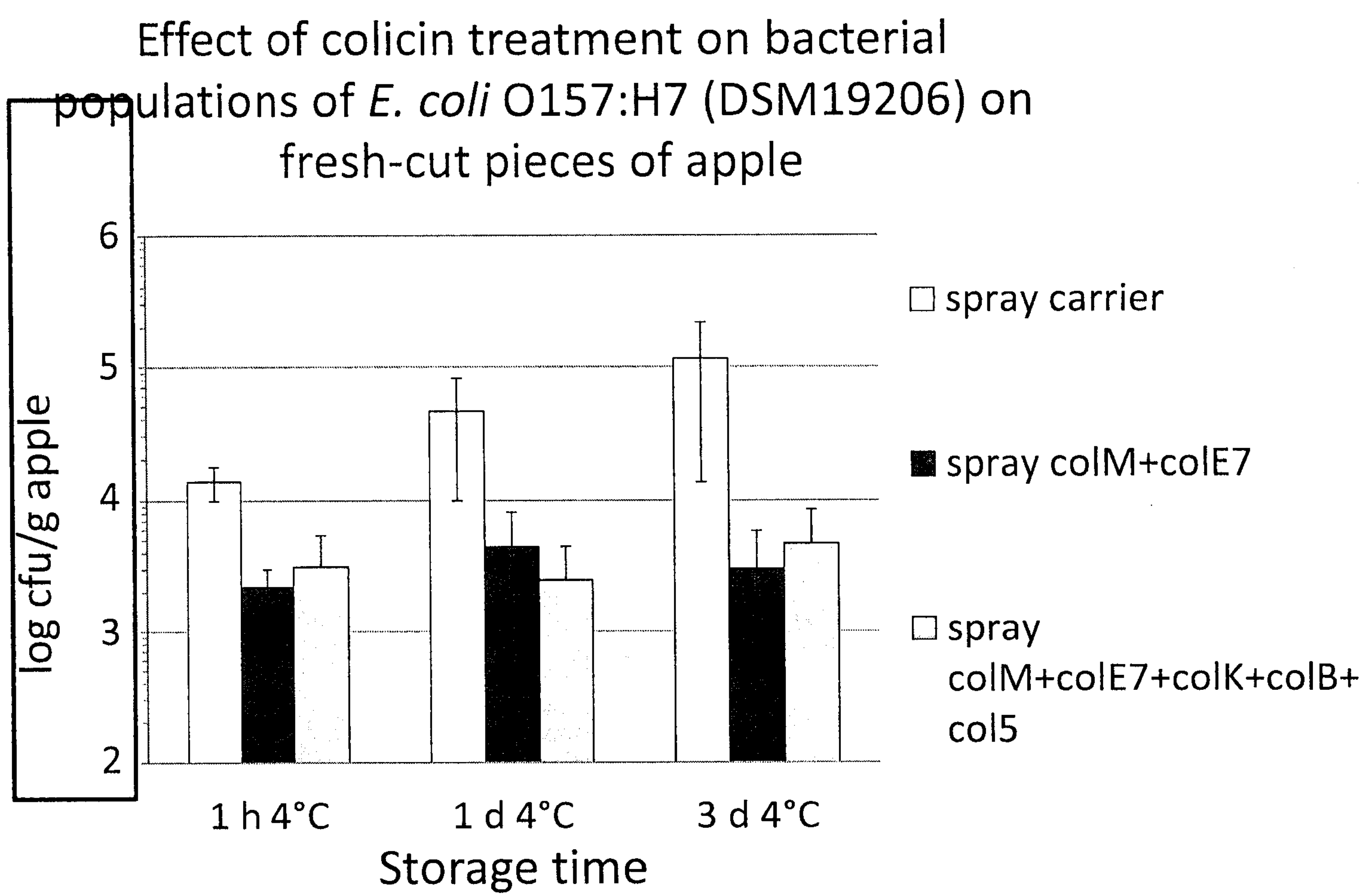

Effect of colicin treatment on bacterial populations of E. coli O157:H7 (DSM19206) on fresh-cut pieces of apple
log cfu/g apple
6
5
4
3
2
1 h 4°C
1 d 4°C
3 d 4°C
Storage time
spray carrier
spray colM+colE7
spray colM+colE7+colK+colB+col5

Fig. 13

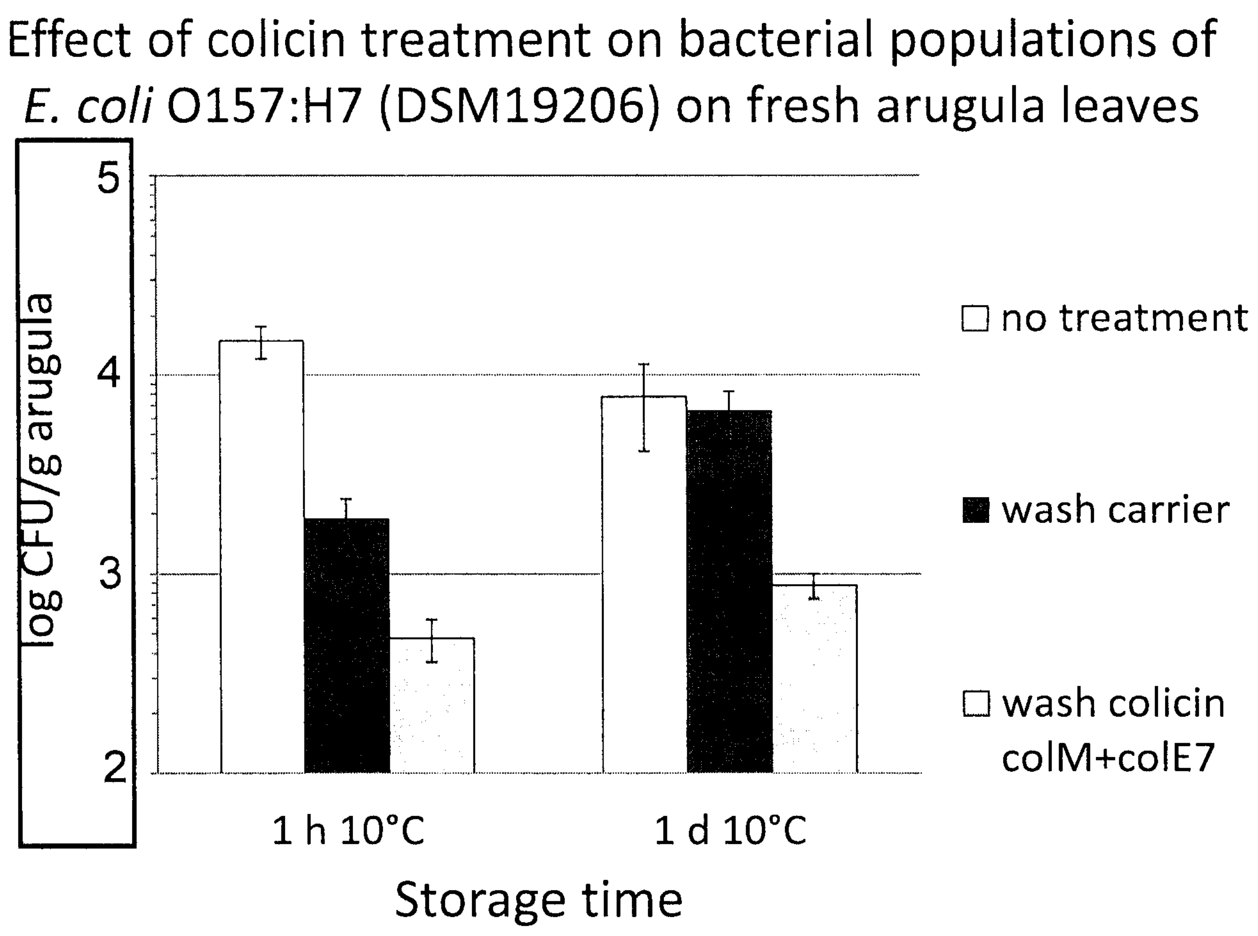

Effect of colicin treatment on bacterial populations of
E. coli O157:H7 (DSM19206) on fresh arugula leaves
log CFU/g arugula
5
4
3
2
1 h 10°C
1 d 10°C
Storage time
no treatment
wash carrier
wash colicin colM+colE7

Fig. 14

Effect of colicin treatment on bacterial populations of *E. coli* O157:H7 (DSM19206) on beef steak meat

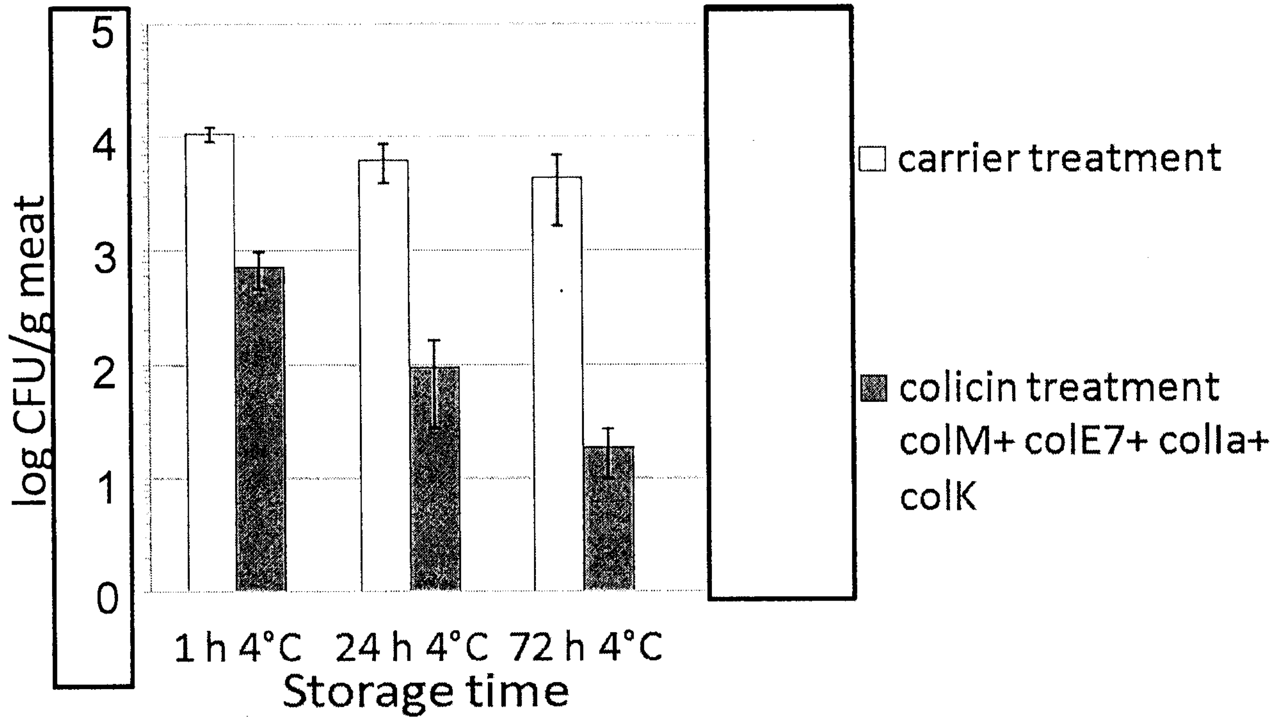


Fig. 15

Effect of colicin treatment on bacterial populations of *E. coli* O157:H7 (DSM19206) on ground beef meat

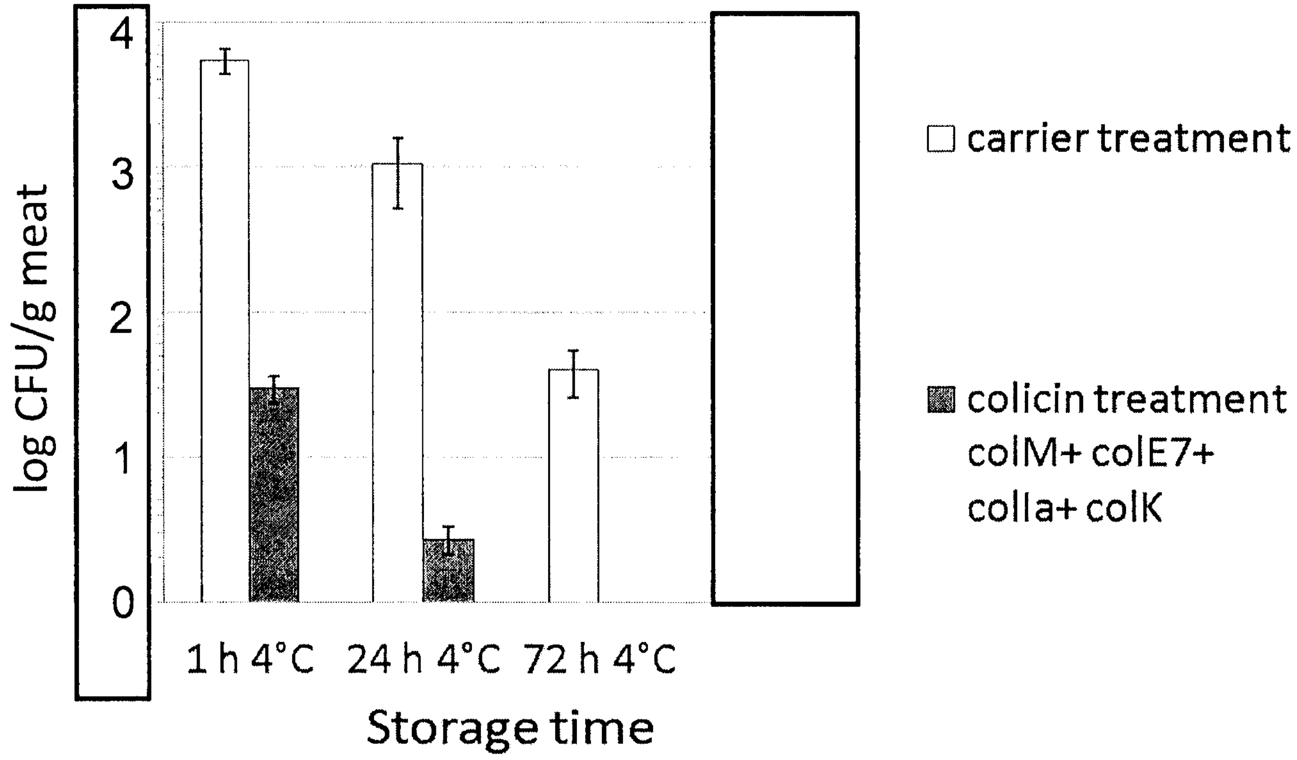


Fig. 16

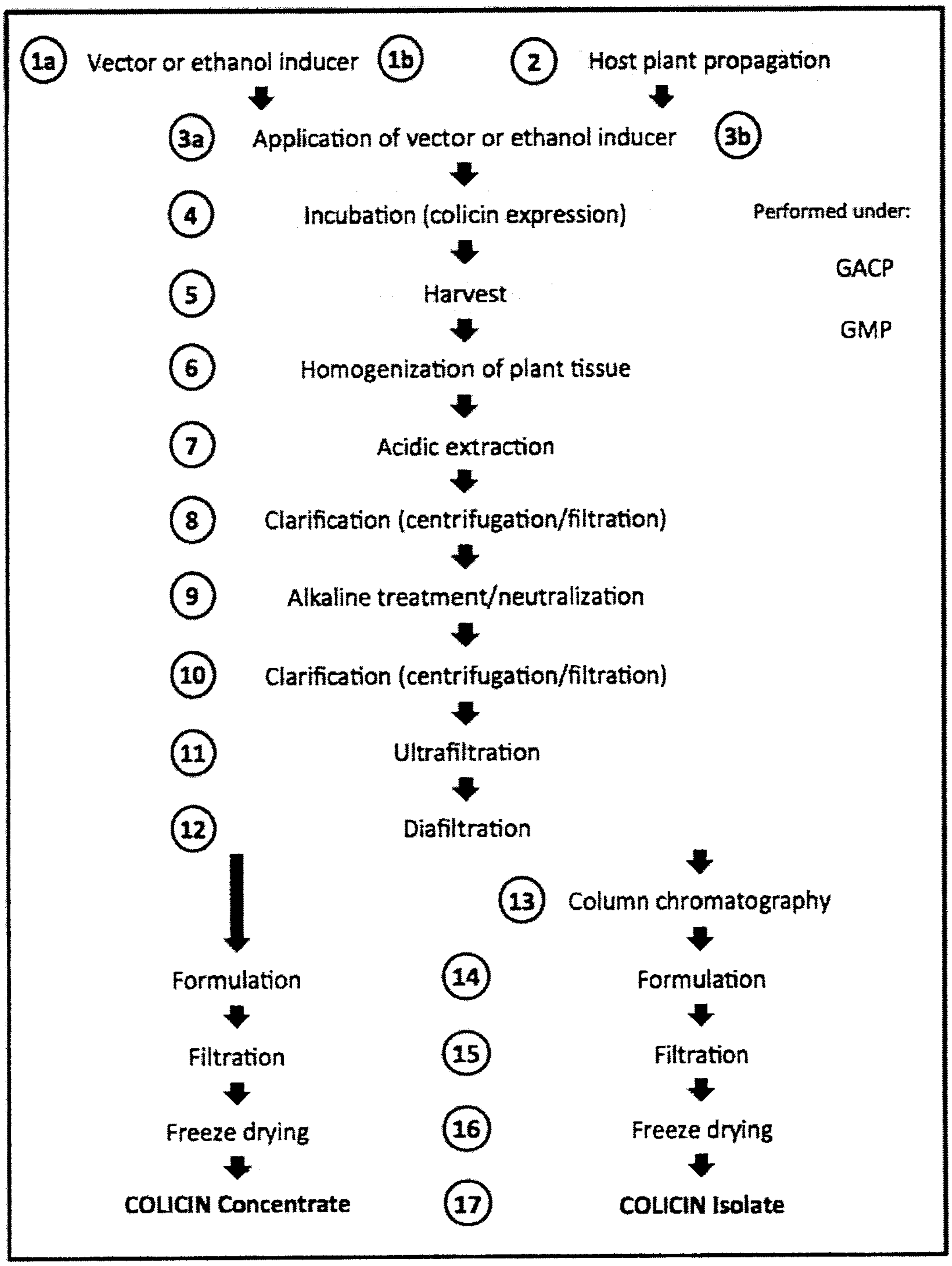

1a Vector or ethanol inducer 1b
2 Host plant propagation
3a Application of vector or ethanol inducer 3b
4 Incubation (colicin expression)
Performed under:
GACP
GMP
5 Harvest
6 Homogenization of plant tissue
7 Acidic extraction
8 Clarification (centrifugation/filtration)
9 Alkaline treatment/neutralization
10 Clarification (centrifugation/filtration)
11 Ultrafiltration
12 Diafiltration
13 Column chromatography
Formulation
14
Formulation
Filtration
15
Filtration
Freeze drying
16
Freeze drying
COLICIN Concentrate
17
COLICIN Isolate

Fig. 17

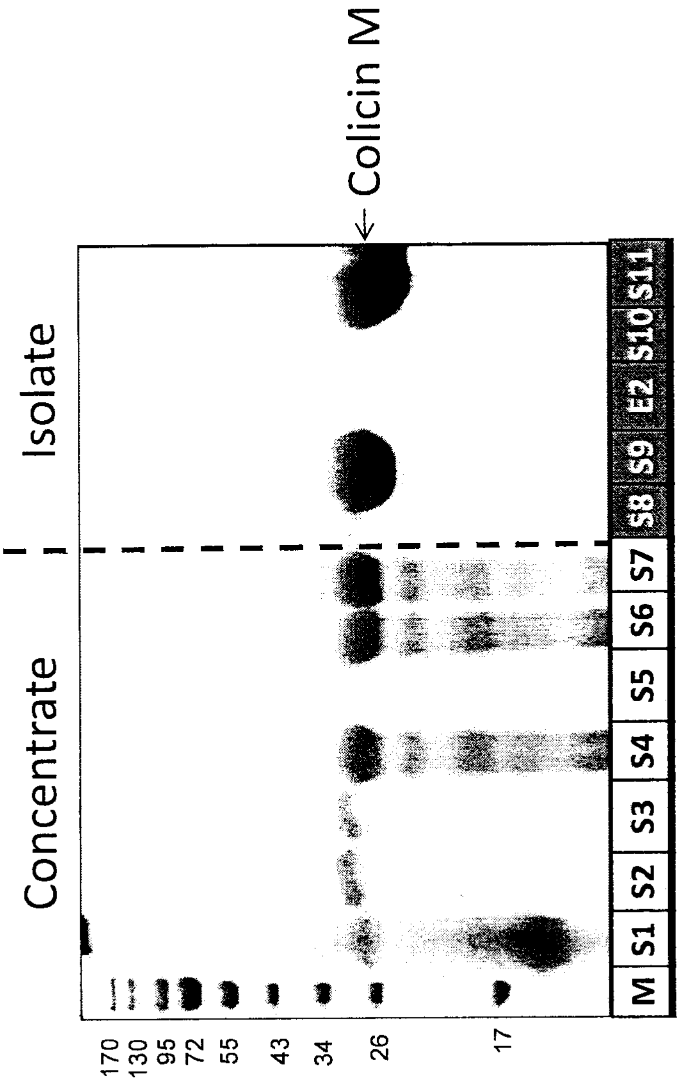
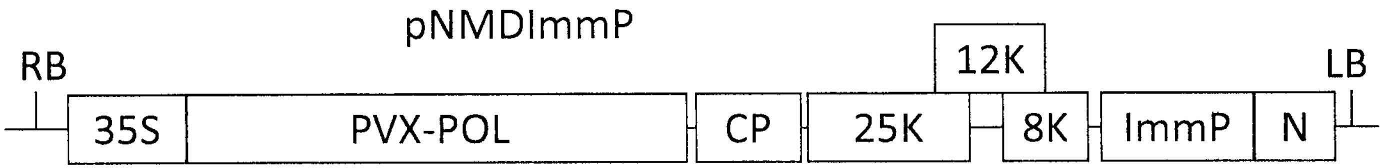

Concentrate
Isolate
Colicin M
M
S1
S2
S3
S4
S5
S6
S7
S8
S9
E2
S10
S11
170
130
95
72
55
43
34
26
17

Fig. 18

Nr	Colicin	Plasmid construct	Intron position
1	colE5	pNMD25880	G468
2	colE8	pNMD25891	G513
3	colE9	pNMD25901	V546
4	cloacin DF13	pNMD15351	G528
5	colA	pNMD25831	no intron
6	colS4	pNMD25856	no intron
7	col10	pNMD25848	no intron
8	colR	pNMD25813	no intron
9	col28b	pNMD25871	no intron
10	colY	pNMD26490	V451
11	colIb	pNMD25861	no intron

Fig. 19A



Nr	Immunity protein	Plasmid construct
1	ImmE5	pNMD25911
3	ImmE8	pNMD25920
4	ImmE9	pNMD25931
5	ImmDF13	pNMD15371

Fig. 19B

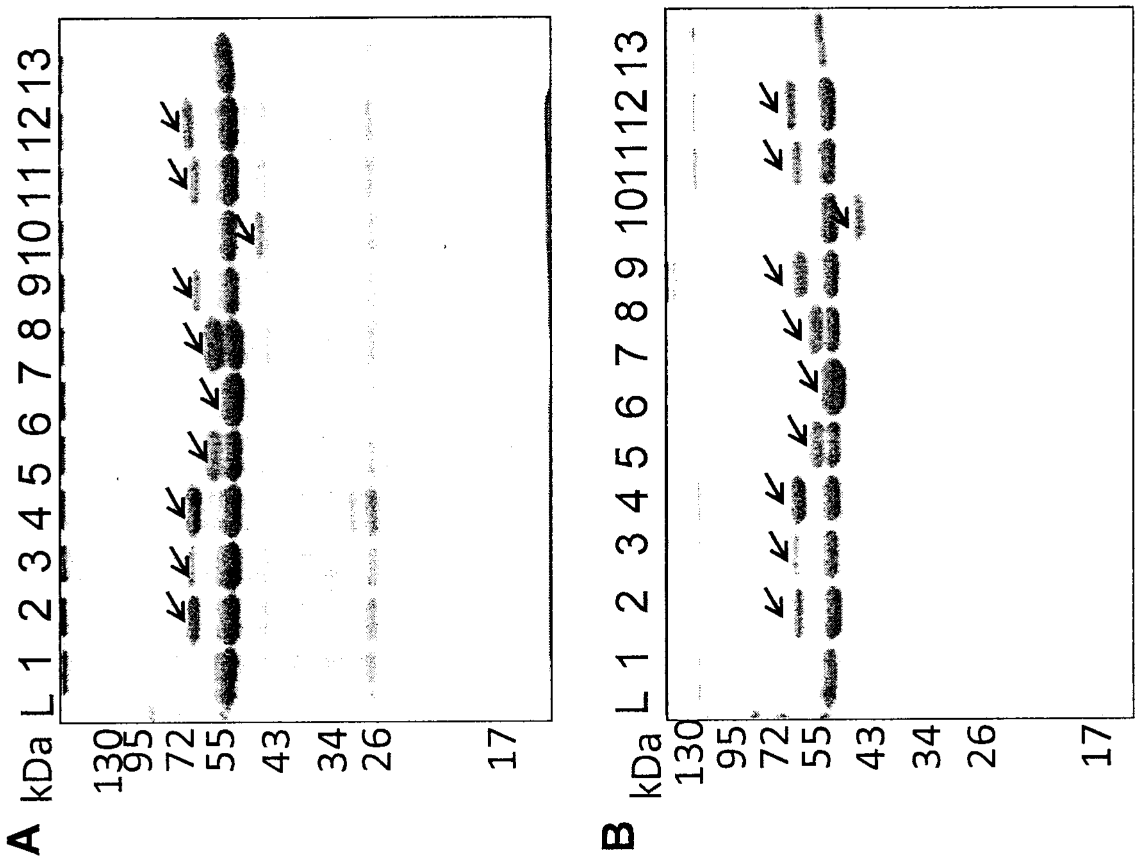
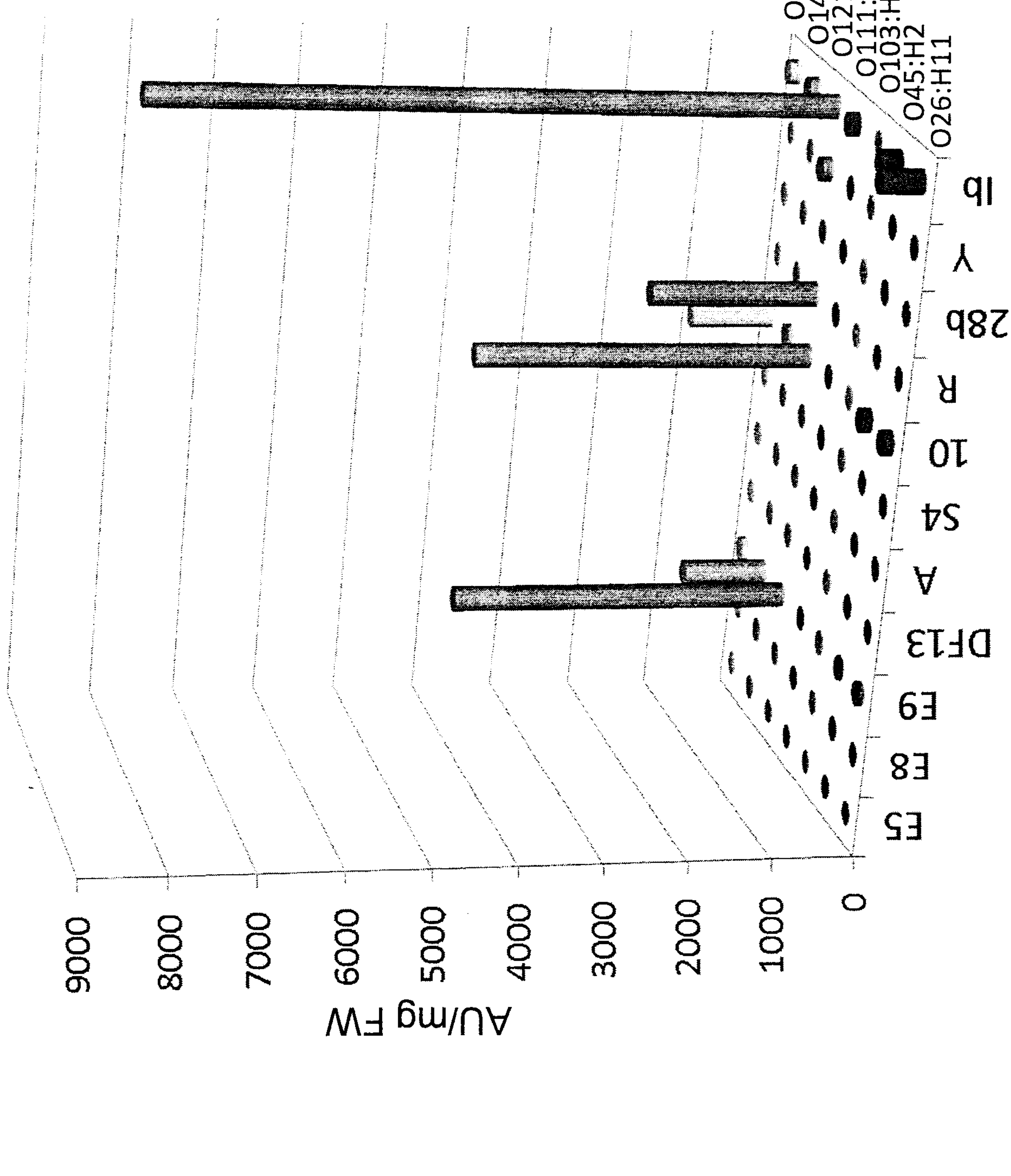

A
kDa
130
95
72
55
43
34
26
17
L 1 2 3 4 5 6 7 8 9 10 11 12 13
B
kDa
130
95
72
55
43
34
26
17
L 1 2 3 4 5 6 7 8 9 10 11 12 13

Fig. 20

Fig. 21


AU/mg FW
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
E5
E8
E9
DF13
A
S4
10
R
28b
Y
Ib
O157:H7
O145:NM
O121:H19
O111:H8
O103:H11
O45:H2
O26:H11

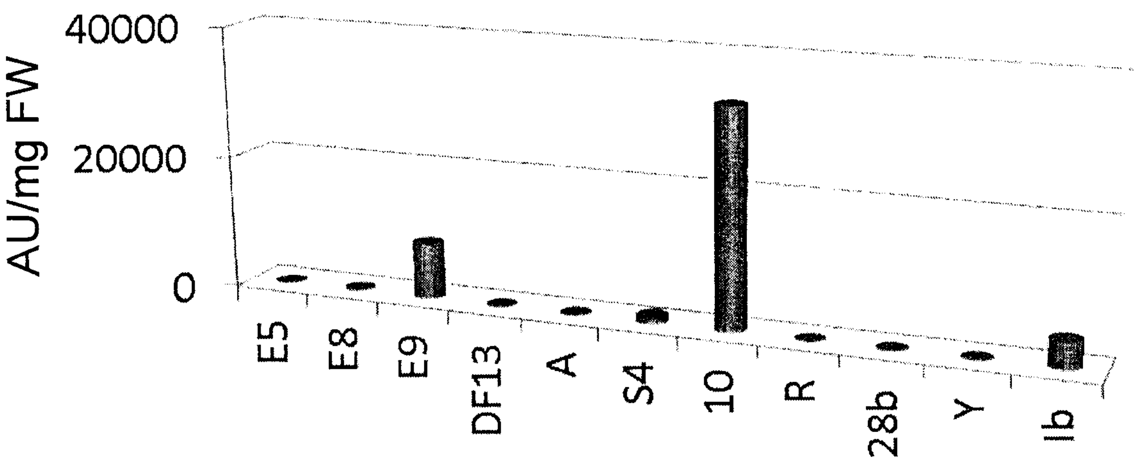
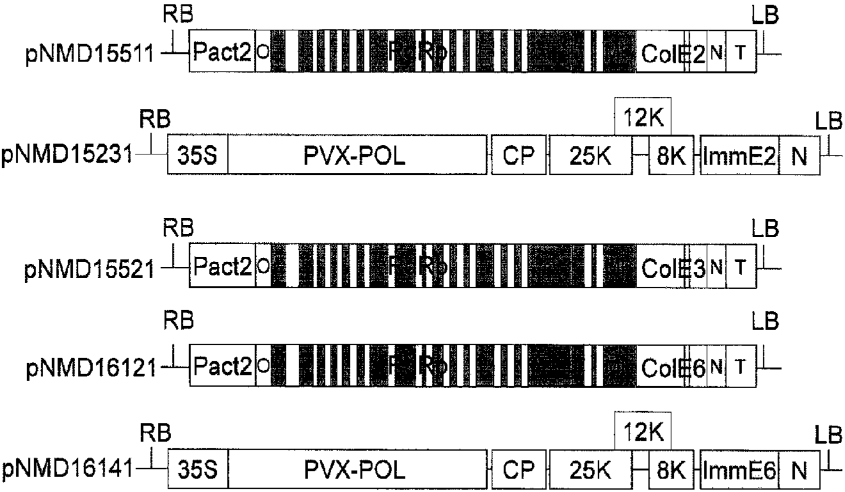


Fig. 22



pNMD15511
RB
Pact2
ColE2
N
T
LB
pNMD15231
RB
35S
PVX-POL
CP
25K
12K
8K
ImmE2
N
LB
pNMD15521
RB
Pact2
ColE3
N
T
LB
pNMD16121
RB
Pact2
ColE6
N
T
LB
pNMD16141
RB
35S
PVX-POL
CP
25K
12K
8K
ImmE6
N
LB