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(54) Title: COMPOSITIONS AND METHODS FOR TREATING INTESTINAL BACTERIAL INFECTIONS

(57) Abstract: The present invention relates to compositions and methods for treating infection by pathogenic bacteria. In particular, the present invention relates to compositions and methods for targeting virulence factors of pathogenic *E. coli*.



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COMPOSITIONS AND METHODS FOR TREATING INTESTINAL BACTERIAL INFECTIONS

This application claims priority to U.S. Provisional Patent Application No. 61/616,707,
5 filed March 28, 2012, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to compositions and methods for treating infection by
pathogenic bacteria. In particular, the present invention relates to compositions and methods for
10 targeting virulence factors of pathogenic *E. coli*.

BACKGROUND OF THE INVENTION

The intestine of animals is colonized by a large number of commensal microorganisms.
Millions of years of co-evolution have led this host-microbe interaction into a symbiotic
15 relationship in which the microbiota contributes to many host physiological processes. A key role
of the intestinal microbiota is protection against

Enteropathogenic *E. coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) are pathogenic *E.*
colonization and invasion by pathogens that often enter the intestinal tract. *coli* that are food- and
waterborne pathogens that are non-invasive attaching and effacing (A/E) bacteria. As a result of
20 the infection and ensuing intestinal inflammation, EPEC and EHEC cause severe diarrhea and
other complications, leading to the death of hundreds of thousands of children worldwide each
year. In addition, infection in adults can be lethal and cause significant morbidity. Unfortunately,
antibiotic therapy for EPEC and EHEC infections is not effective and can result in worsening of
intestinal symptoms.

25 New strategies to treat colitis caused by A/E pathogens are needed.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods for treating infection by
pathogenic bacteria. In particular, the present invention relates to compositions and methods for
30 targeting virulence factors of pathogenic *E. coli*.

For example, in some embodiments, the present invention provides a method of inhibiting
a biological activity of a virulence factor of a pathogenic *E. coli* (or other bacteria), comprising:
contacting the virulence factor or a cell expressing the virulence factor with a composition that
inhibits a biological activity of the virulence factor. In some embodiments, the pathogenic *E. coli*

is EHEC or EPEC. In some embodiments, the composition is a small molecule or an antibody that specifically binds to the virulence factor. In some embodiments, the small molecule is a salicylidene acyle hydrazine compound (e.g., ME0054, ME055, ME097, ME0114, or ME0214) or aurodox. In some embodiments, the antibody binds specifically to a LEE factor (e.g., intimin).

- 5 In some embodiments, the inhibiting treats infection by the pathogenic *E. coli*. In some embodiments, the inhibiting results in commensal *E. coli* out competing pathogenic *E. coli*.

Additional embodiments are described herein.

DESCRIPTION OF THE FIGURES

- 10 Figure 1 shows that microbiota is required for eradication of *C. rodentium*. A,B, SPF and GF mice (n= 7) were infected orally with 109cfu of *C. rodentium* and pathogen load in feces (A) and mouse survival (B) were determined over the indicated time. C, Cells were isolated from the large intestine of SPF or GF mice on day 0, 12 and 22 after oral infection with *C. rodentium*, and stained for CD11b, F4/80, and Gr-1mAb. D, Flow cytometry analysis for inflammatory
15 monocytes, neutrophils and CD3+ T cells in total lamina propria mononuclear cells (LPMCs). E, Histological analysis of the distal colon of SPF and GF mice infected orally with 109cfu of *C. rodentium* on day 12, 22 and 42 post-infection. F, Histopathological scores evaluating the infectious colitis phenotype induced by *C. rodentium* on day 12, 22 and 42 post-infection.

- Figure 2 shows expression and role of *ler* during *C. rodentium* infection in SPF and GF
20 mice.

- A, *ler* mRNA levels were determined by qPCR in fecal pellets of SPF and GF mice infected with *C. rodentium* at the indicated days post-infection. B, Expression of *ler* in fecal pellets of SPF and GF mice infected with the reporter *ler-lux* *C. rodentium* strain at the indicated day post-infection. C, Bioimaging of *ler* expression in the intestine of SPF and GF mice infected with the *ler-luxC. rodentium* strain. D-E, SPF and GF mice (n= 5) were infected orally with 109cfu of wild-type
25 and Δ *ler* mutant *C. rodentium*, and pathogen load in feces (D) and mouse survival (E) were determined over indicated time. F, GF mice were infected with wild-type and Δ *ler* mutant *C. rodentium*. At day 3 or day 21 post infection, mice were co-housed with SPF mice (1:1).

- Figure 3 shows localization of *C. rodentium* to intestinal niches is mediated by LEE
30 virulence factors. A, GF mice were infected with wild-type or Δ *ler* mutant *C. rodentium*. B, For FISH, tissues were fixed in Carnoy's fixation. C, Transmission Electron Micrographs of *C. rodentium*-infected GF mice cecum at day 5 and day 21 post infection. D-E, GF mice were infected orally with *C. rodentium*. Bioimaging of *ler* expression of tissue-attached *C.*

rodentium in the cecum (top) and colon (bottom) in GF mice (D). *C.rodentium* burden and *ler-lux* luminescence in feces, cecum, and colon (E).

Figure 4 shows that similar catabolic preferences for saccharides determine the competing ability of commensal bacteria with the enteric pathogen. A, GF mice were infected with wild-type *C. rodentium* (Cr). At day 21 post infection, commensal *E. coli* (Ec) or *B. thetaiotaomicron* (Bt) or *B. vulgatus* (Bv) were inoculated. B, Total *Enterobacteria* (*C. rodentium* and *E. coli*) and *Bacteroides* culture in feces at day 0 and day 14 of competing experiment. C, Diluted fecal samples were plated onto McConkey plates with or without kanamycin. D, Carbohydrate catabolic profiles of *C. rodentium* and commensal bacteria strains. E, GF mice were infected with wild-type *C. rodentium* (Cr). F, *C. rodentium* mono-associated GF mice (day 21) were fed with a simple sugar diet (ssDiet) for 7 days.

Figure 5 shows antimicrobial peptide expression in intestinal epithelial cells.

Figure 6 shows *tir* mRNA expression in stool samples were collected on day 7 and day 12 (also on day 22 and day 42 in GF mice) after *C. rodentium* infection.

Figure 7 shows that bioluminescence of *C. rodentium* *ler-lux* strain represents the endogenous *ler* expression. A. Schematic representation of the *ler-lux* reporter operon fusion between the *C. rodentium* *ler* regulatory region from positions -260 to +216 (14) and the *Photobacterium luminescens* *luxCDABE* operon in pCS26-Pac (15). B. Expression of *ler-lux* in wild type *C. rodentium* under *in vitro* growth conditions. (Left panel) luminescence of *ler-lux* in bacteria grown in DMEM (filled circle) or LB medium (open circle). (Right panel) bacteria proliferation in DMEM (filled circle) or in LB medium (open circle). C. Endogenous *ler* mRNA expression in bacteria grown in DMEM or LB medium.

Figure 8 shows that down-regulation of *ler*-virulence is reversible. Pathogen load in feces (A) were determined over the indicated time, and histology was assessed at day 12 (B). Arrows denote marked submucosal edema and infiltration of acute inflammatory cells.

Figure 9 shows that a functional type III secretion system is not required for robust growth in GF mice, but essential to compete with the commensal microbiota. A, B, SPF and GF mice (n=5) were infected orally with 10⁹cfu of wild-type and ΔescN (T3SS) mutant *C. rodentium*, and pathogen load in feces (A) and mouse survival (B) were determined over indicated time.

Figure 10 shows that *ler*-regulated virulence expression before conventionalization in GF mice. A, Bacterial RNAs were purified from feces, and *ler* expression was assessed by qPCR. B, Expression of *ler* in fecal pellets of GF mice infected with the *ler-lux* reporter *C. rodentium* strain at the indicated day post-infection.

Figure 11 shows that intestinal inflammation does not enhance colonization of *C. rodentium* lacking a functional type 3 secretion system. A, Experimental protocol of DSS-induced colitis. B, Weight (left) and disease activity index (DAI: right) of DSS-treated and untreated mice which are infected with wild-type *C. rodentium* or Δ escN mutant. C, Bacteria number in feces 2 days after *C. rodentium* or Δ escN mutant inoculation. D, SPF C57BL/6 mice were infected with *C. rodentium* (ampicillin resistant) or Δ escN mutant (kanamycin resistant), respectively, or coinfectd with both strains.

Figure 12 shows that GF mice develop *C. rodentium*-specific immunoglobulins after infection.

Figure 13 shows that germ-free mice were able to develop Th1 and Th17 immune responses. A, Intracellular production of IFN- γ , and IL-17A was assessed in isolated colonic CD4+ T cells from SPF and GF mice on day 0, 12 and 22 after infection with *C. rodentium*. B, Th1 and Th17 cytokines production by isolated LP cells from SPF and GF mice on day 0, 12 after infection with *C. rodentium*.

Figure 14 shows Ler-dependent virulence factor-specific IgG production in *C. rodentium* infected mice. A. Serum IgG. B. Intestinal content IgG.

Figure 15 shows intimin specific IgG production in the intestine of *C. rodentium* infected mice.

DEFINITIONS

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

As used herein, the terms “detect”, “detecting” or “detection” may describe either the general act of discovering or discerning or the specific observation of a detectably labeled composition.

The term “derivative” of a compound, as used herein, refers to a chemically modified compound wherein the chemical modification takes place either at a functional group of the compound or backbone.

As used herein, the term “subject” refers to organisms to be treated by the methods of the present invention. Such organisms preferably include, but are not limited to, mammals (e.g., murines, simians, equines, bovines, porcines, canines, felines, and the like), and most preferably includes humans. In the context of the invention, the term “subject” generally refers to an individual who will receive or who has received treatment for a condition characterized by bacterial infection.

The term “diagnosed,” as used herein, refers to the recognition of a disease by its signs and symptoms (*e.g.*, resistance to conventional therapies), or genetic analysis, pathological analysis, histological analysis, and the like.

As used herein the term, “*in vitro*” refers to an artificial environment and to processes or reactions that occur within an artificial environment. *In vitro* environments include, but are not limited to, test tubes and cell cultures. The term “*in vivo*” refers to the natural environment (*e.g.*, an animal or a cell) and to processes or reaction that occur within a natural environment.

As used herein, the term “host cell” refers to any eukaryotic or prokaryotic cell (*e.g.*, mammalian cells, avian cells, amphibian cells, plant cells, fish cells, and insect cells), whether located *in vitro* or *in vivo*.

As used herein, the term “cell culture” refers to any *in vitro* culture of cells. Included within this term are continuous cell lines (*e.g.*, with an immortal phenotype), primary cell cultures, finite cell lines (*e.g.*, non-transformed cells), and any other cell population maintained *in vitro*, including oocytes and embryos.

As used herein, the term “effective amount” refers to the amount of a therapy (*e.g.*, a therapeutic agent described herein) sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages and is not limited intended to be limited to a particular formulation or administration route.

As used herein, the term “co-administration” refers to the administration of at least two agent(s) (*e.g.*, a compound of the present invention) or therapies to a subject. In some embodiments, the co-administration of two or more agents/therapies is concurrent. In some embodiments, a first agent/therapy is administered prior to a second agent/therapy. Those of skill in the art understand that the formulations and/or routes of administration of the various agents/therapies used may vary. The appropriate dosage for co-administration can be readily determined by one skilled in the art. In some embodiments, when agents/therapies are co-administered, the respective agents/therapies are administered at lower dosages than appropriate for their administration alone. Thus, co-administration is especially desirable in embodiments where the co-administration of the agents/therapies lowers the requisite dosage of a known potentially harmful (*e.g.*, toxic) agent(s).

As used herein, the term “toxic” refers to any detrimental or harmful effects on a cell or tissue as compared to the same cell or tissue prior to the administration of the toxicant.

As used herein, the term “pharmaceutical composition” refers to the combination of an active agent with a carrier, inert or active, making the composition especially suitable for diagnostic or therapeutic use *in vivo*, *in vivo* or *ex vivo*.

As used herein, the term “pharmaceutically acceptable carrier” refers to any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions (*e.g.*, such as an oil/water or water/oil emulsions), and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants. (*See e.g.*, Martin, Remington's Pharmaceutical Sciences, 15th Ed., Mack Publ. Co., Easton, PA [1975]).

As used herein, the term “pharmaceutically acceptable salt” refers to any pharmaceutically acceptable salt (*e.g.*, acid or base) of a compound of the present invention which, upon administration to a subject, is capable of providing a compound of this invention or an active metabolite or residue thereof. As is known to those of skill in the art, “salts” of the compounds of the present invention may be derived from inorganic or organic acids and bases. Examples of acids include, but are not limited to, hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, lactic, salicylic, succinic, toluene-*p*-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, naphthalene-2-sulfonic, benzenesulfonic acid, and the like. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts.

As used herein, the term “sample” is used in its broadest sense. In one sense, it is meant to include a specimen or culture obtained from any source, as well as biological and environmental samples. Biological samples may be obtained from animals (including humans) and encompass fluids, solids, tissues, and gases. Biological samples include blood products, such as plasma, serum and the like. Environmental samples include environmental material such as surface matter, soil, water, crystals and industrial samples. Such examples are not however to be construed as limiting the sample types applicable to the present invention.

As used herein, the term “modulate” refers to the activity of a compound (*e.g.*, a compound described herein) to affect (*e.g.*, to promote or retard) an aspect of cellular function, including, but not limited to, bacterial growth and the like.

The term “test compound” refers to any chemical entity, pharmaceutical, drug, and the like, that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function, or otherwise alter the physiological or cellular status of a sample (*e.g.*, bacterial infection). Test compounds comprise both known and potential therapeutic compounds. A test compound can be determined to be therapeutic by using the screening methods of the present invention. A “known therapeutic compound” refers to a therapeutic compound that has been shown (*e.g.*, through

animal trials or prior experience with administration to humans) to be effective in such treatment or prevention. In some embodiments, "test compounds" are agents that target virulence genes.

As used herein, the term "purified" or "to purify" refers to the removal of components (e.g., contaminants) from a sample. For example, antibodies are purified by removal of
5 contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind to the target molecule. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind to the target molecule results in an increase in the percent of target-reactive immunoglobulins in the sample. In another example, recombinant polypeptides are expressed in bacterial host cells and the polypeptides are purified
10 by the removal of host cell proteins; the percent of recombinant polypeptides is thereby increased in the sample.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods for treating infection by
15 pathogenic bacteria. In particular, the present invention relates to compositions and methods for targeting virulence factors of pathogenic *E. coli* and other bacteria.

I. Pathogenic *E. coli*

It is generally unclear, during the course of an enteric infection, how commensal bacteria
20 and pathogens interact. This includes uncertainty regarding how virulence strategies allow pathogens to obtain an environmental niche when competing with the microbiota. Similarly, it is unclear whether commensals play a role, not only in colonization resistance, but also in the clearance of pathogens from the intestines of an infected host. Enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are important causes of watery diarrhea and
25 mortality worldwide (1, 2). These Gram-negative bacteria are food- and waterborne non-invasive pathogens which attach to and colonize the intestinal tract by inducing characteristic attaching and effacing (A/E) lesions on the intestinal epithelium, leading to transient enteritis or colitis in humans (1, 2). The genome of EPEC and EPEC and related pathogens harbor the locus for enterocyte effacement (LEE) which is critical for bacterial colonization and the ability to cause
30 pathology (3, 4). Recently, it was reported that infection with *Citrobacter rodentium*, a natural A/E bacterial pathogen of mice that is widely used to model human infections with EPEC and EHEC (5, 6), is associated with a significant but reversible decrease in the total number of commensal bacteria in the mouse colon (7, 8). However, the role of the microbiota in *C. rodentium* colonization is unclear.

Experiments conducted during the course of development of embodiments of the present invention demonstrated the role of virulence factors in growth of pathogenic *E. coli*. Accordingly, embodiments of the present invention provide compositions and methods for treating infection by pathogenic *E. coli* comprising inhibiting virulence factor genes and proteins.

II. Therapeutic Applications

In some embodiments, the present invention provides therapies for pathogenic *E. coli* (e.g., EPEC and/or EHEC). In some embodiments, therapies target virulence factors of EPEC and/or EHEC.

A. Small Molecules

In some embodiments, small molecule inhibitors of virulence factors are utilized. In some embodiments, small molecule therapeutics are salicylidence acyle hydrazine compounds. Salicylidence acyl hydrazide compounds that have been shown to reduce the expression of T3SS effectors including those from A/E pathogens at ~1-20 μ M range (33). Examples of salicylidence acyle hydrazine compounds include, but are not limited to, ME0054, ME055, ME097, ME0114, and ME0214. These compounds do not inhibit bacteria growth, but can inhibit expression of several LEE virulence genes including Ler and formation of A/E lesions on bovine epithelial cells (33).

In some embodiments, small molecules are aurodox or related compounds. Aurodox is a linear polyketide compound that is a specific T3SS inhibitor of EPEC and *C. rodentium* (34) and targets multiple LEE virulence factors. Aurodox is commercially available (e.g., from Enzo Life Sciences, Inc. Farmingdale, NY) and has been shown to protect mice from *C. rodentium* infection in vivo (34).

C. Antibody Therapy

In some embodiments, the present invention provides antibodies or antigen binding partners thereof that target virulence factors of EPEC and/or EHEC. Any suitable antibody (e.g., monoclonal, polyclonal, or synthetic) may be utilized in the therapeutic methods disclosed herein. In some embodiments, the antibodies used for therapy are humanized antibodies. Methods for humanizing antibodies are well known in the art (See e.g., U.S. Pat. Nos. 6,180,370, 5,585,089, 6,054,297, and 5,565,332; each of which is herein incorporated by reference).

In some embodiments, antibodies with defined specificity against LEE factors are used to inhibit Ler and pathogen colonization. In some embodiments, antibodies are raised against

intimin (e.g., the C-terminal 382 amino acid extracellular domain) from *C. rodentium*. In some embodiments, antibodies against LEE factors other than intimin are utilized. For example, in some embodiments, extracts of *C.rodentium* expressing LEE virulence factors and total RNA purified from the blood are utilized

5 In preferred embodiments, antibody based therapeutics are formulated as pharmaceutical compositions as described below. In preferred embodiments, administration of an antibody composition of the present invention results in a measurable decrease in disease (e.g., decrease or elimination of infection by pathogenic *E. coli*).

10 The present invention also includes pharmaceutical compositions and formulations that include the antibody compounds of the present invention as described below.

D. Pharmaceutical Compositions

15 The present invention further provides pharmaceutical compositions (e.g., comprising pharmaceutical agents that inhibit virulence factors of pathogenic *E.coli*). The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary (e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral

20 administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration.

25 Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

 Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

30 Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions that may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

 Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be

generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the acid(s) of the formulation.

Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from

measurements of drug accumulation in the body of the patient. The administering physician can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC₅₀s found to be effective in *in vitro* and *in vivo* animal models or based on the examples described herein. In general, dosage is from 0.01 µg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly. The treating physician can estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the subject undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 µg to 100 g per kg of body weight, once or more daily, to once every 20 years.

In some embodiments, the present invention provides therapeutic methods comprising one or more compositions described herein in combination with an additional agent (e.g., an antibiotic).

III. Drug Screening Applications

In some embodiments, the present invention provides drug screening assays (e.g., to screen for drugs that target virulence factors of pathogenic *E. coli*). In other embodiments, candidate compounds are antibodies or small molecules that specifically bind to a virulence factor and inhibit its biological function.

In one screening method, candidate compounds are evaluated for their ability to alter expression of virulence genes by contacting a compound with pathogenic *E. coli* and then assaying for the effect of the candidate compounds on expression of virulence genes. In some embodiments, the effect of candidate compounds on expression of a virulence gene is assayed for by detecting the level of mRNA expressed by the cell. mRNA expression can be detected by any suitable method.

In other embodiments, the effect of candidate compounds on expression of virulence genes is assayed by measuring the level of polypeptide encoded by the virulence genes. The level of polypeptide expressed can be measured using any suitable method.

Specifically, the present invention provides screening methods for identifying modulators, i.e., candidate or test compounds or agents (e.g., proteins, antibodies, peptides, peptidomimetics, peptoids, small molecules or other drugs) which have an inhibitory (or stimulatory) effect on, for example, pathogenic *E. coli* virulence gene expression or activity, or have a stimulatory or inhibitory effect on, for example, the expression or activity of a virulence

gene. Compounds thus identified can be used to modulate the activity of target gene products (e.g., virulence genes) either directly or indirectly in a therapeutic protocol, to elaborate the biological function of the target gene product, or to identify compounds that disrupt normal target gene interactions. Compounds that inhibit the activity or expression of virulence genes in the treatment of infection by pathogenic *E. coli* (e.g., EPEC and/or EHEC).

The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone, which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, e.g., Zuckennann *et al.*, J. Med. Chem. 37: 2678-85 [1994]); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are preferred for use with peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam (1997) Anticancer Drug Des. 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.*, Proc. Natl. Acad. Sci. U.S.A. 90:6909 [1993]; Erb *et al.*, Proc. Nad. Acad. Sci. USA 91:11422 [1994]; Zuckermann *et al.*, J. Med. Chem. 37:2678 [1994]; Cho *et al.*, Science 261:1303 [1993]; Carrell *et al.*, Angew. Chem. Int. Ed. Engl. 33:2059 [1994]; Carell *et al.*, Angew. Chem. Int. Ed. Engl. 33:2061 [1994]; and Gallop *et al.*, J. Med. Chem. 37:1233 [1994].

Libraries of compounds may be presented in solution (e.g., Houghten, Biotechniques 13:412-421 [1992]), or on beads (Lam, Nature 354:82-84 [1991]), chips (Fodor, Nature 364:555-556 [1993]), bacteria or spores (U.S. Pat. No. 5,223,409; herein incorporated by reference), plasmids (Cull *et al.*, Proc. Nad. Acad. Sci. USA 89:18651869 [1992]) or on phage (Scott and Smith, Science 249:386-390 [1990]; Devlin Science 249:404-406 [1990]; Cwirla *et al.*, Proc. Natl. Acad. Sci. 87:6378-6382 [1990]; Felici, J. Mol. Biol. 222:301 [1991]).

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

Example 1

A. Materials and Methods

Animals

- 5 Specific pathogen free (SPF) C57BL/6 mice were purchased from Jackson Laboratories. GF wild-type mice were bred and maintained at the Germ-free Animal Core Facility of the University of Michigan. GF mice were maintained in flexible film isolators and were checked weekly for germ-free status by aerobic and anaerobic culture. The absence of microbiota was verified by microscopic analysis of stained cecal contents to detect unculturable contamination.
- 10 All animal studies were performed according to approved protocols by the University of Michigan Committee on the Use and Care of Animals.

C. rodentium infection

- The kanamycin (Km)-resistant wild-type *Citrobacter rodentium* strain DBS120 (pCRP1::Tn5) was a gift of Dr. David Schauer, Massachusetts Institute of Technology. The isogenic *C. rodentium* Δ ler mutant strain has been described(4). For inoculations, bacteria were grown overnight in Luria-Bertani (LB) broth supplemented with Km (50 μ g/ml) with shaking at 37°C. Mice were infected by oral gavage with 0.2 ml of PBS containing approximately 1×10^9 CFU of *C. rodentium*. To determine bacterial numbers in the feces, fecal pellets were collected
- 20 from individual mice, homogenized in cold PBS and plated at serial dilutions onto MacConkey agar containing 50 μ g/ml Km, and the number of CFU was determined after overnight incubation at 37°C. Mice were sacrificed at various time points post-infection (p.i.), and colons were flushed with PBS and used for colonic cell isolation or fixed in 10% formalin and then processed for H&E staining.

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Flow cytometric analysis

- Cell surface fluorescence was assessed using a FACSCalibur analyzer and analyzed using FlowJo software (TreeStar). Dead cells were excluded with 7-AAD staining. Non-specific Ab binding was blocked with anti-CD16/32 Ab. FITC- PE- or APC-conjugated mAb against CD11b
- 30 (M1/70), Gr-1 mAb (RB6-8C5), F4/80 (BM8) and CD3 (145-2C11) were from eBioscience. Isotype-matched antibodies (eBioscience) were used for control staining.

Measurement of *ler* expression

A plasmid containing a *ler-lux* transcriptional fusion was constructed by cloning a fragment spanning the *C. rodentium* *ler* regulatory region from position -260 to +216 with respect to the *ler* transcriptional start site (14), in plasmid pCS26-Pac containing the promoterless *Photorhabdus luminescens luxCDABE* operon (15). The resulting plasmid *pler-lux* was transformed into wild type *C. rodentium* by electroporation. For determination of the levels of *ler* expression in feces, fecal pellets were suspended in PBS at 10 mg/ml, and luminescence emitted from *ler-lux*-expressing bacteria was measured using a LMax luminometer (Molecular Device). For *in vivo* bioluminescence imaging (BLI), the entire gastrointestinal tissues were immediately removed and placed into the light-tight chamber of the CCD camera system (IVIS200, Xenogen). Luminescence emitted from *lux*-expressing bacteria in the tissue was quantified using the software program living image (Xenogen). Quantitative real time RT-PCR (qPCR) for *ler* and *tir* was performed using a SYBR green PCR master mix and the StepOne Real-time PCR system (Applied Biosystems) and normalized to the expression of the kanamycin resistance gene (*Km*).

Relative expression of virulence factors was determined as fold expression when compared to that of bacteria cultured in LB culture. The following primer sets were used: *ler*; 5'-AATATACCTGATGGTGCTCTTG-3' and 5'-TTCTTCCATTCAATAATGCTTCTT-3'. *tir*; 5'-TACACATTCGGTTATTCAGCAG-3' and 5'-GACATCCAACCTTCAGCATA-3'. *rrsA* (16SRNA); 5'-AGGCCTTCGGGTTGTAAAGT-3' and 5'-ATTCCGATTAACGCTTGCAC-3'. *Km* (kanamycin resistant protein); 5'-CTGAATGAACTGCAGGACGA-3' and 5'-ATACTTTCTCGGCAGGAGCA-3'.

Immunostaining for Tir

Briefly, 5 µm paraffin sections were deparaffinized by heating at 55–65°C for 10 min, cleared with xylene, rehydrated through an ethanol gradient to water. Sections were blocked using the appropriate blocking buffer (either 2% Goat or Donkey Serum in PBS containing 1% bovine serum albumin (BSA), 0.1% Triton-X100 (Sigma), and 0.05% Tween 20, and 0.05% sodium azide. Rat anti-sera generated against *C. rodentium* specific Tir (1:5K; gift from W. Deng) was used as the primary antibody. Epifluorescent labeling for was carried out with the appropriate secondary antibody using AlexaFluor 568-conjugated goat anti-rat IgG. Tissues were mounted using ProLong Gold Antifade reagent (Molecular Probes/Invitrogen) that contains 4',6'-diamidino-2-phenylindole (DAPI) for DNA staining. Sections were viewed on a Zeiss AxioImager microscope. Images were obtained using a Zeiss AxioImager microscope equipped with an AxioCamHRm camera operating through AxioVision software (Version 4.4).

Fluorescence in-situ hybridization

Carnoy's-fixed paraffin-embedded sections were deparaffinized and rehydrated as described above. Sections were incubated overnight at 37°C in the dark with Texas red-conjugated EUB338 general bacterial probe (5'-GCT GCC TCC CGT AGG AGT-3') and an AlexaFluor 488 conjugated GAM42a probe (5'-GCC TTC CCA CAT CGT TT-3') that recognizes bacteria that belong to the γ -Proteobacter class diluted to a final concentration of 2.5 ng/ μ l each in hybridization solution (0.9 M NaCl, 0.1 M Tris pH 7.2, 30% Formamide, 0.1% SDS). Sections were then washed once in the dark with hybridization solution for 15 minutes with gentle shaking. This step was repeated once with wash buffer (0.9 M NaCl, 0.1 M TRIS pH 7.2), and sections were placed in dH₂O, and then mounted using ProLong Gold Antifadereagent with DAPI (Molecular Probes) and imaged as described above. For quantification studies, the methods were carried as previously described

Transmission Electron Microscopy

GF wild-type mice were orally infected with *C. rodentium*. At day 5 and 21 post infection, cecums from infected mice were collected and fixed with 2.5 % glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4). Fixed tissues were then 1 % osmium tetroxide in 0.1 M Sorensen's buffer, sequentially dehydrated through graded alcohols and propylene oxide, and then infiltrated in Spurr's or Epon. Ultrathin sections were cut with a diamond knife, stained, and examined with Philips CM-100 transmission electron microscope.

Carbohydrate growth array

Mouse-derived commensal bacteria used for competition experiments with *C. rodentium* were *Escherichia coli* (strain dn15.6244.1), *Bacteroides thetaiotaomicron* (strain dnklv9) and *B. vulgatus* (strain dnklv7) (22). For gnotobiotic mouse inoculations, *C. rodentium* and *E. coli* were grown aerobically overnight in LB broth with shaking. Both *Bacteroides* species were grown at 37°C overnight in pre-reduced chopped-meat broth (Beckton Dickinson) under an atmosphere of 5% H₂, 5% CO₂ and 90% N₂ in an anaerobic growth chamber (Coy Manufacturing, Grass Lake, MI). Gnotobiotic mice were fed either an autoclaved conventional PICO LAB rodent diet 5L0D (PMI Nutrition International, LLC), or an irradiated simple sugar diet, a customized version of Harlan TD.08810 in which starch and maltodextrin were replaced with sucrose (64% final w/w). For growth profiling in a custom carbohydrate growth array, each species was grown as indicated above and inoculated into 2x carbohydrate-free minimal medium as described previously (26), except that 0.5% chopped-meat extract was added to the medium for *B. vulgatus* and *E. coli*. To

quantify the amount of growth on each carbohydrate tested, the maximum absorbance (600nm) was measured for each culture after 4d of anaerobic growth, then both the starting absorbance on each substrate and the amount of growth, if any, in minimal medium lacking a carbon source were subtracted. Growth data was recorded at 10-25 min intervals for the entire 4d period and each positive growth curve was manually inspected to validate the presence of an exponential growth profile. Because each species grew to varying overall levels in the anaerobic media conditions, the growth ability on each substrate was normalized to the average growth ability of a given species on all substrates for which it demonstrated growth. By this normalization strategy, values >1 indicate above average growth; values <1 indicate below average growth.

Measurement of *C. rodentium*-reactive immunoglobulin

C. rodentium-specific Ig was quantified by coating 96-well plates with heat-killed *C. rodentium* as described (18) using alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG, IgM or IgA Abs (Southern Biotechnology Associates, Birmingham, AL). Plates were developed using p-nitrophenyl phosphate substrate (Southern Biotechnology Associates) and OD405 values determined.

Isolation and stimulation of colonic CD4⁺ T cells.

Colonic CD4⁺ T cells were isolated from total colonic cells as described (25), using mouse CD4⁺ cell MACS (MiltenyiBiotec). Purified T cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of GolgiStop (BD Pharmingen), and the cells producing IFN- γ and IL-17A were then stained with FITC-labeled anti-mouse IFN- γ mAb (eBioscience) and PE-labeled anti-mouse IL-17A mAb (eBiosciences), and analyzed by flow cytometry. For ELISA, total LP cells were stimulated plate-bound anti-CD3 Ab (10 μ g/ml) and soluble anti-CD28 Ab (2 μ g/ml) for 48 h. IFN- γ , IL-17A and IL-22 in the supernatants were measured.

Measurement of anti-microbial peptide expression

At day 0 and day12 after *C. rodentium* infection, colons were removed from SPF and GF mice. Colons were opened, washed with PBS and incubated in 10mM EDTA/PBS for 30 min at room temperature with gentle shaking. After EDTA treatment, colonic tissues were cut into small pieces and put into HBSS. Tissues were then shaken intensely for 10 sec, and epithelial cells were collected using a 70 μ m cell strainer. RNAs were isolated from colonic epithelial cells using Total RNA Kit I (Omega Bio-tek), and cDNA was synthesized using High Capacity RNA-to-

cDNA Kit (Applied Biosystems), according to the manufacturer's instructions. Quantitative real time RT-PCR (qPCR) was performed using a SYBR green PCR master mix and StepOne Real-time PCR system (Applied Biosystems). Relative mRNA expression was calculated by the ΔC_t method and normalized to the expression of β -actin. The following primer sets were used:

- 5 RegIII γ ; 5'-TCAGGTGCAAGGTGAAGTTG-3' and 5'-GGCCACTGTTACCACTGCTT-3'.
 β Def-1; 5'-AGGTGTTGGCATTCTCACAAG-3' and 5'-GCTTATCTGGTTTACAGGTTCCC-3'.
 β Def-2; 5'-TATGCTGCCTCCTTTTCTCA-3' and 5'-GACTTCCATGTGCTTCCTTC-3'. β Def-3; 5'-GTCTCCACCTGCAGCTTTTAG-3' and 5'-AGGAAAGGAACTCCACAAGTGC-3'.
 10 β Def-4; 5'-GCAGCCTTTACCCAAATTATC-3' and 5'-ACAATTGCCAATCTGTCTGAA-3'.
 β -actin; 5'-AAGTGTGACGTTGACATCCG-3' and 5'-GATCCACATCTGCTGGAAGG-3'.

Statistical analyses

- 15 Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software Inc.). Differences between two groups were evaluated using Student's *t* test (parametric) or Mann-Whitney *U* test (non-parametric). For the multiple comparisons, statistical analysis was performed using one-way ANOVA (parametric) or Kruskal-Wallis test (non-parametric), and then the Dunnett's or Bonferroni test for parametric samples, or Dunn's test for non-parametric samples as a post-hoc test. Survival between groups of mice was compared using Log-rank (Mantel-Cox) test. Differences at $P < 0.05$ were considered significant.

B. Results

- To assess the role of the microbiota in enteric infection, germ-free (GF) and specific pathogen-free (SPF) C57BL/6 mice reared in standard animal housing conditions were orally inoculated with *C. rodentium*. Consistent with previous results (5), the pathogen rapidly colonized the intestine of conventional SPF mice reaching maximal concentration in the feces on day 7-10 post infection (108cfu/gram), followed by a decline by day 12 and becoming undetectable by day 22 (**Fig. 1A**). In contrast, GF mice harbored 10-fold more *C. rodentium* on day 7-10 post infection, but unlike SPF mice, they were unable to clear *C. rodentium* even by day 42 when the experiments were terminated (**Fig. 1A**). Notably, all GF infected mice remained alive despite high and persistent pathogen burdens (**Fig. 1B**).

Infection of mice with *C. rodentium* triggers the recruitment of neutrophils and macrophages in the intestine (9-12). To determine whether the failure of GF mice to clear the

pathogen was associated with impaired inflammatory responses, the recruitment of acute inflammatory cells in the intestine of SPF and GF mice after infection by flow cytometry was monitored. In infected animals, there was an increase in the influx of neutrophils (CD11b+Gr1^{high}F4/80⁻) to the colon on day 12 when inflammation is known to peak, but neutrophil numbers were reduced by day 22; however, this was comparable in SPF and GF mice (Fig. 1C and 1D). Likewise, the recruitment of inflammatory macrophages (CD11b+Gr1^{high}F4/80⁺) and CD3⁺ T cells was similar in SPF and GF mice (Fig. 1C and 1D). Consistently, histological analysis revealed comparable pathology score on day 12 post-infection which declined on day 22 in GF and SPF mice (Fig. 1E and 1F). Because the microbiota is known to trigger the production of several anti-microbial peptides in the colon (13), their induction in the intestine after infection with *C. rodentium* in SPF and GF mice was assessed. Comparable mRNA expression of the anti-microbial peptides RegIII γ , β -defensin-1, β -defensin-3, and β -defensin-4 after oral infection with *C. rodentium* in the colon of SPF and GF mice was found (Fig. 5). These results indicate that the presence of microbiota is required for the elimination of *C. rodentium*, and that persistence of the pathogen in the intestine of GF mice is not associated with an impaired inflammatory or anti-microbial response to the bacterium.

The expression of most LEE genes in *C. rodentium* and other A/E pathogens is controlled by Ler (LEE encoded regulator), a 15 kDa protein belonging to the H-NS family of nucleoid-associated proteins (3, 4, 14). Because GF mice remained healthy despite robust pathogen colonization, *ler* expression in SPF and GF mice was compared by real-time quantitative (qPCR) during the early (day 7) and late phase (day 12) of infection to determine whether colonizing bacteria express this critical regulatory gene. It was found that *ler* was expressed in the feces on day 7 post-infection in both GF and SPF mice (Fig. 2A), which correlated with robust growth of *C. rodentium* in the intestine (Fig. 2D). Expression of *ler* and *tir*, a Ler-regulated LEE gene and critical virulence factor that is also required for pathogen colonization, were both down-regulated on day 12 after infection in both GF and SPF mice and were not expressed at day 42 in GF mice despite the continued high level of pathogen colonization (Fig. 2A, D and fig. 6). To directly monitor the expression of *ler* in the mouse intestine, a bioluminescent reporter *C. rodentium* strain in which the *ler* promoter is fused to the *luxCDABE* operon of *Photobacterium luminescens* was utilized (Fig. 7) (15). Consistent with the qPCR results, *ler-lux* expression was detected in the feces on day 5, but down-regulated by day 7-12 post-infection in both SPF and GF mice (Fig. 2B). *ler-lux* expression was visualized on day 5 post-infection in the ileum, cecum and distal colon/rectum, but was down-regulated on day 14 in both SPF and GF mice (Fig. 2C), even though the bacterial load had not declined at day 14 (Fig. 1A). The *ler* luminescent signal was

~10-fold higher in GF mice than in SPF mice which correlated with a comparably higher pathogen load in GF mice (**Fig. 1A**). If the *C. rodentium* was harvested from infected GF mice at day 21 and reinoculated into conventional SPF mice, the pathogen robustly grew in the intestines of the newly infected mice, infecting the epithelium and triggering colonic inflammation (**Fig. 8**), indicating that down-regulation of *Ler* was not due to selection of avirulent mutant bacteria in the intestine. These results indicate that *Ler*-dependent expression of LEE virulence genes occurs early during infection in both GF and conventional SPF mice, but is down-regulated prior to the decline of pathogen load in the intestine of conventional mice.

Since expression of virulence LEE genes is essential for pathogen colonization in the intestine of conventional SPF mice (4), it was next investigated whether LEE virulence was required for colonization in GF mice. To assess this, GF and conventional SPF mice were orally infected with wild-type *C. rodentium* and an isogenic strain deficient in *Ler* (Δler), the master regulator of LEE genes (4). Wild-type, but not the Δler mutant strain, colonized SPF mice (**Fig. 2D**). The Δler mutant grew robustly in GF mice, reaching numbers similar to the wild-type pathogen (**Fig. 2D**). Both SPF and GF mice remained viable after infection with wild-type and Δler *C. rodentium* (**Fig. 2E**). These results demonstrate that in the absence of microbiota, *Ler*-dependent LEE virulence is not required for *C. rodentium* colonization and growth in the intestine. To determine whether *Ler* regulates the ability of the microbiota to out-compete the pathogen, GF mice were orally infected with wild-type and Δler mutant *C. rodentium* and the infected GF mice were naturally colonized with commensal microbes by cohousing them at day 3 or day 21 post-infection with conventional SPF mice. The burden of wild-type *C. rodentium* in GF mice harboring the pathogen for 21 days declined significantly by day 3 and was further reduced by 5-6-logs by day 7 after cohousing, similarly to the decline observed in mice infected with the Δler mutant for 3 and 21 days (**Fig. 2F and Figure 9**). In contrast, the microbiota could not out-compete the wild-type bacterium on day 3 post-infection upon co-housing with SPF mice (**Fig. 2F**), consistent with the observation that *ler* expression is active in wild-type *C. rodentium* at day 3 post-infection (Figure 10). The results indicate that *Ler*-regulated virulence is important to determine the the intestinal localization of *C. rodentium* and the ability of the pathogen to out-compete the microbiota.

C. rodentium induces marked inflammation in the distal colon which requires *Ler*-mediated expression of virulence factors including the T3SS (4). If the T3SS promotes outgrowth of *C. rodentium* by enabling the pathogen to colonize a niche at or near the epithelium, it would be expected that impaired colonization of the $\Delta escN$ mutant could not be rescued when inflammation is provided by other means. To test this, colitis was induced in mice by oral

administration of 2.5 % dextran sulfate sodium (DSS), a chemical that directly damages the colonic epithelium (17) for 5 days and then infected the mice with wild-type or $\Delta escN$ mutant *C. rodentium*. Administration of DSS enhanced the colonization of the wild-type bacterium by 3 logs but not that of the $\Delta escN$ mutant (**Fig. 11**). In addition, co-infection of wild-type *C.*

rodentium that induces inflammation with the $\Delta escN$ mutant strain did not rescue colonization by the T3SS mutant strain (**Fig. 11**). These results indicate that inflammation promotes colonization of wild-type *C. rodentium*, but not in the absence of a functional T3SS.

Recent studies have indicated that the adaptive immune system and specifically IgG production and Th17 responses against the pathogen are important for the intestinal eradication of *C. rodentium* (16-19). The observation that the adaptive immune system is critical for pathogen eradication prompted an examination of the role of the microbiota in the induction of specific T-cell and B-cell responses against *C. rodentium*. To directly test this, SPF and GF mice were orally infected with *C. rodentium* and the development of immunoglobulin and T-cell immune responses against the pathogen were assessed. The production of IgG, IgM, and IgA against *C. rodentium* in the serum was unimpaired in GF and SPF mice (**Fig. 12**). In line with these results, the generation of colonic IL-17 and interferon- γ -producing T cells in response to *C. rodentium* was comparable in SPF and GF mice (**Fig. 13**). Thus, unimpaired induction of adaptive immunity does not explain the failure of GF mice to clear the pathogen.

During the early phase of infection, *C. rodentium* is known to colonize and infect the apical surface of the intestinal epithelium, a site largely devoid of commensals (20). To determine whether Ler expression controls the intestinal localization of the bacterium, GF mice were orally infected with wild-type or Δler *C. rodentium* and cecum and rectum were harvested on day 5 when pathogen growth and *ler* expression is highest and on day 21 when *ler* expression is down-regulated. At day 5 post-infection, Tir, a Lerdependent factor, and a marker of *C. rodentium* attachment to the epithelium was seen on the intestinal surface in the cecum of GF mice infected with the wild-type *C. rodentium*, but not with the Δler strain (**Fig. 3A**). In contrast, Tir labeling was not detected on day 21 post-infection on the intestinal surface of mice infected with either wild-type or mutant *C. rodentium* (**Fig. 3A**). Consistently, the wild-type pathogen was largely associated with the epithelium whereas the Δler *C. rodentium* localized to the intestinal lumen on day 5 post-infection as determined by dual fluorescence in-situ hybridization (**Fig. 3B**). On day 21 post-infection, however, neither wild-type or Δler *C. rodentium* detected on the epithelium, but instead they localized to the intestinal lumen (**Fig. 3B**). The differential localization of the pathogen was independently verified by transmission electron microscopy that revealed *C. rodentium* at or near the intestinal epithelium on day 5, but not on day 21 post-

infection (**Fig. 3C**). *Δler C. rodentium* did not localize at or near the epithelium on day 5 post-infection (**Fig. 3C**). Consistent with these results, Ler-expressing *C. rodentium* tightly associated to the cecum and colon was abundant in the early phase of infection in GF mice, but dramatically decreased in the later phase of the infection, despite that pathogen burdens in feces were comparable (**Fig. 3D and 3E**). The results indicate that the localization of the pathogen differs in the early and late phase of infection and this is controlled by LEE virulence expression.

The intestine harbors a large number of bacterial species (21). Upon infection with *C. rodentium* there is a significant decrease in the total number of anaerobic commensal bacteria, but an increase of aerobic γ -Proteobacteria such as *E. coli* (7, 8). To determine whether commensal bacteria exhibit different abilities to out-compete *C. rodentium*, GF mice were orally infected with *C. rodentium* and the infected GF mice were naturally colonized on day 21 post-infection with either *E. coli* or one of two different anaerobic *Bacteroides* species, all isolated from the intestine of SPF mice (22). The burden of *C. rodentium* in GF mice declined about 200-fold by day 3 and more than 500-fold by day 14 upon colonization with *E. coli*, but not at all, with *B. thetaiotaomicron* or *B. vulgatus* (**Fig. 4A**). Secondary administration of *E. coli* to GF mice colonized with *B. thetaiotaomicron* or *B. vulgatus* also reduced the number of *C. rodentium* in the feces about 500-fold (**Fig. 4A**). In contrast, colonization of mice harboring *E. coli* with *B. thetaiotaomicron* or *B. vulgatus* was not effective in further reducing the burden of *C. rodentium* in the feces (**Fig. 4A**). Assessment of total *Enterobacteria* (*E. coli* and *C. rodentium*) and *Bacteroides* on day 14 post colonization with individual commensal bacteria showed comparable number of bacteria $\sim 10^{10}$ cfu/gram in the feces of GF mice (**Fig. 4B**). Before inoculation of commensal bacteria (d0), all *Enterobacteria* detected in the feces were *C. rodentium* (**Fig. 4C**). In contrast, > 99% of the total *Enterobacteria* in the *E. coli* inoculated group were *E. coli* after 14 days of colonization (**Fig. 4C**). 100% of total *Enterobacteria* were *C. rodentium* in *B. thetaiotaomicron* or *B. vulgatus* inoculated groups, since *Bacteroides* strains were unable to grow under this condition (**Fig. 4C**). These results indicate that the differing abilities of *E. coli* vs *B. thetaiotaomicron* or *B. vulgatus* to out-compete *C. rodentium* cannot be explained simply by differences in bacterial colonization and suggest that *E. coli* and *C. rodentium* compete for similar niches in the mouse gut. To explore this concept further, the growth abilities of each of these species was analyzed on a custom carbohydrate growth array that contains most of the common mono- and polysaccharides present in plant and animal tissue (**Fig. 4D and table 1**). Both *C. rodentium* and *E. coli* exhibited optimal growth on monosaccharides (**Fig. 4D**). They were both generally deficient in the ability to grow on more complex polysaccharides; although, *C. rodentium* exhibited a small amount of growth on several different polysaccharides (**Fig. 4D**),

indicating that it possesses some capacity to target more complex carbohydrate structures present in the gut. In contrast, both *B. thetaiotaomicron* and *B. vulgatus* exhibited broad abilities to catabolize both mono- and polysaccharides (**Fig. 4D**). These results indicate that commensal populations differ in their ability to out-compete *C. rodentium* in the mouse intestine and this correlates with their ability to grow on structurally distinct carbohydrates. To further assess if competition for glycans between commensals and *C. rodentium* is important for pathogen eradication, GF mice on a conventional maintenance diet containing both mono- and polysaccharides were orally infected with *C. rodentium* and on day 21 post-infection the infected GF mice were colonized with *B. thetaiotaomicron* for 7 days. On day 7 post-colonization with *B. thetaiotaomicron*, the mice were divided into two groups that were fed the conventional diet or a simple sugar diet containing monosaccharides but not polysaccharides. The burden of *C. rodentium* in GF mice on the simple sugar diet declined ~ 200-fold by day 3, whereas no decline was observed when the mice were fed the conventional diet (**Fig. 4E**). The results were not explained by reduced *C. rodentium* burden in GF mice on the simple sugar diet because in the absence of colonization with *B. thetaiotaomicron*, the number of *C. rodentium* in feces was comparable between animals fed with the conventional and simple sugar diets (**Fig. 4F**).

The results indicate that during the early phase of infection, *C. rodentium* expresses Ler-dependent LEE genes that mediate their ability to infect the intestinal epithelium and trigger inflammatory responses (4), which in turn reduce the total numbers of colonic commensal bacteria, but increase the density of γ -Proteobacteria (7, 8). These events allow the pathogen to compete with the commensal microbiota by promoting colonization of unique intestinal niches that support *C. rodentium* growth. This model is supported by the finding that avirulent Δler *C. rodentium* cannot colonize the intestines of conventional SPF mice, but grows robustly in GF mice. Thus, Ler-mediated virulence is not required for colonization and growth of the pathogen in the intestine per se, but rather is critical in colonizing the epithelial surface, allowing *C. rodentium* to compete among the indigenous microbiota. Furthermore, it was shown that Ler controls the localization of the pathogen to epithelial niches that are largely devoid of commensals and may provide nutritional sources allowing the pathogen to out-compete the commensal microbiota. LEE virulence is down-regulated by day 12 of infection in the intestines of infected mice and that reduced expression correlates with *C. rodentium* clearance. The concomitant reduction of LEE virulence in GF mice upon *ler* inactivation explains the finding that microbiota-free mice can sustain robust and prolonged colonization of the pathogen without obvious adverse effects. Upon *ler* down-regulation, *C. rodentium*-mediated intestinal inflammation recedes, allowing the recovery of the intestinal microbiota, which then out-

competes and eradicates the pathogen not expressing LEE genes. It was found that *E. coli*, but not *B. thetaiotaomicron* and *B. vulgatus* exhibited broad abilities to catabolize both mono- and polysaccharides, consistent with previous reports of the broad glycan-degrading potential of various *Bacteroidetes* species (23, 24). The list of tested growth substrates only includes carbohydrates, and not other potential nutrients (peptides, amino acids, and lipids) that could be targeted by these species. Nevertheless, the similar catabolic preferences for monosaccharides by *C. rodentium* and *E. coli*, combined with their close phylogenetic relationship, support the idea that they would compete most directly for similar nutrient pools *in vivo*. In contrast, the *Bacteroides* species tested have ample metabolic alternatives (*i.e.*, plant or animal polysaccharides) they can utilize that may limit their direct competition with *C. rodentium* *in vivo*. Because γ -Proteobacteria such as *E. coli* specifically accumulates after *C. rodentium* infection, these results indicate that this change in commensal populations benefit the host by increasing the number of commensals that can out-compete the pathogen. An important role for glycans in determining the ability of commensals to out-compete *C. rodentium* is supported by the finding that *B. thetaiotaomicron* can reduce the intestinal burden of the pathogen only when the mice are fed a simple sugar diet. Under these conditions, *B. thetaiotaomicron* cannot use polysaccharides and is forced to compete with *C. rodentium* for available monosaccharides or another common nutrient that both *C. rodentium* and *B. thetaiotaomicron* rely on in the absence of dietary polysaccharides. These findings clarify the tenuous nature of A/E pathogens, in terms of their survival/colonization within hosts, and therefore nutrient or probiotic shifting of the microbiota to promote commensals that directly compete with pathogens for food sources may be a useful therapeutic approach.

Substrate	<i>C. rodentium</i>	<i>E. coli</i>	<i>B. thetaiotaomicron</i>	<i>B. vulgatus</i>
Monosaccharides:				
arabinose	1.84 $\pm$ ^{0.01}	0.46 $\pm$ ^{0.04}	1.27 $\pm$ ^{0.005}	0.37 $\pm$ ^{0.03}
cellobiose	1.26 $\pm$ ^{0.34}	0.24 $\pm$ ^{0.22}	0.05 $\pm$ ^{0.03}	No growth
fructose	1.36 $\pm$ ^{0.12}	1.36 $\pm$ ^{0.08}	1.24 $\pm$ ^{0.03}	1.30 $\pm$ ^{0.06}
fucose	no growth	0.89 $\pm$ ^{0.11}	0.68 $\pm$ ^{0.04}	1.81 $\pm$ ^{0.11}
galactose	1.69 $\pm$ ^{0.004}	1.30 $\pm$ ^{0.06}	1.51 $\pm$ ^{0.05}	1.01 $\pm$ ^{0.19}
galacturonic acid	1.36 $\pm$ ^{0.07}	1.19 $\pm$ ^{0.04}	0.93 $\pm$ ^{0.02}	No growth
glucosamine	1.43 $\pm$ ^{0.08}	1.19 $\pm$ ^{0.07}	0.73 $\pm$ ^{0.01}	1.01 $\pm$ ^{0.33}
glucose	1.50 $\pm$ ^{0.07}	1.60 $\pm$ ^{0.08}	1.45 $\pm$ ^{0.05}	1.11 $\pm$ ^{0.28}
glucuronic acid	0.75 $\pm$ ^{0.13}	1.06 $\pm$ ^{0.13}	1.04 $\pm$ ^{0.02}	No growth
mannose	1.43 $\pm$ ^{0.07}	1.34 $\pm$ ^{0.06}	1.38 $\pm$ ^{0.07}	1.13 $\pm$ ^{0.01}
N-acetylgalactosamine	no growth	0.85 $\pm$ ^{0.02}	0.91 $\pm$ ^{0.02}	1.33 $\pm$ ^{0.05}
N-acetylglucosamine	2.07 $\pm$ ^{0.74}	1.31 $\pm$ ^{0.01}	1.19 $\pm$ ^{0.002}	0.95 $\pm$ ^{0.05}
N-acetylneuraminic acid	0.96 $\pm$ ^{0.03}	0.91 $\pm$ ^{0.01}	no growth	1.03 $\pm$ ^{0.08}
rhamnose	1.53 $\pm$ ^{0.04}	0.99 $\pm$ ^{0.11}	0.79 $\pm$ ^{0.01}	1.51 $\pm$ ^{0.09}
ribose	1.21 $\pm$ ^{0.13}	1.13 $\pm$ ^{0.04}	1.36 $\pm$ ^{0.02}	0.42 $\pm$ ^{0.01}
xylose	1.43 $\pm$ ^{0.05}	1.26 $\pm$ ^{0.05}	1.57 $\pm$ ^{0.10}	1.09 $\pm$ ^{0.09}
Plant polysaccharides:				
arabinogalactan	no growth	no growth	0.96 $\pm$ ^{0.03}	0.41 $\pm$ ^{0.01}
amylopectin (maize)	no growth	no growth	1.12 $\pm$ ^{0.02}	1.83 $\pm$ ^{0.004}
amylopectin (potato)	no growth	no growth	1.25 $\pm$ ^{0.0004}	1.87 $\pm$ ^{0.007}
arabinan	no growth	no growth	1.14 $\pm$ ^{0.02}	0.52 $\pm$ ^{0.49}
barley β -glucan	no growth	no growth	no growth	no growth
fructooligosaccharides	0.60 $\pm$ ^{0.02}	0.51 $\pm$ ^{0.02}	1.30 $\pm$ ^{0.04}	1.47 $\pm$ ^{0.08}
galactomannan	no growth	no growth	no growth	no growth
glucomannan	no growth	no growth	0.41 $\pm$ ^{0.005}	0.22 $\pm$ ^{0.01}
inulin	no growth	no growth	0.65 $\pm$ ^{0.04}	1.27 $\pm$ ^{0.04}
oat spelt xylan	no growth	no growth	0.19 $\pm$ ^{0.01}	0.32 $\pm$ ^{0.02}
polygalacturonic acid	no growth	no growth	0.75 $\pm$ ^{0.05}	0.51 $\pm$ ^{0.03}
pectic galactan (lupin)	0.65 $\pm$ ^{0.19}	no growth	1.15 $\pm$ ^{0.09}	0.44 $\pm$ ^{0.02}
pectic galactan (potato)	0.59 $\pm$ ^{0.06}	no growth	1.07 $\pm$ ^{0.006}	0.58 $\pm$ ^{0.03}
rhamnogalacturonan I	0.36 $\pm$ ^{0.01}	no growth	0.78 $\pm$ ^{0.003}	no growth
wheat arabinoxylan	0.33 $\pm$ ^{0.02}	no growth	no growth	no growth
xyloglucan	no growth	no growth	no growth	no growth
Animal polysaccharides:				
chondroitin sulfate	0.25 $\pm$ ^{0.01}	no growth	0.87 $\pm$ ^{0.03}	0.20 $\pm$ ^{0.02}
glycogen	0.36 $\pm$ ^{0.05}	no growth	1.26 $\pm$ ^{0.01}	1.81 $\pm$ ^{0.003}
heparin	no growth	no growth	0.20 $\pm$ ^{0.002}	no growth
hyaluronan	no growth	no growth	1.15 $\pm$ ^{0.06}	no growth
mucin O-glycans	no growth	no growth	1.21 $\pm$ ^{0.02}	0.98 $\pm$ ^{0.002}
Other polysaccharides:				
α -mannan	0.42 $\pm$ ^{0.05}	no growth	0.85 $\pm$ ^{0.03}	no growth
alginate	no growth	no growth	no growth	no growth
carrageenan	no growth	no growth	no growth	no growth
dextran	no growth	0.68 $\pm$ ^{0.25}	1.31 $\pm$ ^{0.01}	no growth
laminarin	0.29 $\pm$ ^{0.03}	no growth	no growth	no growth
levan	no growth	no growth	1.11 $\pm$ ^{0.08}	no growth
lichenin	no growth	no growth	0.90 $\pm$ ^{0.06}	no growth
porphyran	no growth	no growth	no growth	no growth
pullulan	no growth	no growth	1.26 $\pm$ ^{0.01}	1.49 $\pm$ ^{0.03}

Example 2

This example describes methods of screening inhibitors of *E. coli* virulence genes.

Use of small molecule inhibitors of LEE virulence to enhance *C. rodentium*

5 eradication by the microbiota

Two classes of small molecules that have been shown to inhibit LEE virulence factors in A/E enteric pathogens are used. The first class are salicylidene acyl hydrazide compounds that have been shown to reduce the expression of T3SS effectors including those from A/E pathogens at ~1-20 μ M range. Five compounds of this class (ME0054, ME055, ME097, MEO114, and
10 ME0214) are obtained from Dr. Mikael Elofsson of Umea University in Sweden. These

compounds do not inhibit bacteria growth, but can inhibit expression of several LEE virulence genes including Ler and formation of A/E lesions on bovine epithelial cells.

The second class of small-molecules is represented by aurodox, a linear polyketide compound that is a specific T3SS inhibitor of EPEC and *C. rodentium* and therefore targets multiple LEE virulence factors. Aurodox is commercially available and has been shown to protect mice from *C. rodentium* infection in vivo, although its effect on bacterial loads and mechanism by which improves survival were not investigated.

First, each compound is tested at 10, 30 and 60 mg/Kg-1 or saline (mock treatment) in groups of mice (n=6). In the case of aurodox, 25 mg/Kg-1 given orally for 4 consecutive days was found to be effective in inhibiting infection-associated lethality. Thus, SPF B6 mice are given orally each compound in 5% DMSO or saline in 5% DMSO at day 0 with a gavage needle and infected 2 hrs later orally with 10⁹ cfu of *C. rodentium*. Administration of the compound via the same route is given daily for 3 more days. Pathogen loads are monitored in feces at day 3, 5, 7, 12 and 14 after infection. LEE virulence gene expression (ler and two T3SS effectors tir and intimin) is quantitated on day 1, 3, 5, and 7 post-infection in fecal pellets when LEE virulence genes are induced by real-time qPCR.

Use of antibodies against LEE virulence factors to enhance *C. rodentium* eradication by the microbiota

The advantage of small-molecules to inhibit LEE virulence is that they are relatively inexpensive which is useful important for treatment of A/E pathogen-associated colitis in underdeveloped countries. However, small-molecules may target additional pathogen or host signaling pathways. Therefore, antibodies with defined specificity against LEE factors are used to inhibit Ler and pathogen colonization. Two types of approaches and antibodies are tested. Rabbits were immunized with the purified C-terminal 382 amino acid extracellular domain of intimin from *C. rodentium* in CFA and after boosting, high titer antibodies against the protein were obtained. Intimin mediates the intimate interaction of the pathogen with the host epithelium and *C. rodentium* mutant lacking intimin cannot colonize SPF mice. Furthermore, *C. rodentium*-infected mice develop anti-intimin antibodies in the intestinal content and serum (Figure 15). However, mice and humans infected with enteric A/E pathogens develop antibodies against several LEE factors in addition to intimin (Figure 14). Therefore, it is possible that single or combination of antibodies against LEE factors other than intimin might be involved in the regulation of Ler expression or be more effective in reducing Ler-mediated virulence in vivo.

Llamas were immunized with purified intimin or membrane extracts of *C. rodentium* expressing LEE virulence factors and total RNA purified from the blood was used to generate a single domain heavy-chain antibody (nanobody) phage display library. The approach to generate nanobodies using phage display library is now well established and has been successful against a wide variety of antigens including after immunization with extracts from whole bacteria. Unlike most mammals, Camelidae including llamas develop heavy-chain antibodies devoid of light chain which can be manipulated genetically and exhibit high affinity for antigen, tissue penetration and stability. Because the antigen-binding site is reduced to a single variable domain, PCR amplification of the these fragments containing antigenic diversity can be cloned into a phage display vector and electroporated into *E. coli* to obtain a library of 10^6 - 10^7 individual colonies. *E. coli* are infected with helper phage to generate phage particles expressing individual single variable domain antibodies. Nanobodies targeting intimin will be selected by panning the phage libraries against immobilized intimin, and by screening individual clones for intimin binding using ELISA. Intimin-binding nanobodies are screened for an inhibitory effect on TIR – intimin binding and antagonistic nanobodies are purified to milligram scale for the use in *C. rodentium* infection studies in mice. To enrich for antibodies specific for LEE virulence factors, $\sim 5 \times 10^8$ viral particles are incubated with immunotubes pre-coated with extracts from Ler-deficient *C. rodentium* (negative selection) and non-binding clones are panned against extracts from Ler-expressing bacteria (positive selection) to generate a library enriched for antibody clones reacting with LEE virulence factors. The enriched library for LEE virulence factors is then split into individual clones and clones reacting with extracts from wild-type *C. rodentium* are further screened with extracts from Ler-deficient bacteria to select antibody clones that recognize Ler-dependent factors. Nanobodies binding LEE virulence factors are purified and screened for an antagonistic effect on *C. rodentium* virulence.

In order to determine which *C. rodentium* surface factors regulate LEE virulence, the nanobody library is screened for individual *C. rodentium* binding clones that display a similar regulatory function observed from the mouse polyclonal antibodies. For this, the *ler-lux C. rodentium* reporter strain is incubated with nanobody extracts from positive *C. rodentium* binding clones (initially pooled per 10-20) and screen for clones that influence Ler expression as read out by luminescence assay. Positive pools are split into individual clones to identify the unique clones that demonstrate Ler regulation. Upon identification of Ler-active nanobodies, their molecular target on the *C. rodentium* surface is pulled down by co-immunoprecipitation and/or tandem affinity purification, and identified using mass spectrometry.

The same strategy is used to test the ability of polyclonal rabbit and llama monoclonal antibody clones to inhibit LER expression and to promote the eradication of *C. rodentium* in vivo. It is first tested whether administration of rabbit anti-intimin antibodies inhibit Ler expression and pathogen colonization in the intestine of pathogen-infected mice. The rabbit sera with high titer anti-intimin antibodies is used to purify specific IgG using an intimin-affinity column. Because oral administration of antibodies is known to be effective in inducing regulatory activity in the gut, it is tested whether oral administration of purified rabbit IgG against intimin or control IgG can affect the virulence and colonization of *C. rodentium* in vivo. In these experiments, conventional SPF mice (n=6) are infected orally with the *ler-lux C. rodentium* reporter strain and the mice are given orally anti-intimin IgG (40 mg/kg) or control IgG on the day 0, 1 and 2 post infection. Pathogen burden in feces and Ler expression in the feces and the intestine of individual mice is measured by bioluminescence on day 1, 3, 7, 12, 15 post-infection and compared with that of mock-treated mice. It is contemplated that inhibition of LEE virulence by specific antibodies will promote pathogen eradication in a microbiota-dependent manner given that the loads of Ler-deficient *C. rodentium* are not reduced at all in GF mice. To test this, anti-intimin antibody or control IgG is given orally to groups of SPF and GF mice infected with the *ler-lux C. rodentium* reporter strain and pathogen load and Ler expression is measured in the feces as outlined above.

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All publications, patents, patent applications and accession numbers mentioned in the above specification are herein incorporated by reference in their entirety. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications and variations of the described compositions and methods of the invention will be apparent to those of ordinary skill in the art and are intended to be within the scope of the following claims.

20

CLAIMS

We claim:

1. A method of inhibiting a biological activity of a virulence factor of a pathogenic
5 *E. coli*, comprising: contacting said virulence factor or a cell expressing said virulence factor
with a composition that inhibits a biological activity of the virulence factor.

2. The method of claim 1, wherein said pathogenic *E. coli* is selected from the group
consisting of enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC).

10 3. The method of claim 1, wherein said composition is selected from the group
consisting of a small molecule and an antibody that specifically binds to said virulence factor.

4. The method of claim 3, wherein said small molecule is a salicylidence acyle
15 hydrazine compound.

5. The method of claim 4, wherein said salicylidence acyle hydrazine compound is
selected from the group consisting of ME0054, ME055, ME097, ME0114, and ME0214.

20 6. The method of claim 3, wherein said small molecule is aurodox.

7. The method of claim 3, wherein said antibody binds specifically to a LEE factor.

8. The method of claim 7, wherein said LEE factor is intimin.

25 9. The method of claim 1, wherein said inhibiting treats infection by said pathogenic
E. coli.

10. The method of claim 9, wherein said inhibiting results in commensal *E. coli* out
30 competing said pathogenic *E. coli*.

Figure 1

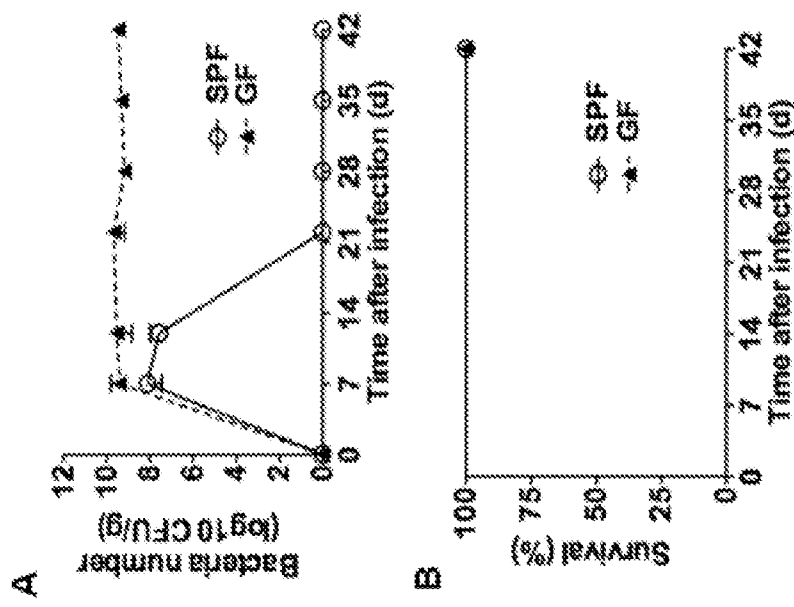
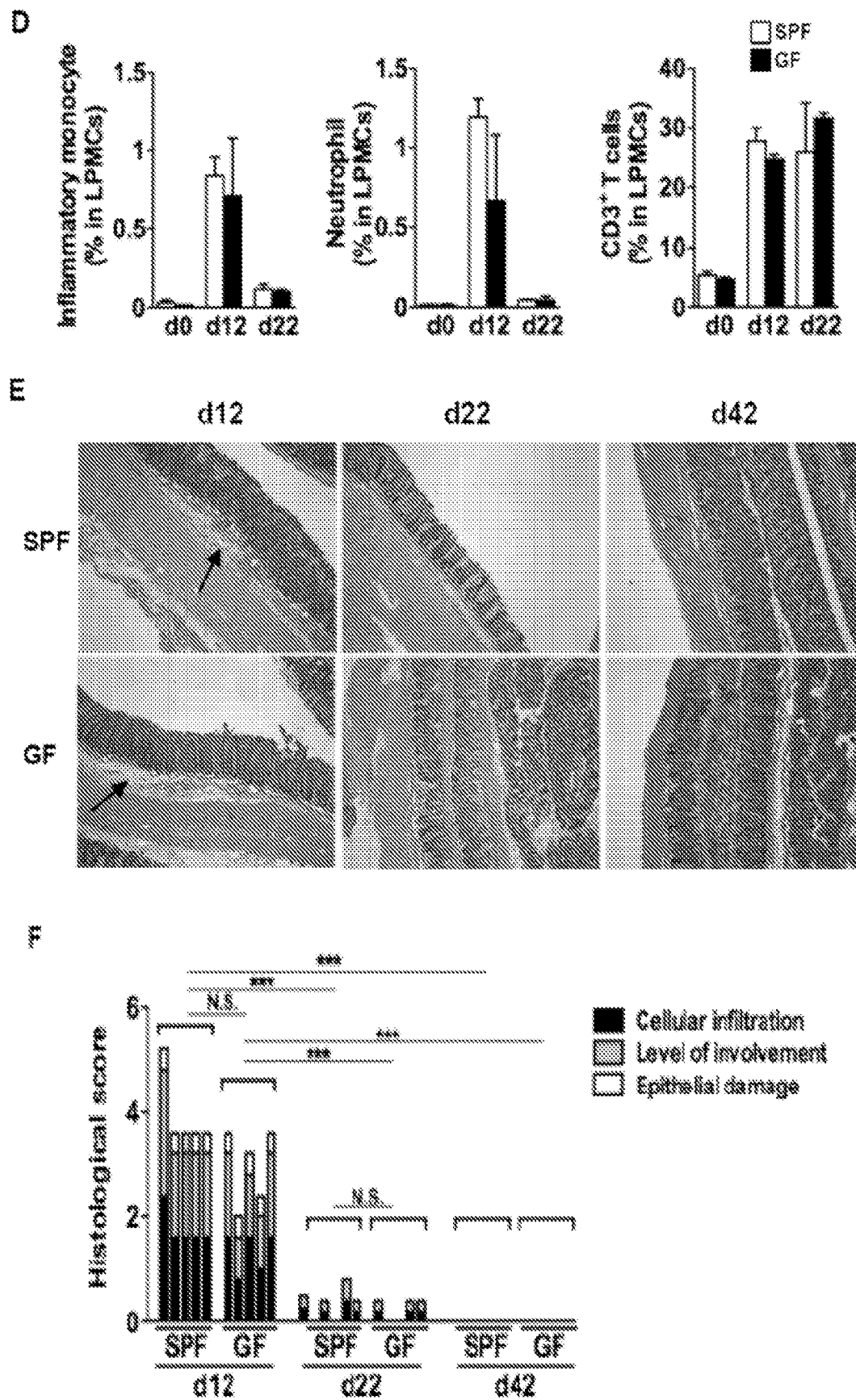


Figure 1 (cont.)



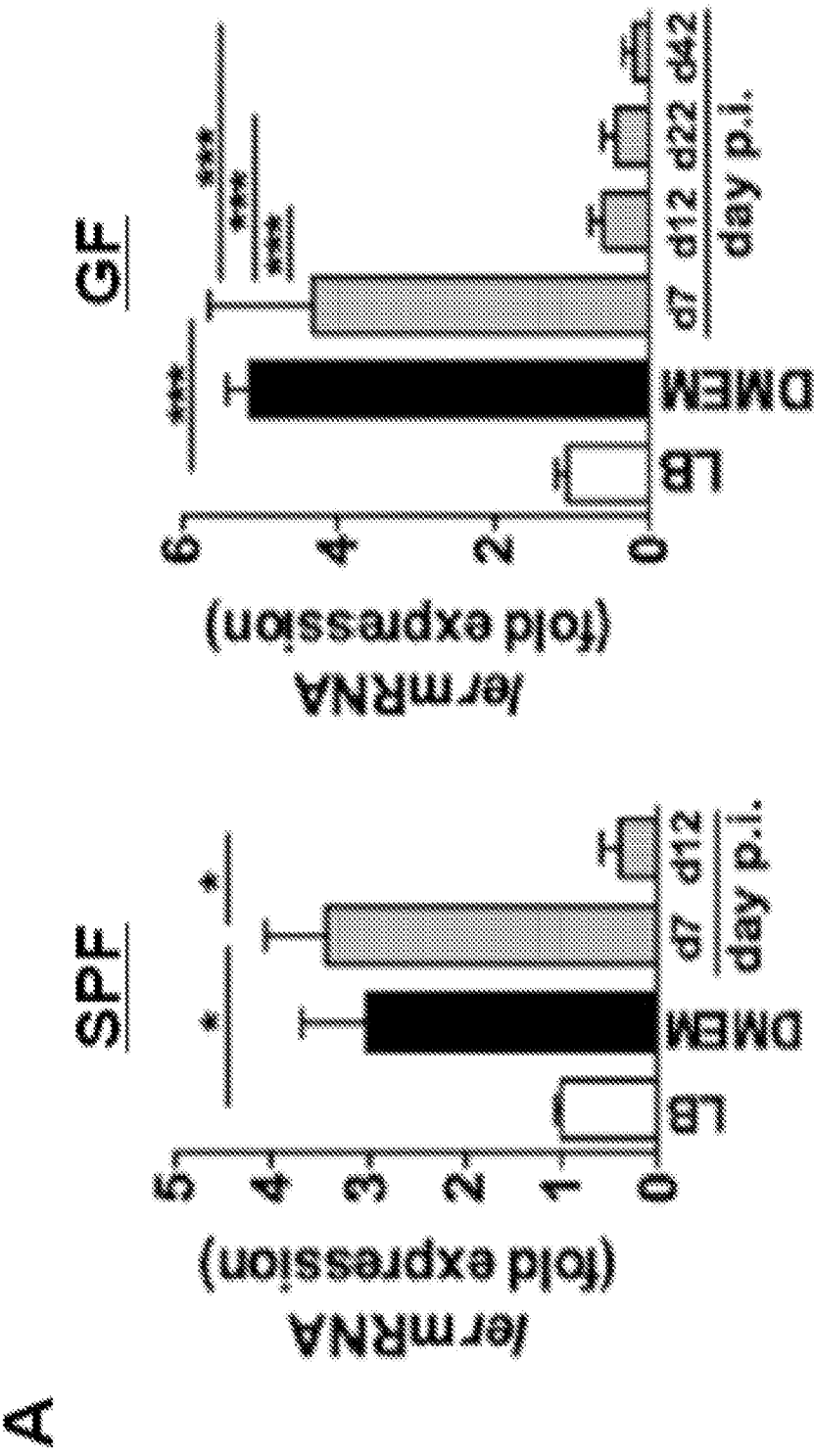
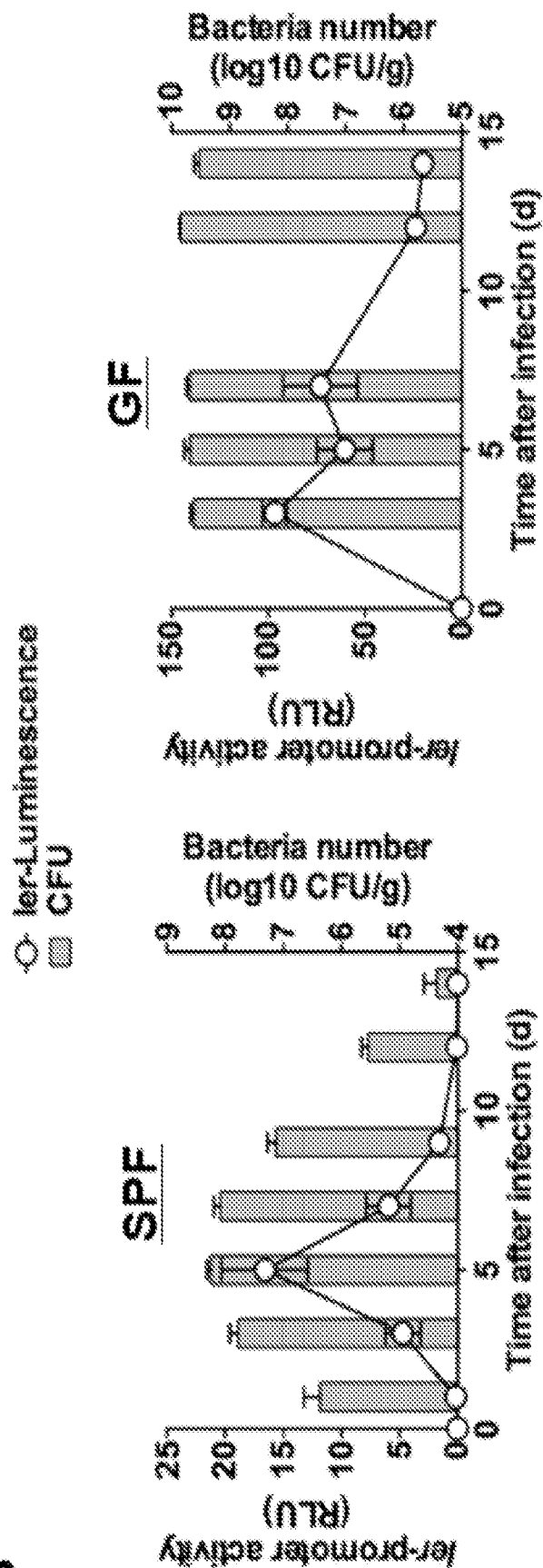


Figure 2

Figure 2 (cont.)

B



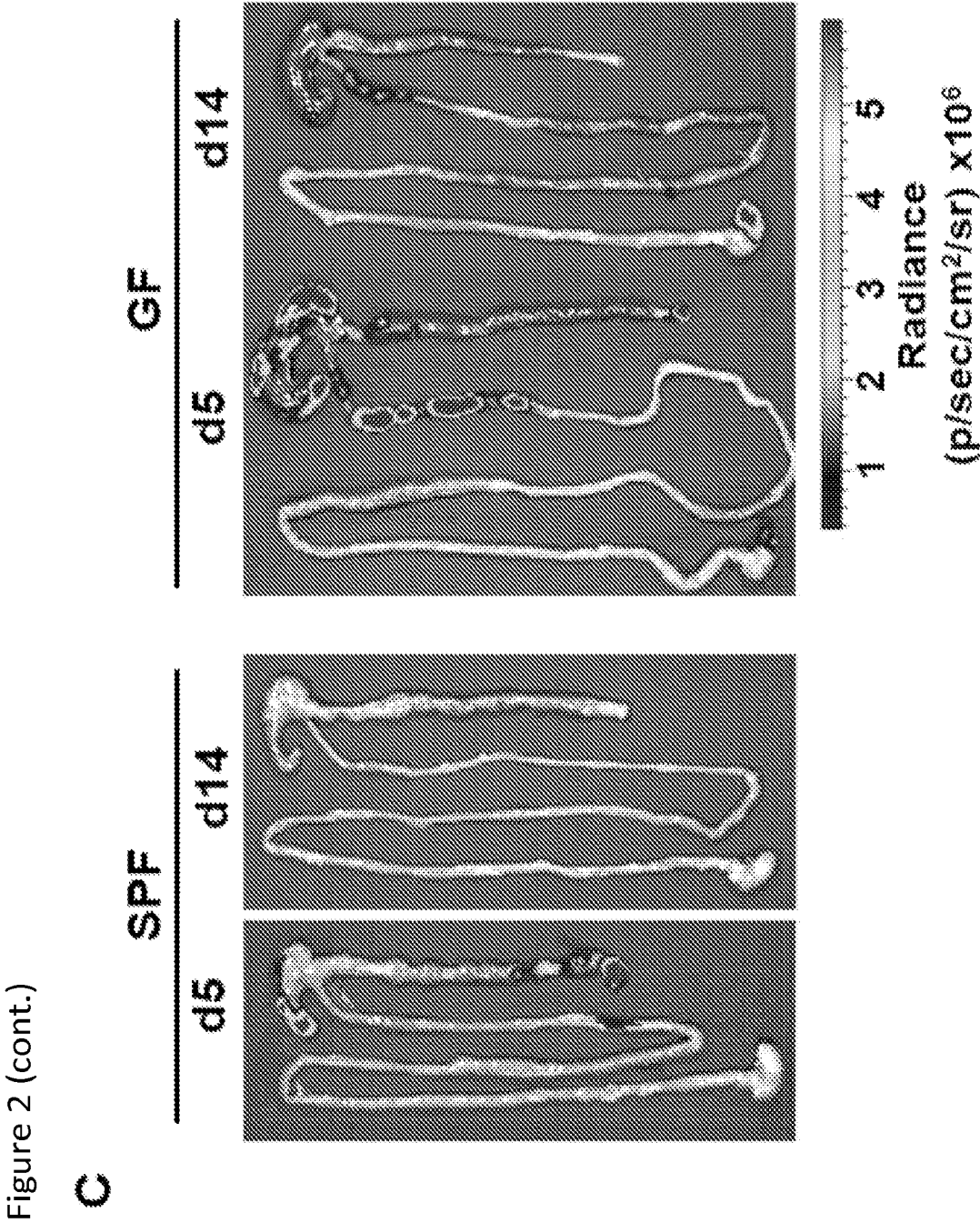


Figure 2 (cont.)

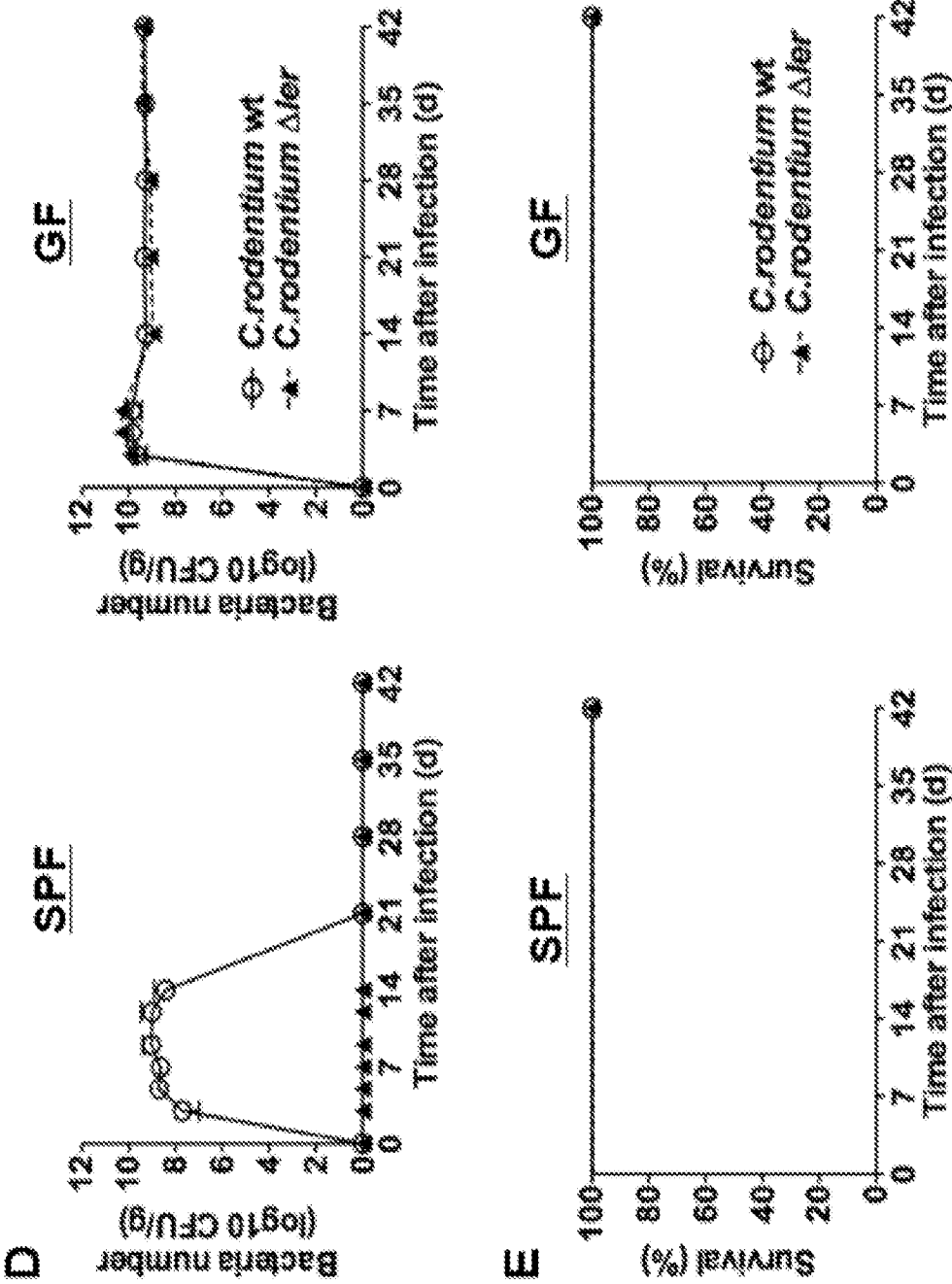


Figure 2 (cont.)

F

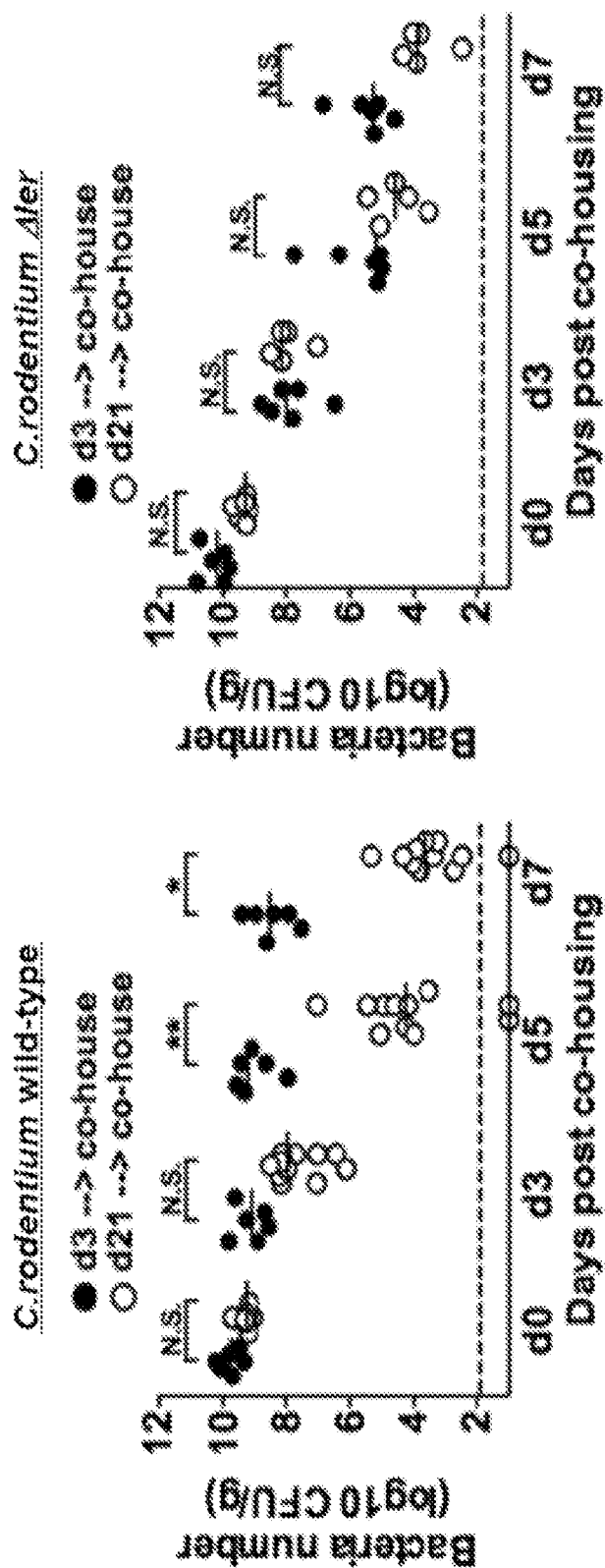


Figure 3

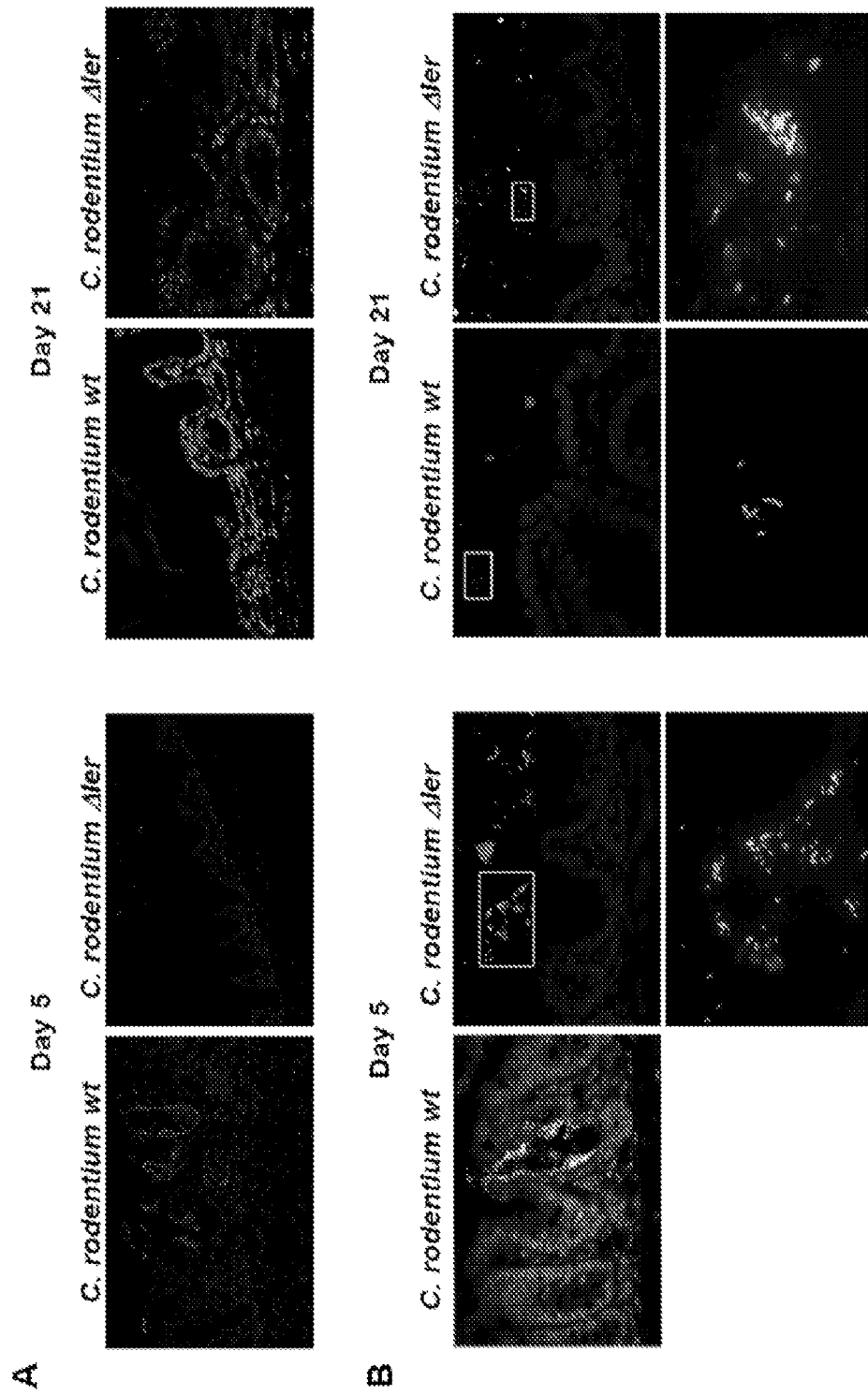


Figure 3 (cont.)

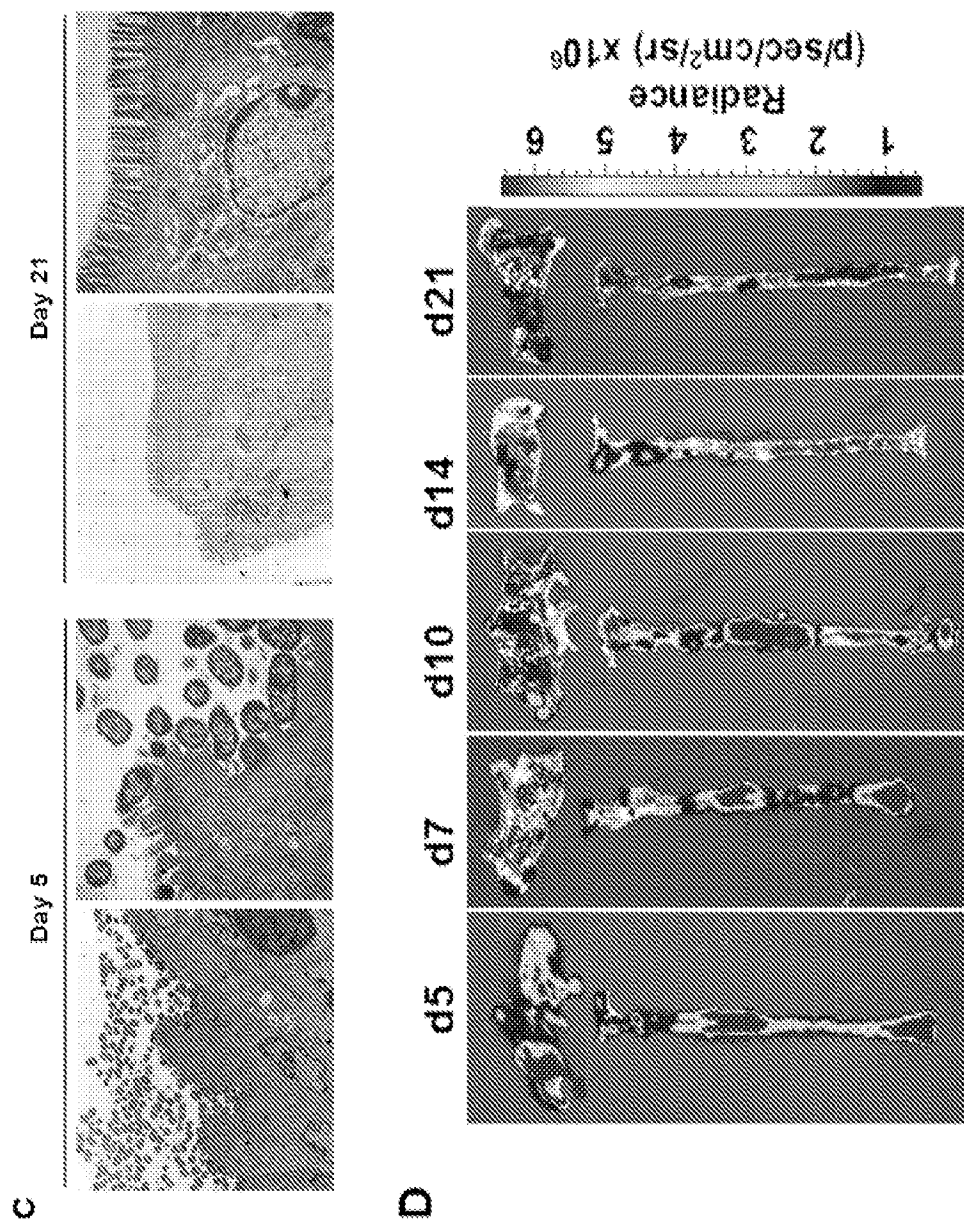


Figure 3 (cont.)

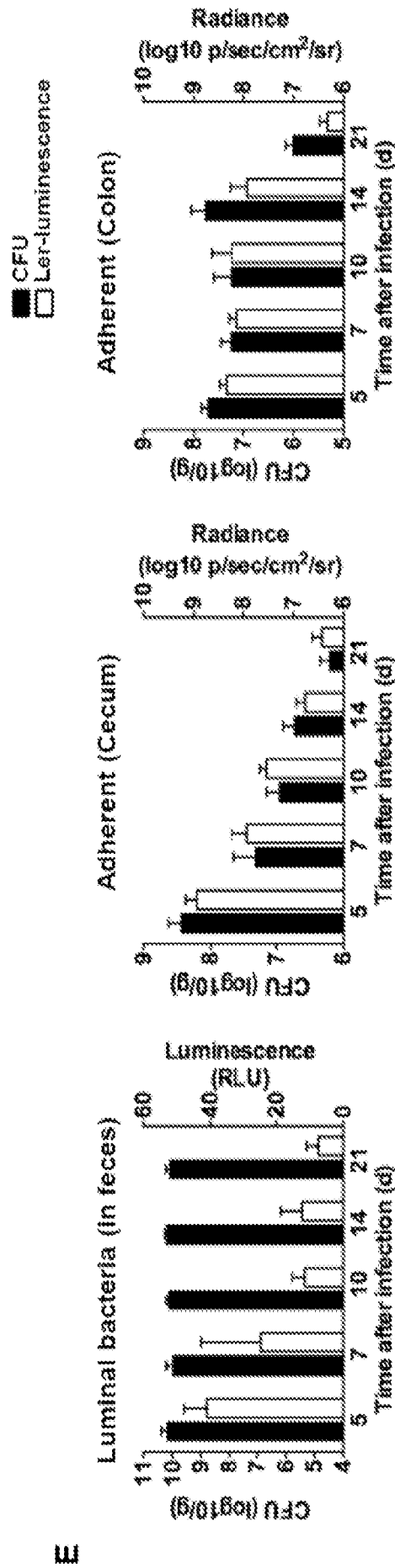


Figure 4

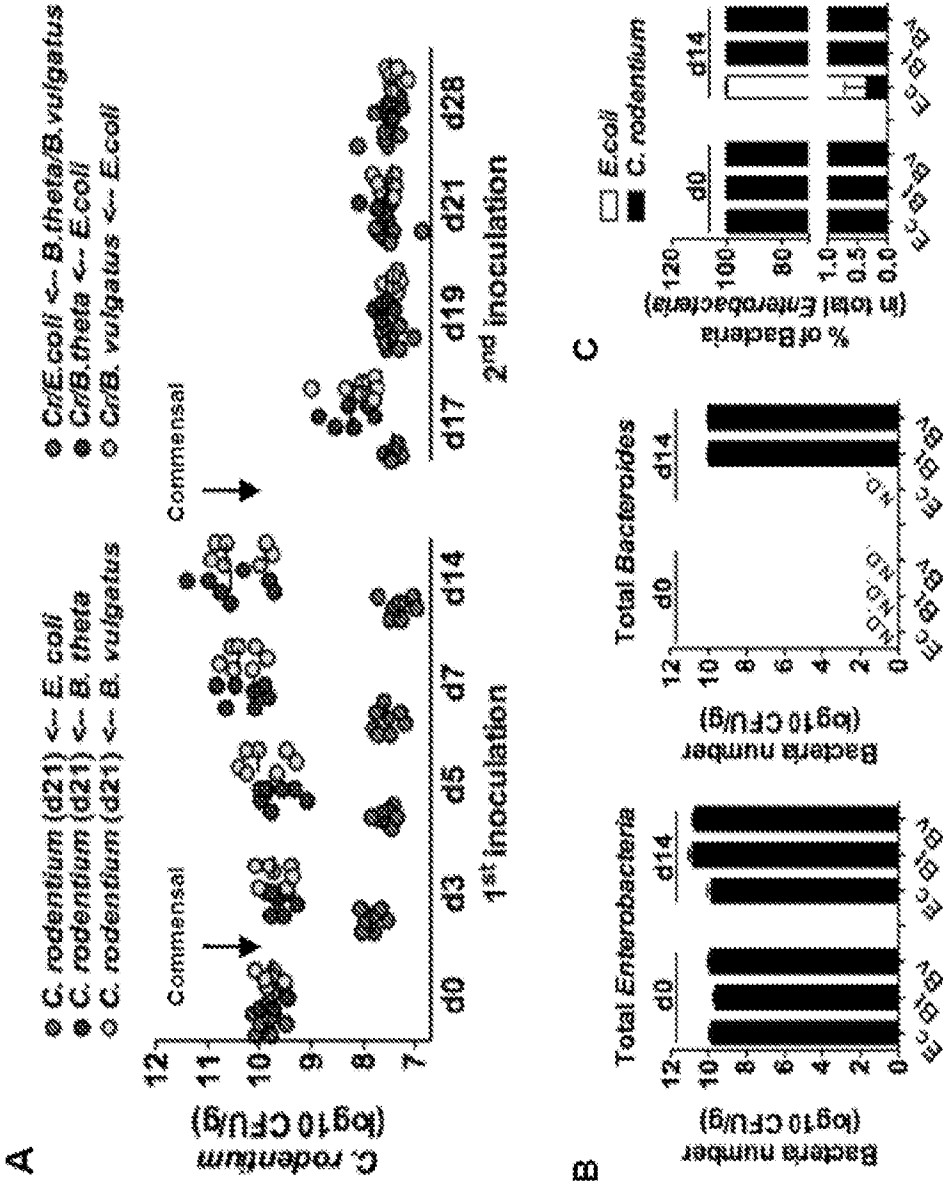
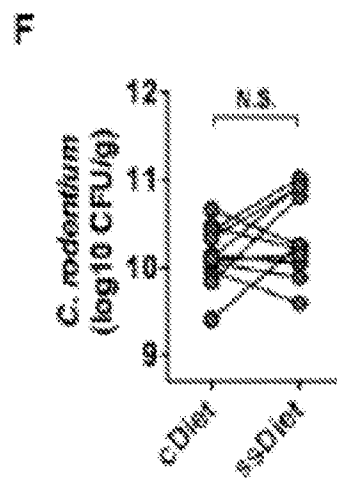
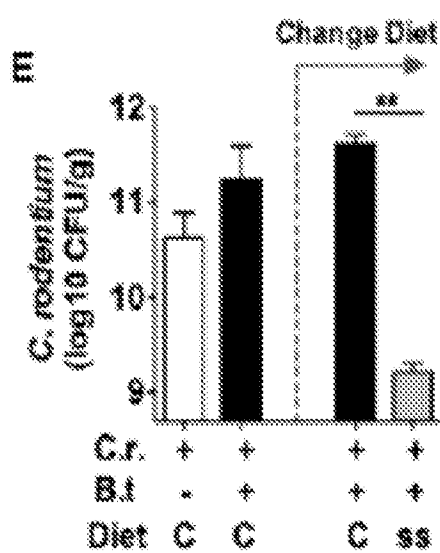
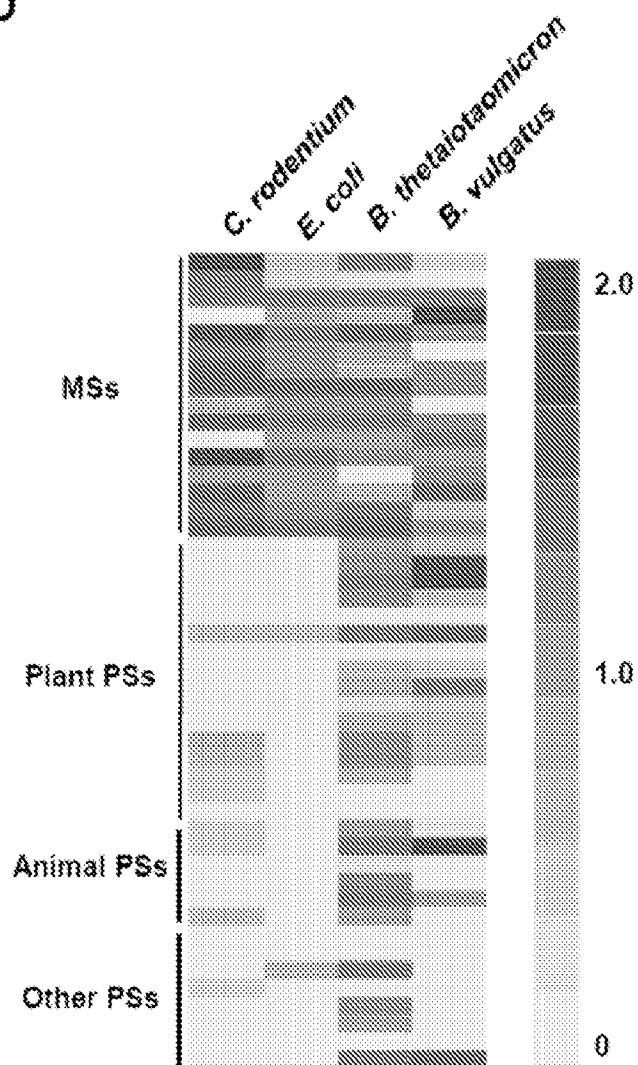


Figure 4 (cont.)

D



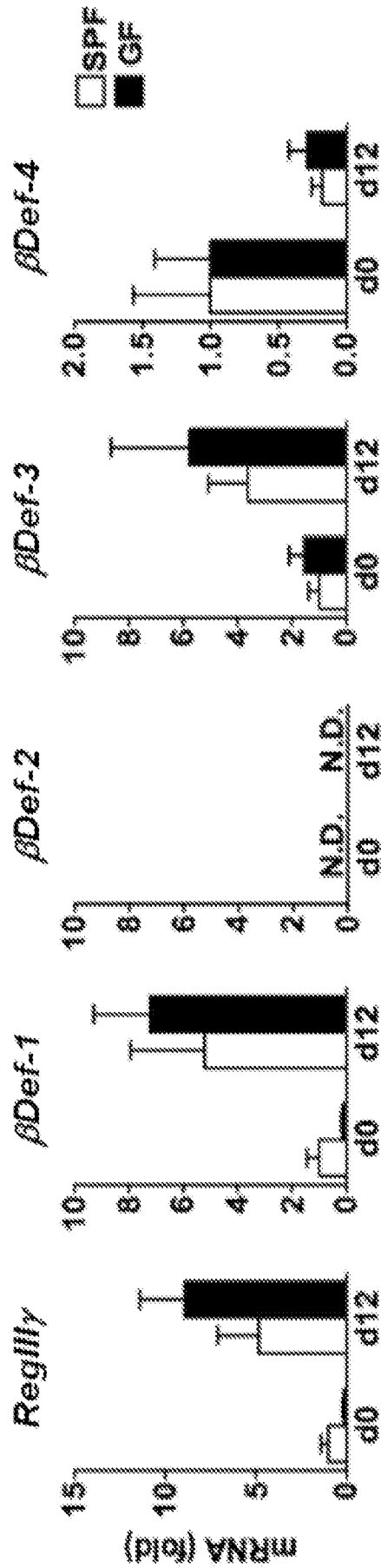


Figure 5

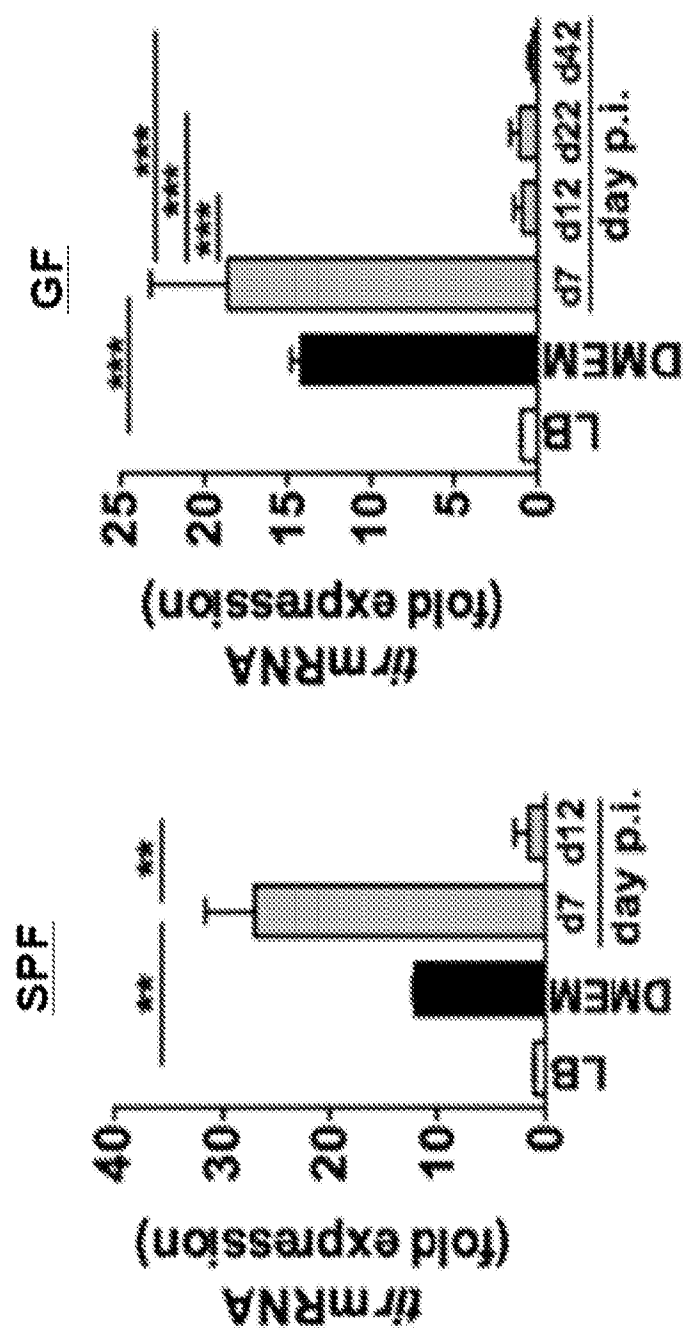
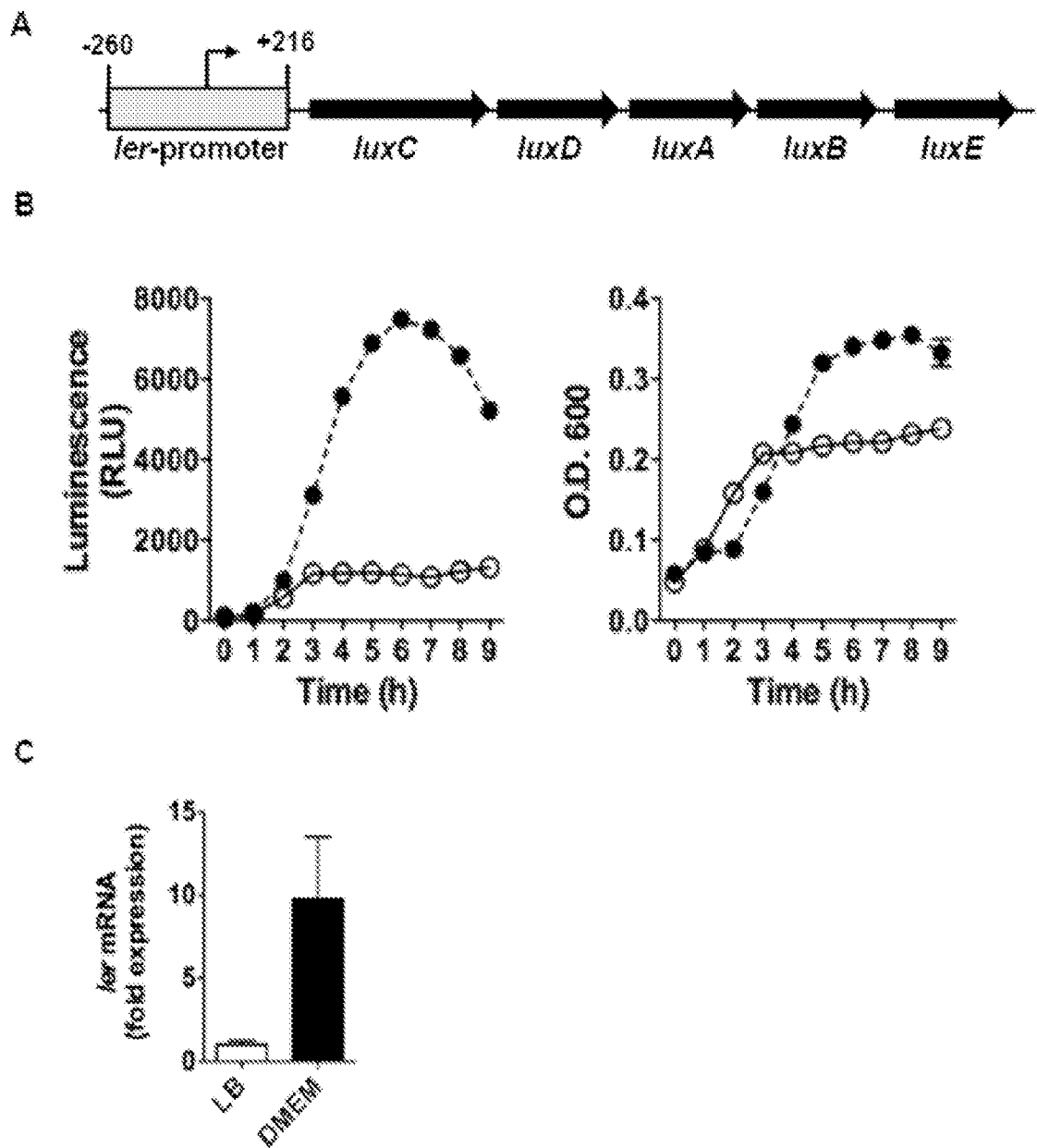


Figure 6

Figure 7



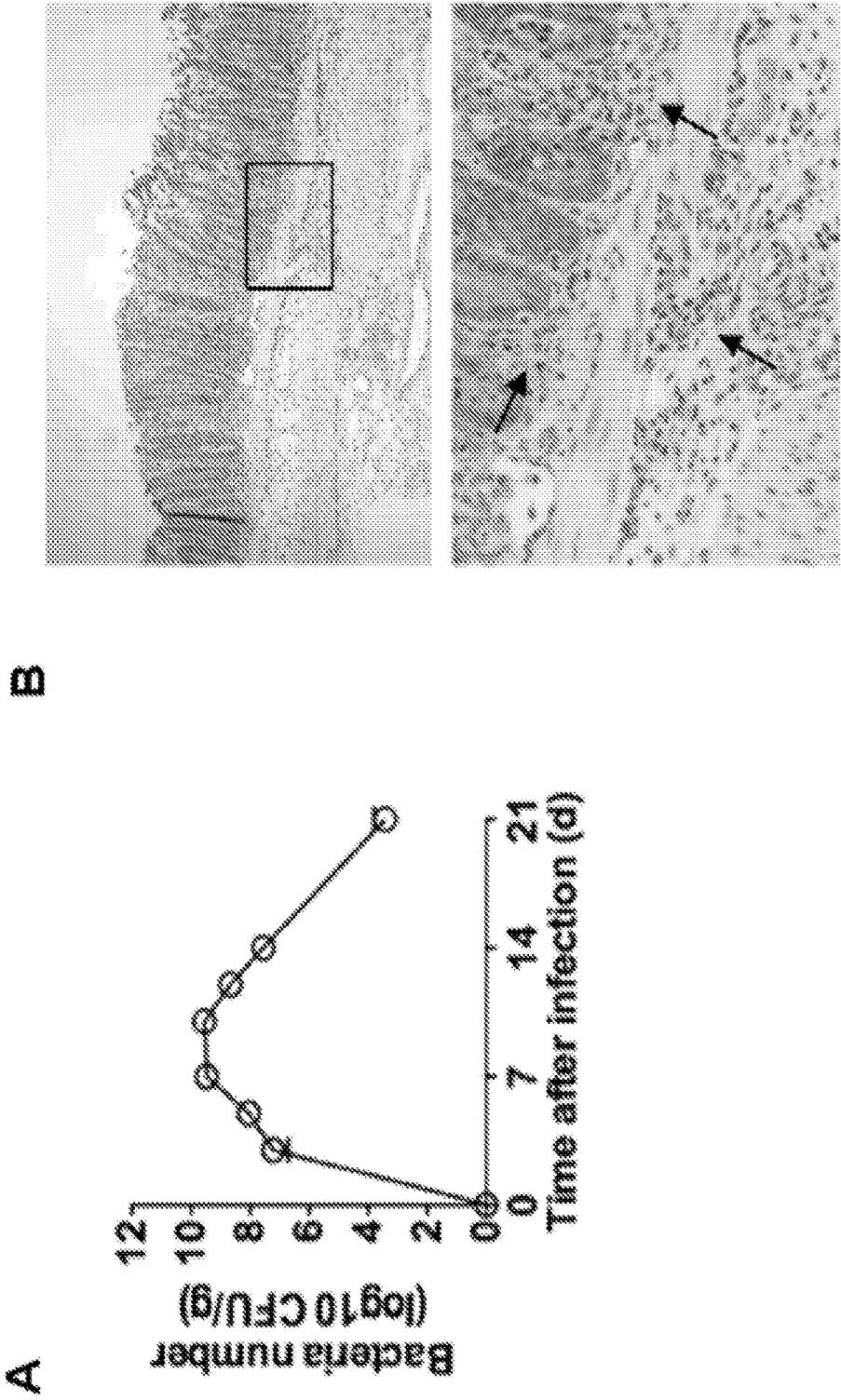
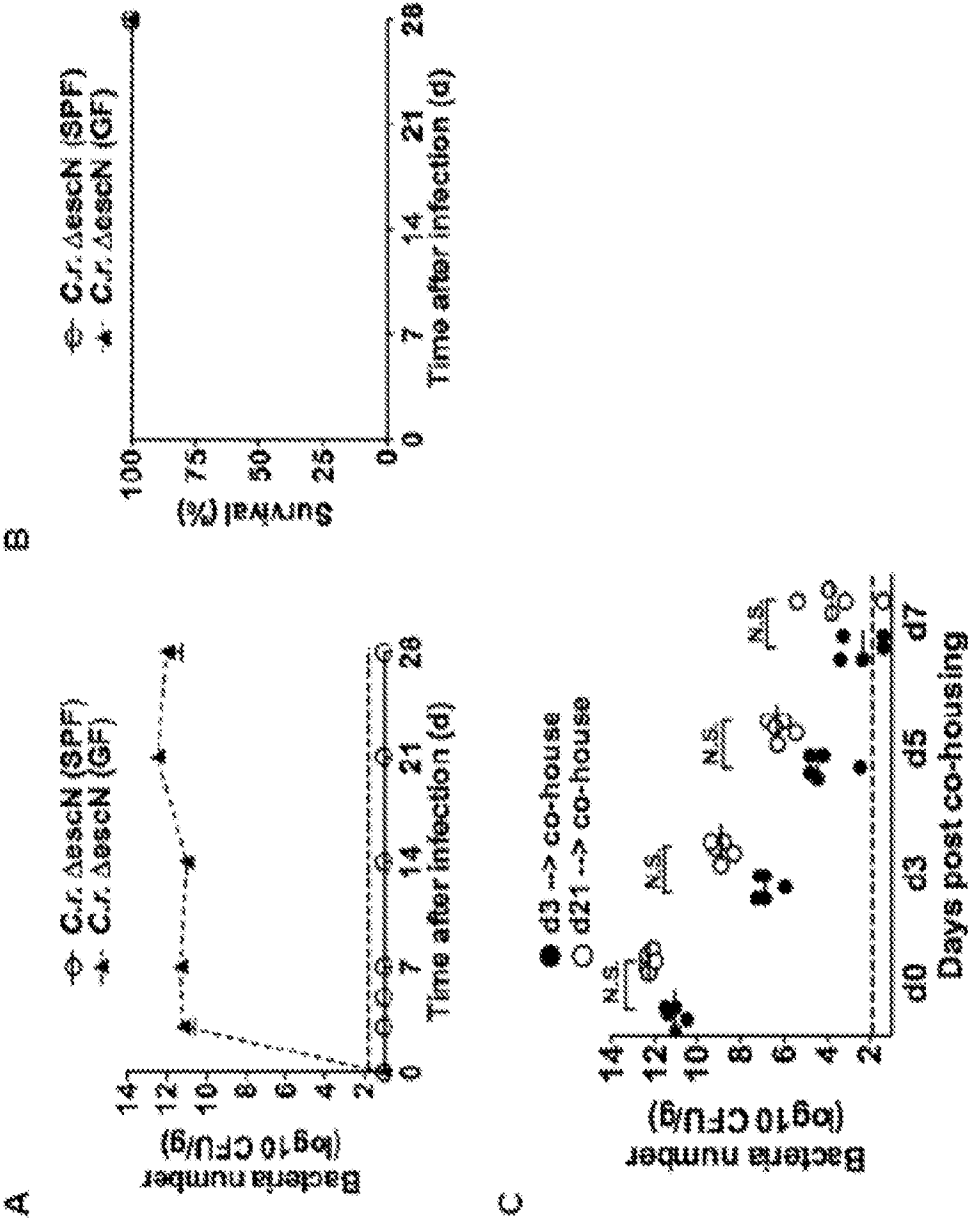


Figure 8

Figure 9



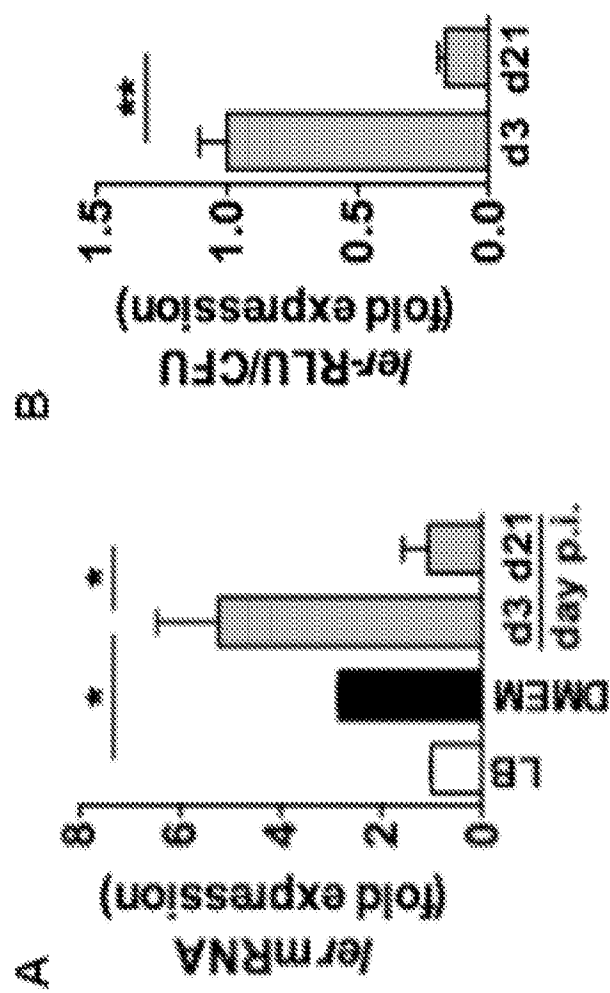


Figure 10

Figure 11

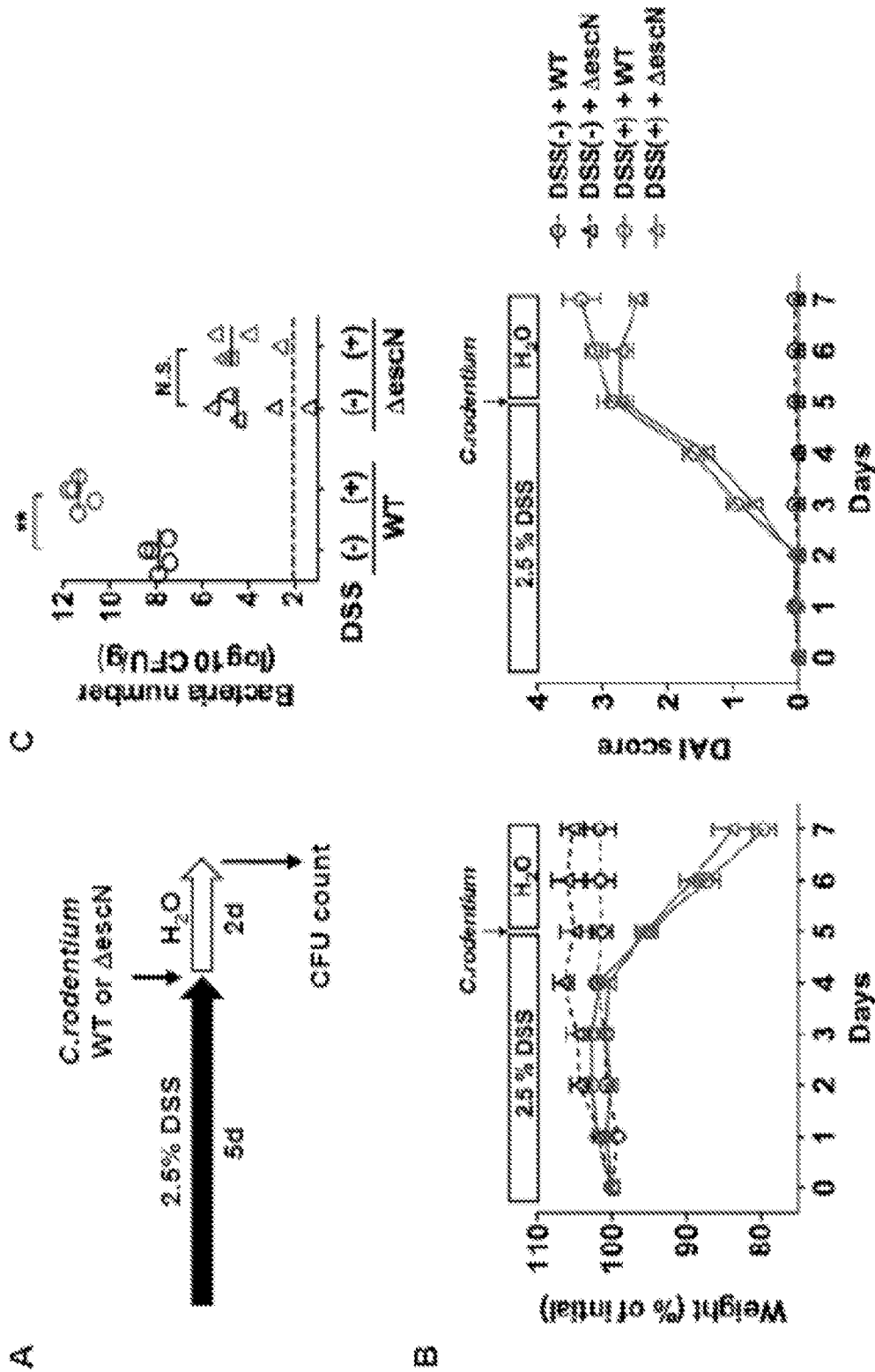


Figure 11 (cont.)

D

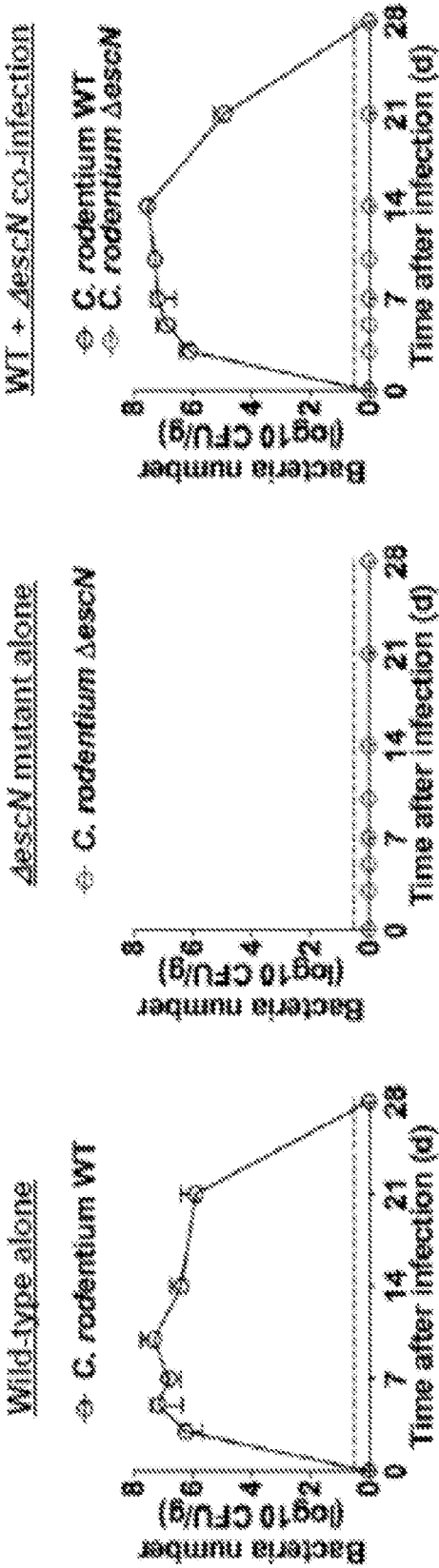


Figure 12

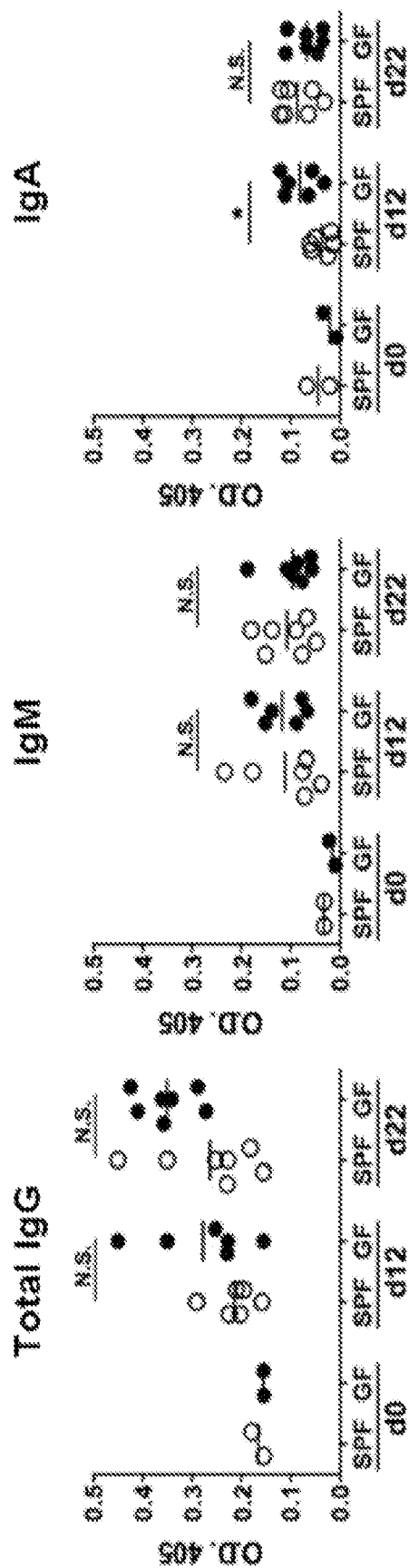
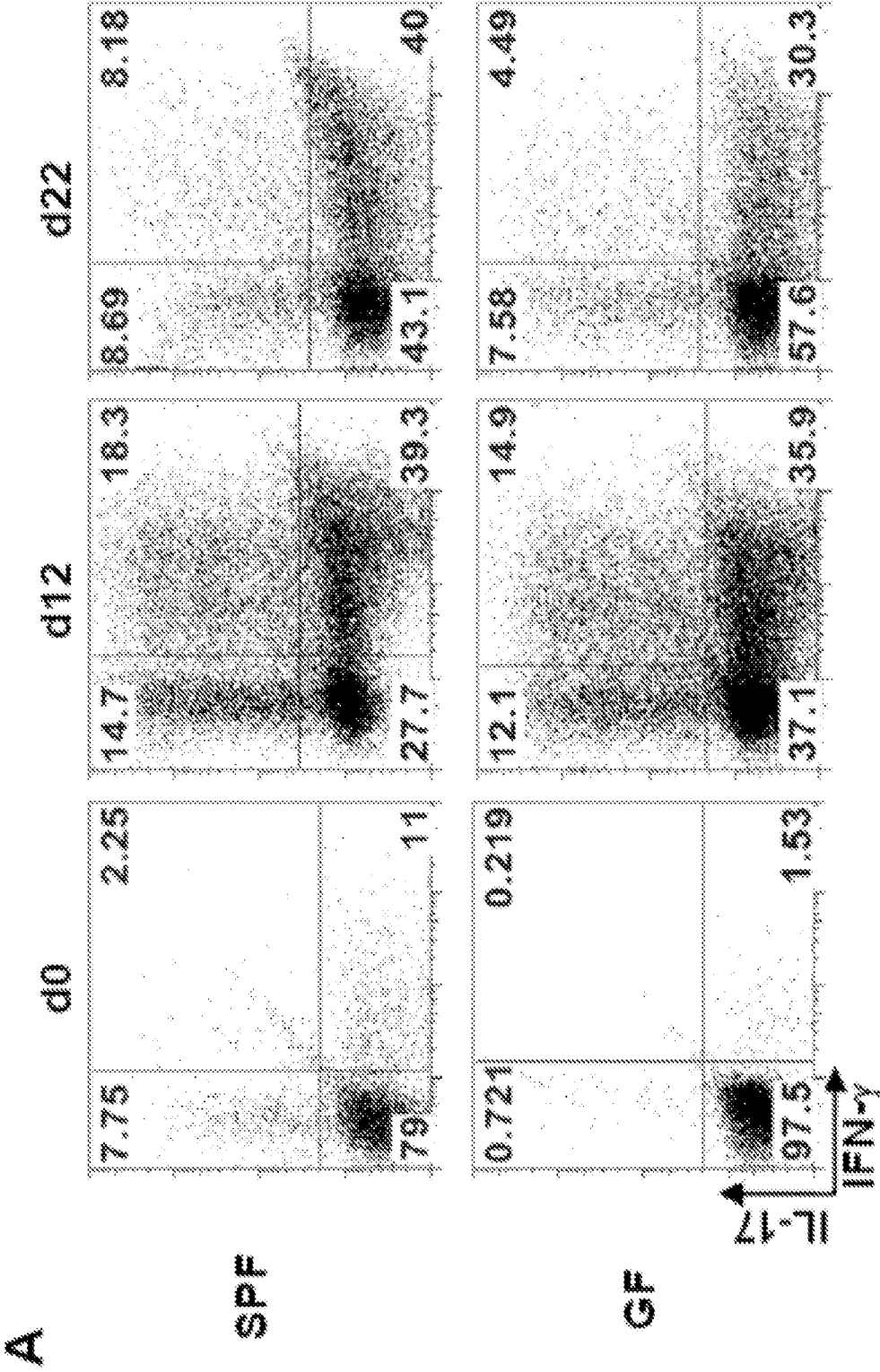


Figure 13



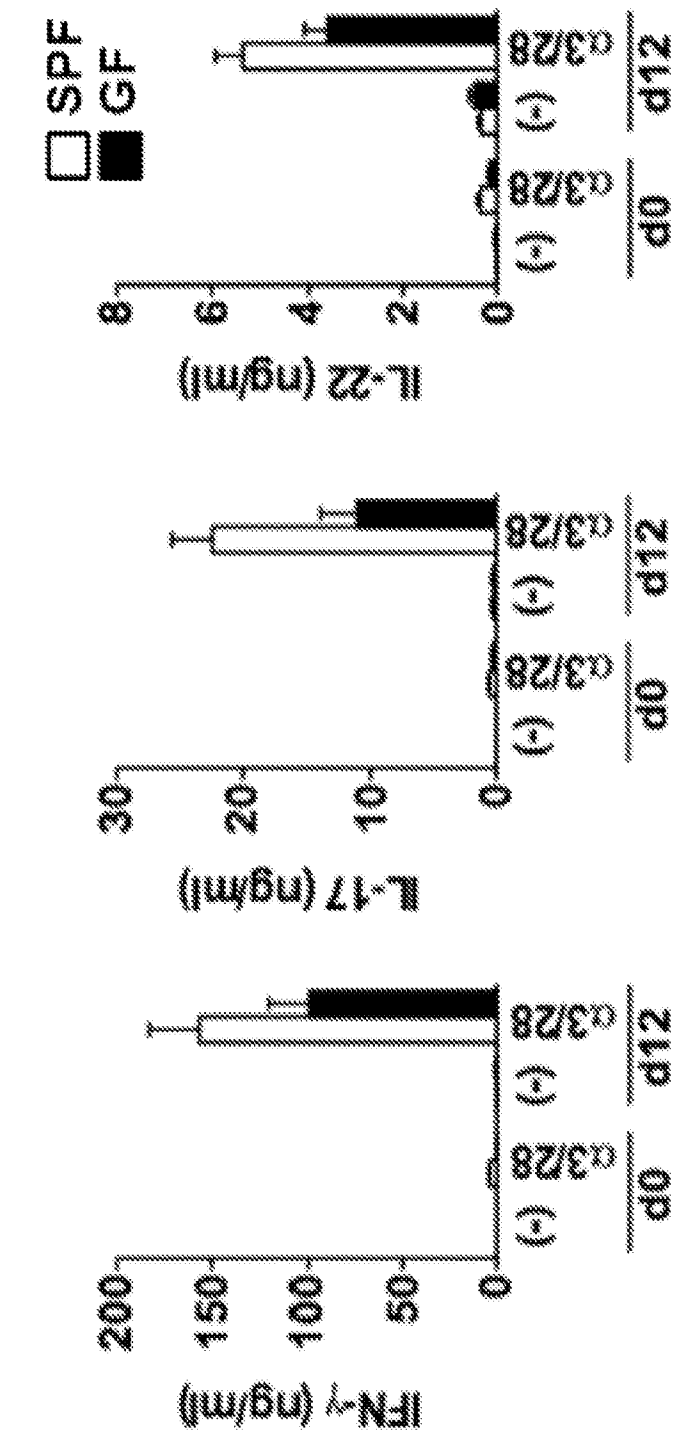
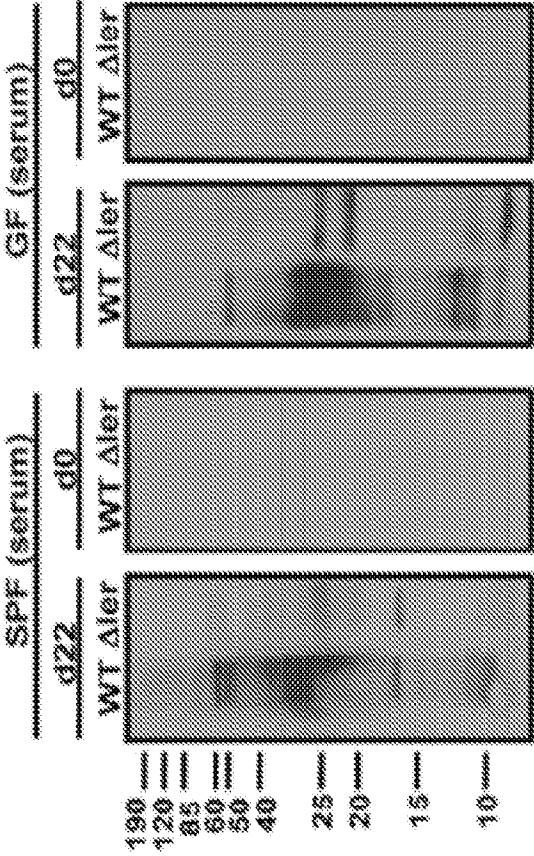


Figure 13 (cont.)

Figure 14

A. Serum IgG



B. Intestinal content IgG

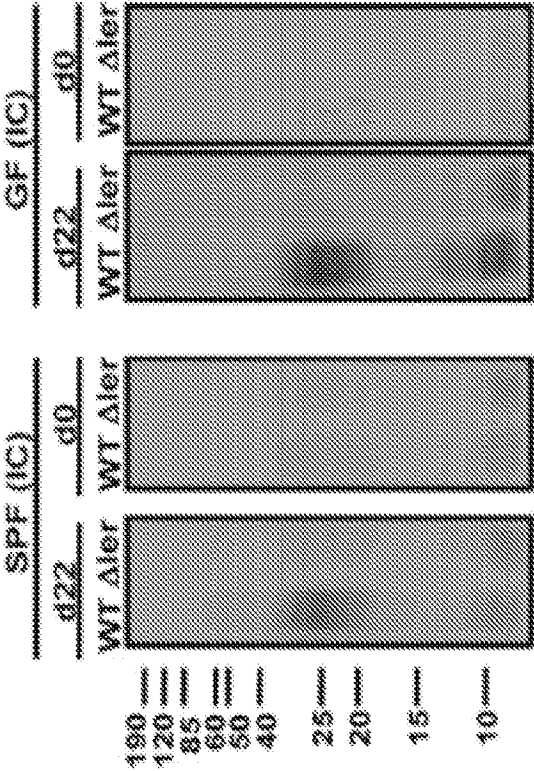


Figure 15

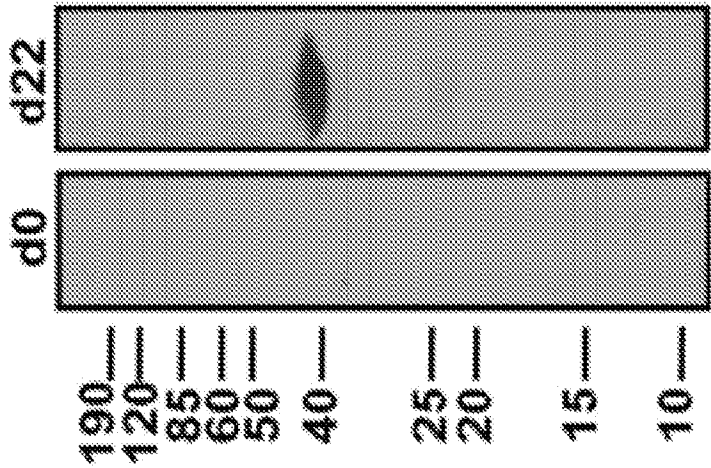


Fig. 11. Intimin-specific IgG production in the intestine of *C. rodentium* infected mice. Purified extracellular domain of *C. rodentium* intimin was loaded, and blotted with intestinal content of the mice before (day 0) and after (day 22) oral infection of *C. rodentium*. Peroxidase conjugated rabbit anti-mouse IgG antibody was used as a secondary antibody

A. CLASSIFICATION OF SUBJECT MATTER**A61K 38/17(2006.01)i, A61K 38/16(2006.01)i, A61K 38/10(2006.01)i, A61K 38/18(2006.01)i, A61K 48/00(2006.01)i, A61**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 38/17; A61K 31/4412; A61K 38/16; A61K 38/10; A61K 38/18; A61K 48/00; A61P 31/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: pathogenic, E. coli, inhibition, infection, salicylidene acyle hydrazine, aurodox and intimin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TREE et al., 'Characterization of the effects of salicylidene acylhydrazide compounds on type iii secretion in Escherichia coli O157:h7' Infection and Immunity, Vol.77, No.10, pp.4209-4220 (2009) See abstract; pages 4210, 4214, 4216, 4218; figures 1-3; and table 3.	1-5,7-10
Y		6
Y	CHINALI, 'Synthetic analogs of aurodox and kirromycin active on elongation factor tu from Escherichia coli' The Journal of Antibiotics, Vol.34, No.8, pp.1039-1045 (1981) See abstract; pages 1040-1041; and figure 2.	6
A	WO 2009-061491 A2 (UNIVERSITY OF MASSACHUSETTS) 14 May 2009 See abstract and claims 1-5.	1-10
A	CARVALHO et al., 'Antibody against the carboxyl terminus of intimin alpha reduces enteropathogenic escherichia coli adherence to tissue culture cells and subsequent induction of actin polymerization' Infection and Immunity, Vol.73, No.4, pp.2541-2546 (2005) See the whole document.	1-10



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HILLGREN et al., `Synthesis of [4-(2-hydroxyphenyl)thiazol-2-yl]methanones as potential bioisosteres of salicylidene acylhydrazides` Molecules, Vol.15, No.9, pp.6019-6034 (2010) See the whole document.	1-10

Information on patent family members

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