

## MUCOADHESIVE MICROORGANISM

### CROSS REFERENCE TO RELATED APPLICATIONS

[001] This application claims benefit of the filing date of U.S. Provisional Application  
5 No. 62/394,024, filed September 13, 2016.

### REFERENCE TO SEQUENCE LISTING

[002] The instant application contains a Sequence Listing which has been submitted  
electronically in ASCII format and is hereby incorporated by reference in its entirety. Said  
ASCII copy, created on September 11, 2017, is named 205350-0031-00-WO\_SL.txt and is  
10 122,643 bytes in size.

### BACKGROUND

[003] Genetically modified microorganisms (*e.g.*, bacteria) have been used to deliver  
therapeutic molecules to mucosal tissues. *See, e.g.*, Steidler, L., *et al.*, *Nat. Biotechnol.* 2003,  
21(7): 785-789; Robert S. and Steidler L., *Microb. Cell Fact.* 2014, 13 Suppl. 1: S11; Braat *et*  
15 *al.*, *Clin. Gastroenterol. Hepatol.* 2006, 4(6):754-759; and Steidler *et al.*, *Science* 2000,  
289(5483):1352-1355.

[004] There is a need in the art for microbial (*e.g.*, bacterial) strains with improved  
pharmacokinetic and pharmacodynamics properties, and a need for efficacious, targeted, and  
controlled methods for the treatment of various diseases treatable with such genetically  
20 modified bacteria. The present disclosure addresses these needs.

### BRIEF SUMMARY

[005] The present disclosure provides microorganisms (*e.g.*, bacteria or yeast) with  
enhanced cell-adhesion and/or mucin-binding properties. For example, the present disclosure  
provides bacteria exhibiting increased *in vitro* binding to Caco-2 cells, and increased *in vitro*  
25 binding to mucins. Such microorganisms (*e.g.*, bacteria) can be used, *e.g.*, to deliver  
bioactive polypeptides to the gastrointestinal tract of a mammalian subject, while the  
described genetic modifications allow for the modulation of gastrointestinal retention and  
transit times of the microorganism (*e.g.*, bacterium). The described technology allows for the  
modulation of pharmacokinetic and pharmacodynamic properties of the bioactive  
30 polypeptides expressed by the microorganism (*e.g.*, bacterium). For example, expression,

secretion and anchoring of a fusion protein containing a trefoil factor (TFF) and a cell-adhesion polypeptide, such as CmbA (*see, e.g., Jensen et al., Microbiology* 2014, 160(4):671-681) (*e.g.,* TFF3-CmbA) in the cell wall of a lactic acid bacterium (LAB), such as *Lactococcus lactis*, enables adherence of the bacterium to intestinal epithelial cells, and  
 5 further enables binding of the bacterium to mucins.

### Compositions

[006] In some aspects, the present disclosure provides microorganisms (*e.g.,* a bacteria or yeast) comprising an exogenous nucleic acid encoding a fusion protein comprising a cell-adherence polypeptide. In some examples, the current disclosure provides a microorganism  
 10 (*e.g.,* bacterium or yeast) comprising an exogenous nucleic acid encoding a fusion protein, wherein the exogenous nucleic acid encoding the fusion protein contains a sequence encoding a cell-adherence polypeptide. In some examples, the cell-adherence polypeptide is selected from the group consisting of cell and mucus-binding protein A (CmbA) (*see, e.g., Jensen et al., Microbiology* 2014, 160(4):671-681), mucus binding protein or mub domain proteins  
 15 (Mub) (*see, e.g., Boekhorst et al., Microbiology* 2006, 152(1):273-280), mucus adhesion promoting protein (MapA) (*see, e.g., Miyoshi et al., Biosci. Biotechnol. Biochem.* 2006, 70(7):1622-8), lactococcal mucin binding protein (MbpL) (*see, e.g., Lukić et al., Appl. Environ. Microbiol.* 2012, 78(22):7993-8000). A cell-wall anchoring peptide, such as *Staphylococcus aureus* protein A anchor fragment (SpaX) may be added (*see, e.g., Steidler et al., Appl. Environ. Microbiol.* 1998, 64(1):342-5). In some examples, the current disclosure provides a microorganism (*e.g.,* bacterium or yeast) comprising an exogenous nucleic acid encoding a fusion protein, wherein the fusion protein includes a CmbA polypeptide. In some examples, the CmbA polypeptide is CmbA from *Lactobacillus reuteri*. *See, e.g.,* ATCC PTA6474, *e.g.,* as disclosed in Jensen *et al., supra*. In some examples according to any of the  
 25 above embodiments, the cell-adherence polypeptide is a CmbA polypeptide having an amino acid sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 1. In other examples according to any of the above embodiments, the cell-adherence polypeptide is a CmbA polypeptide encoded by an exogenous nucleic acid sequence that is at least 90%, at least 92%, at least 95%, at least  
 30 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 2.

[007] In other examples according to any of the above embodiments, the fusion protein comprises a mucin-binding polypeptide, such as a trefoil factor (TFF) polypeptide (*e.g.,*

TFF1, TFF2, or TFF3) or a MucBP polypeptide (see, e.g., Lukic *et al*, *Appl. Environ. Microbiol.* 2012, 78(22):7993-8000). Thus, in some examples, the current disclosure provides a microorganism (e.g., a bacterium or yeast) comprising an exogenous nucleic acid encoding a fusion protein, wherein the fusion protein contains a cell-adherence polypeptide (e.g., a CmbA polypeptide) and a mucin-binding polypeptide (e.g., a TFF polypeptide). In some examples, the TFF polypeptide is a human TFF polypeptide (e.g., hTFF1, hTFF2, or hTFF3). In some examples according to any of the above embodiments, the mucin-binding polypeptide is a human TFF3 polypeptide having an amino acid sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 3. In other examples according to any of the above embodiments, the mucin-binding polypeptide is a human TFF3 polypeptide encoded by an exogenous nucleic acid sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 4. In some examples, the TFF polypeptide is a mammalian TFF polypeptide, such as a cow, pig, sheep, dog, cat, or horse TFF. In further examples, the TFF is an amphibian TFF. Exemplary TFF polypeptides are disclosed, e.g., in Conlon *et al.*, *Peptides* 2015, 72:44-49, and cited references therein, each of the disclosures are incorporated herein by reference in their entirety. In other examples, the TFF polypeptide is a trefoil-like domain. Exemplary polypeptides according to this embodiment are disclosed in Fujita *et al.*, *Mol. Reprod. Dev.* 2006, 75(7):1217-1228, the disclosure of which is incorporated herein by reference in its entirety.

[008] In some examples according to any of the above embodiments, the current disclosure provides a bacterium (e.g., a lactic acid bacterium, such as *Lactococcus lactis*) comprising an exogenous nucleic acid encoding a fusion protein comprising (1) a mucin-binding polypeptide selected from a TFF polypeptide (e.g., human TFF1, human TFF2, or human TFF3) and a MucBP polypeptide; and (2) a cell-adherence polypeptide selected from a CmbA polypeptide, a Mub polypeptide, a MapA polypeptide, an MbpL polypeptide, and a SpaX polypeptide. In some examples according to this embodiment, the fusion protein contains a CmbA polypeptide (e.g., *Lactobacillus reuteri* CmbA) and a TFF polypeptide (e.g., human TFF1, human TFF2, or human TFF3).

[009] In some examples according to any of the above embodiments, the exogenous nucleic acid encoding the fusion protein is integrated into the chromosome of the microorganism, e.g., the chromosome of a bacterium. In some examples, the exogenous

nucleic acid encoding the fusion protein is constitutively expressed in the microorganism (*e.g.*, bacterium). In other examples, the exogenous nucleic acid encoding the fusion protein is located on a plasmid.

[0010] In some examples according to any of the above embodiments, the fusion protein is expressed by the microorganism (*e.g.*, bacterium). In other examples the fusion protein is anchored in the cell wall of the microorganism (*e.g.*, bacterium). For example, the fusion protein is displayed on the surface (*i.e.*, outer membrane) of the microorganism (*e.g.*, bacterium).

[0011] In some examples according to any of the above embodiments, the exogenous nucleic acid encoding a fusion protein further includes a secretion leader sequence encoding a secretion signal peptide. In some examples, the secretion leader sequence contains a nucleotide sequence encoding a secretion leader of unidentified secreted 45-kDa protein (SSusp45). Such secretion leader sequence or peptide is referred to herein as SSusp45. In some examples, SSusp45 has an amino acid sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 5. In other examples, SSusp45 is encoded by a nucleic acid sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 6 or SEQ ID NO: 7. Any secretion leader sequence derived from a gram-positive bacterium, *e.g.*, any secretion leader sequence derived from *Lactococcus lactis* is useful in the context of the above embodiments. In further examples according to any of the above embodiments, the secretion signal peptide (*e.g.*, SSusp45) is bound to the mucin-binding polypeptide, such as such as a TFF polypeptide. In some examples according to this embodiment, SSusp45 is bound to a human TFF polypeptide. For examples, the fusion protein may include an amino acid sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 8. In other examples, the fusion protein may be encoded by an exogenous nucleic acid sequence containing a sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 9.

[0012] In other examples, the microorganism (*e.g.*, bacterium) comprises an exogenous nucleic acid encoding a fusion protein containing a TFF polypeptide and a CmbA polypeptide, wherein a secretion signal peptide is bound to the TFF polypeptide (*e.g.*, SSusp45). For example, the fusion protein may include (or consist of) an amino acid

sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 10. In other examples, the fusion protein may be encoded by an exogenous nucleic acid sequence containing (or consisting of) a sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 11.

[0013] In some examples, the secretion signal peptide includes a linker peptide. In some examples, the secretion signal peptide is cleaved from the fusion protein, *e.g.*, when the fusion protein is anchored in a cell wall of the microorganism (*e.g.*, bacterium).

[0014] In some examples, the exogenous nucleic acid encoding the fusion protein is transcriptionally regulated by (*e.g.*, placed under the control of) a promoter that is endogenous to the microorganism (*e.g.*, bacterium). In other examples, expression of the fusion protein is controlled by an exogenous promoter. In some examples, the promoter is selected from a *thyA* promoter (*PthyA*), an *hlla* promoter (*Phlla*), and a *gapB* promoter. In some examples, the nucleic acid encoding a fusion protein is transcriptionally regulated by a *PthyA* promoter. In other examples, the exogenous nucleic acid encoding the fusion protein is transcriptionally regulated by a *Phlla* promoter. Other promoters include those preceding genes *holA*, *soda*, *enoA*, *tufa*, *fbaA*, *acpA*, *ps431*, *malG*, *ptsH*, *dpsA*, *pgk*, *ahpC*, *pdhD*, *pts\_II*, *pfk*, *trePP*, *ptnD*, *pgiA*, *usp45*. Other suitable promoters are described, *e.g.*, in U.S. Patent Application Publication 2014/0105863, the disclosure of which is incorporated herein by reference in its entirety.

[0015] The present disclosure further provides a microorganism (*e.g.*, bacterium) comprising a fusion protein (*e.g.*, anchored in a cell-wall of the microorganism, *e.g.*, bacterium), wherein the fusion protein comprises a TFF polypeptide and a CmbA polypeptide. In some examples, the microorganism (*e.g.*, bacterium) includes an exogenous nucleic acid comprising a secretion leader sequence, a sequence encoding the TFF polypeptide, and a sequence encoding the CmbA polypeptide. In some examples, the secretion leader sequence encodes a secretion signal peptide, which is cleaved from the fusion protein, *e.g.*, when the fusion protein passes the cytoplasmic membrane (*e.g.*, is anchored in the cell wall) of the microorganism (*e.g.*, bacterium).

[0016] In some examples according to any of the above embodiments, the microorganism is a bacterium. In other examples according to any of the above embodiments, such bacterium is a Gram-positive bacterium, *e.g.*, a non-pathogenic Gram-positive bacterium. In

other examples according to any of the above embodiments, the bacterium is a lactic acid bacterium (LAB). Exemplary lactic acid bacteria are disclosed herein, each of which can be used in the context of these embodiments. In some embodiments, the LAB is selected from the group consisting of a *Lactococcus* species (sp.) bacterium, a *Lactobacillus* sp. bacterium, a *Bifidobacterium* sp. bacterium, a *Streptococcus* sp. bacterium, and an *Enterococcus* sp. bacterium. In some examples, the LAB is *Lactococcus lactis*. In other examples, the LAB is selected from *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *hordniae*, and *Lactococcus lactis* subsp. *lactis*. In some examples, the *Lactococcus lactis* is *Lactococcus lactis* subsp. *cremoris*, such as *Lactococcus lactis* strain MG1363.

[0017] In some examples according to any of the above embodiments, the microorganism (e.g., bacterium) comprises an exogenous nucleic acid encoding a fusion protein comprising a TFF polypeptide and a cell-adherence polypeptide (e.g., CmbA). In some examples according to this embodiment, the TFF polypeptide is selected from TFF1, TFF2, and TFF3. In other examples according to this embodiment, the TFF polypeptide is selected from human TFF, mouse TFF, pig TFF, dog TFF, cat TFF, cow TFF, and sheep TFF. In some examples, the TFF polypeptide is human TFF. In other examples, the TFF polypeptide is selected from human TFF1, human TFF2, and human TFF3. In yet other example, the TFF polypeptide is human TFF3. In some example, the TFF polypeptide has an amino acid sequence at least 90%, at least 92%, at least 95%, at least 96%, at least 98%, or at least 99% identical to SEQ ID NO: 3. In other examples, the TFF polypeptide is a TFF variant polypeptide, e.g., a TFF variant polypeptide having enhanced mucin-binding capability when compared to a corresponding wild-type TFF polypeptide. In other examples, the TFF polypeptide is an amphibian or fish TFF polypeptide.

[0018] In some examples according to any of the above embodiments, the microorganism (e.g., bacterium) further contains an exogenous nucleic acid encoding at least one therapeutic polypeptide. In some examples, the therapeutic polypeptide is a cytokine, such as an interleukin (IL). The choice of cytokine is made on the basis of what host responses are sought to be activated or suppressed. In some examples, the cytokine is IL-2, IL-10, or IL-22. In other examples, the therapeutic polypeptide is an antigen. In other examples the therapeutic polypeptide is an antigen and an interleukin, such as IL-2, IL-10, or IL-22. In some examples according to any of these embodiments, the antigen is an autoantigen, e.g., a T1D-specific antigen. Exemplary T1D-specific antigens include proinsulin (PINS), glutamic

acid decarboxylase (GAD65), insulinoma-associated protein 2 (IA-2), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), zinc transporter 8 (ZnT8), chromogranin A, (prepro) islet amyloid polypeptide (ppIAPP), peripherin, citrullinated glucose-regulated protein (GRP), and a combinations thereof. Exemplary amino acid sequences and nucleic acid sequences for the above T1D-specific antigens are disclosed, *e.g.*,  
5 in international patent application publication WO2017/122180, the disclosure of which is incorporated herein by reference in its entirety. In other examples, the antigen is an allergen, such as a tree pollen allergen, a weed pollen allergen, a grass pollen allergen, a food allergen, a dust-mite allergen, a mold allergen, an animal dander allergen, or a combination thereof. In  
10 some examples, the allergen is a weed pollen allergen, *e.g.*, a ragweed pollen allergen. In other examples, the allergen is a tree pollen allergen, such as a birch pollen allergen or a Japanese cedar pollen allergen. In yet other examples, the allergen is a food allergen, such as a peanut allergen, a milk allergen, an egg allergen, a gluten allergen (gliadin epitope), or a combination thereof.

15 [0019] In further examples, the therapeutic polypeptide is an antibody or a fragment thereof. For example, the antibody is a single-domain antibody (*e.g.* camelid or shark antibody) or a nanobody. Exemplary antibodies include cytokine neutralizing antibodies such as antibodies to IL-4, antibodies to IL-5, antibodies to IL-7, antibodies to IL-13, antibodies to IL-15, as well as anti TNF $\alpha$  antibodies to immunoglobulin E (IgE), and any  
20 fragments thereof. In some examples, the therapeutic polypeptide is a fusion protein. For example, the therapeutic polypeptide comprises a soluble receptor, such as a TNF receptor (*e.g.*, soluble TNF receptor 2) and an antibody or an antibody fragment, such as the Fc region of an antibody. In some examples according to these embodiments, the therapeutic polypeptide contains an Fc region of a human immunoglobulin (*e.g.*, human IgG1 Fc). In  
25 some examples, the therapeutic polypeptide comprises soluble TNF receptor 2 fused to human IgG1 Fc). In some examples, the therapeutic polypeptide is etanercept.

[0020] In yet other examples, the therapeutic polypeptide is an enzyme or a fragment (*e.g.*, functional fragment) thereof, *e.g.*, a phenylalanine ammonia lyase (PAL), an amino acid decarboxylase, or a combination thereof. In one example, the therapeutic polypeptide is  
30 PAL, or a functional fragment thereof.

[0021] In further examples, the therapeutic polypeptide is a glucagon-like peptide 1 (GLP-1), glucagon-like peptide 2 (GLP-2), glucagon, exendin-4, or any combination thereof.

In other examples, the therapeutic polypeptide is a growth factor, such as an epidermal growth factor (EGF), *e.g.*, human EGF or porcine EGF. In yet other examples, the therapeutic polypeptide is a TFF, such as TFF1, TFF2, TFF3, or a combination thereof.

[0022] The therapeutic polypeptide may be a combination of any of the above recited therapeutic polypeptides.

[0023] In some examples according to any of the above embodiments, the exogenous nucleic acid encoding the at least one therapeutic polypeptide is transcriptionally regulated by a promoter selected from a *gapB* promoter (*PgapB*), a *thyA* promoter (*PthyA*), and an *hllA* promoter (*PhllA*). In some examples, the exogenous nucleic acid encoding the at least one therapeutic polypeptide is transcriptionally regulated by (*e.g.*, under the control of) a *gapB* promoter. Other promoters include those preceding genes *holA*, *soda*, *enoA*, *tufa*, *fbaA*, *acpA*, *ps431*, *malG*, *ptsH*, *dpsA*, *pgk*, *ahpC*, *pdhD*, *pts\_II*, *pfk*, *trePP*, *ptnD*, *pgiA*, *usp45*. Other suitable promoters are described, *e.g.*, in U.S. Patent Application Publication 2014/0105863, the disclosure of which is incorporated herein by reference in its entirety.

[0024] In some examples according to any of the above embodiments, the microorganism (*e.g.*, LAB) further comprises a combination of mutations and insertions to promote trehalose accumulation, which enhances LAB survivability against bile salts and drying. For example, these may be

(i) chromosomally-integrated trehalose transporter(s), such as *PhllA*>>transporter 1>> intergenic region>>transporter 2, such as *llmg\_0453* and/or *llmg\_0454*, for uptake of trehalose;

(ii) chromosomally-integrated Trehalose-6-phosphate phosphatase gene (*otsB*; Gene ID: 1036914) positioned downstream of *usp45* (Gene ID: 4797218) to facilitate conversion of trehalose-6-phosphate to trehalose;

(iii) inactivated (*e.g.*, through gene deletion) Trehalose-6-phosphate phosphorylase gene (*trePP*; Gene ID: 4797140); and

(iv) inactivated cellobiose-specific PTS system IIC component (Gene ID: 4796893), *ptcC*, (*e.g.*, tga at codon position 30 of 446; tga30).

[0025] For example, an exogenous nucleic acid encoding a trehalose-6-phosphate phosphatase, *e.g.*, *otsB*, such as *Escherichia coli otsB*. In some examples according to these embodiments, the exogenous nucleic acid encoding the trehalose-6-phosphate phosphatase is

chromosomally integrated. In some examples, the exogenous nucleic acid encoding the trehalose-6-phosphate phosphatase is chromosomally integrated 3' of unidentified secreted 45-kDa protein gene (*usp45*). In some examples according to this embodiment, the LAB comprises a second polycistronic expression cassette comprising a *usp45* promoter, the *usp45* gene (e.g., 3' of the promoter), and the exogenous nucleic acid encoding a trehalose-6-phosphate phosphatase (e.g., 3' of the *usp45* gene). In some examples, the second polycistronic expression cassette further comprises an intergenic region between the *usp45* gene and the exogenous nucleic acid encoding a trehalose-6-phosphate phosphatase. In some examples, the second polycistronic expression cassette is illustrated by:

10 Pusp45>>usp45>>intergenic region>>*otsB*. In some examples according to these embodiments, the intergenic region is *rpmD* as described herein above (e.g., having SEQ ID NO: 8 or SEQ ID NO: 9). The second polycistronic expression cassette may then be illustrated by: Pusp45>>usp45>>*rpmD*>>*otsB*.

[0026] In some examples according to any of the above embodiments, a trehalose-6-phosphate phosphorylase gene (*trePP*) is disrupted or inactivated in the microorganism (e.g., LAB). For example, the *trePP* has been inactivated by removing the *trePP* gene or a fragment thereof, or the *trePP* has been disrupted by inserting a stop codon. Thus, in some examples according to these embodiments, the microorganism (e.g., LAB) lacks *trePP* activity.

20 [0027] In other examples according to any of the above embodiments, a cellobiose-specific PTS system IIC component gene (*ptcC*) has been disrupted or inactivated in the microorganism (e.g., LAB). For example, the *ptcC* has been disrupted by inserting a stop codon, or *ptcC* has been inactivated by removing the *ptcC* or a fragment thereof. Thus, in some examples according to these embodiments, the microorganism (e.g., LAB) lacks *ptcC* activity.

[0028] In other examples according to any of the above embodiments, the LAB further comprises one or more genes encoding one or more trehalose transporter(s). In some examples, the one or more genes encoding the one or more trehalose transporter(s) are endogenous to the LAB. In some examples, the LAB overexpresses the one or more genes encoding the one or more trehalose transporter(s). In some examples according to these

30 embodiments, the one or more genes encoding the one or more trehalose transporter(s) is positioned 3' of an exogenous promoter, e.g., an *hllA* promoter (*PhllA*). For example, the one

or more genes encoding the one or more trehalose transporter(s) are transcriptionally regulated by the *PhlA*. In some examples according to these embodiments, the one or more genes encoding the one or more trehalose transporter(s) is selected from *llmg\_0453*, *llmg\_0454*, and any combination thereof. In some examples, *llmg\_0453* and *llmg\_0454* are transcriptionally regulated by a *PhlA*.

[0029] In some examples, according to any of the above embodiments, the one or more genes encoding one or more trehalose transporter(s) comprises two genes encoding two or more trehalose transporters, wherein an intergenic region is located between the two genes. In some examples, the intergenic region is *rpmD*, e.g., having SEQ ID NO: 8 or SEQ ID NO: 9. In some examples, the microorganism (e.g., LAB) comprises a polycistronic expression cassette comprising two nucleic acid sequences (e.g., genes) encoding two different trehalose transporters (transporter 1 and transporter 2 sequences) and an intergenic region between the two nucleic acids encoding the two different trehalose transporters. Such expression cassette may be illustrated by: *PhlA*>>transporter 1>>intergenic region>>transporter 2. In some examples according to these embodiments, the intergenic region is *rpmD* as described herein above (e.g., having SEQ ID NO: 8 or SEQ ID NO: 9). The polycistronic expression cassette may then be illustrated by: *PhlA*>>transporter1>> *rpmD* >>transporter2.

[0030] Thus, in some embodiments, the LAB comprises, in a single strain, several useful features. In one embodiment, the LAB is *Lactococcus lactis*, comprising:

- (A) a chromosomally integrated promoter>>secretion signal>>mucin and cell adherence fusion protein;
- (B) one or more of a chromosomally-integrated promoter >> secretion signal>>therapeutic protein;
- (C) a combination of mutations and insertions to promote trehalose accumulation, which enhances LAB survivability against bile salts and drying. The mutations are selected from
  - (i) chromosomally-integrated trehalose transporter(s), such as *PhlA*>>transporter 1>> intergenic region>>transporter 2, such as *llmg\_0453* and/or *llmg\_0454*, for uptake of trehalose;

- (ii) chromosomally-integrated Trehalose-6-phosphate phosphatase gene (*otsB*; Gene ID: 1036914) positioned downstream of *usp45* (Gene ID: 4797218) to facilitate conversion of trehalose-6-phosphate to trehalose;
- (iii) inactivated (*e.g.* through gene deletion) Trehalose-6-phosphate phosphorylase gene (*trePP*; Gene ID: 4797140); and
- (iv) inactivated cellobiose-specific PTS system IIC component (Gene ID: 4796893), *ptcC*, (*e.g.* tga at codon position 30 of 446; tga30).

The LAB may also contain an auxotrophic mutation for biological containment, such as *thyA*. One or more of these functions (*i.e.* mucin and cell adherence, therapeutic protein, trehalose accumulation) may be in a polycistronic operon, wherein each gene may be separated by an intergenic region.

[0031] In one embodiment, the LAB is *Lactococcus lactis*, with

- (A) *thyA* mutation, for biological containment
- (B) a chromosomally integrated *PthyA>>SSusp45-hTFF3-cmbA* to export and link to the cell wall a mucus and cell binding fusion protein.
- (C) a chromosomally-integrated *PgapB>>gapB>>intergenic region (such as rpmD)>>SSusp45>>pal*, to secrete mature PAL from LAB;
- (D) chromosomally-integrated trehalose transporter(s), such as *PhlIA>>transporter 1>>intergenic region>>transporter 2*, such as *llmg\_0453* and/or *llmg\_0454*, for uptake of trehalose;
- (E) inactivated (*e.g.* through gene deletion) Trehalose-6-phosphate phosphorylase gene (*trePP*; Gene ID: 4797140);
- (F) chromosomally integrated Trehalose-6-phosphate phosphatase gene (*otsB*; Gene ID: 1036914) (positioned downstream of *usp45* (Gene ID: 4797218) to facilitate conversion of trehalose-6-phosphate to trehalose; and
- (G) inactivated cellobiose-specific PTS system IIC component (Gene ID: 4796893), *ptcC*, (*e.g.* tga at codon position 30 of 446; tga30).

[0032] In some examples according to any of the above embodiments, the microorganism (*e.g.*, bacterium) has an increased gastro-intestinal (GI) transit time when compared to a

corresponding microorganism (*e.g.*, bacterium) not comprising the described genetic modification, *i.e.*, not comprising said exogenous nucleic acid encoding the fusion protein or not comprising said fusion protein. In some examples, the GI transit time is increased by at least about 10 %, at least about 30 %, at least about 50 %, at least about 80 %, or at least about 100 % (to about 2×). In other examples, the GI transit time is increased from at least about 10 % to about 500 %, from at least about 20% to about 400%, from at least about 20% to about 300%, from at least about 20% to about 300%, or from at least about 30% to about 300%.

[0033] In some examples according to any of the above embodiments, the microorganism (*e.g.*, bacterium) exhibits increased *in vitro* mucin-binding capability when compared to a corresponding microorganism (*e.g.*, bacterium) not genetically modified as described herein, *i.e.*, not comprising an exogenous nucleic acid encoding a fusion protein or not comprising a fusion protein. In some examples, the *in vitro* mucin-binding capability is increased by at least about 20 %, at least about 30%, at least about 40%, at least about 50 %, at least about 60%, at least about 70%, at least about 80 %, at least about 90% or at least about 100 % (to about 2×). In other examples, the mucin-binding capability is increased from at least about 10% to about 500%, from at least about 10% to about 400%, from at least about 10% to about 300%, from at least about 10% to about 200%, from at least about 20 % to about 200 %, from at least about 20% to about 300 %, or from at least about 20% to about 500 %.

[0034] Mucin-binding capabilities can be measured in accordance with any art-recognized method, *e.g.*, those described herein. In some examples, *in vitro* mucin-binding capability is measured by contacting and binding the microorganism (*e.g.*, a bacterium) to immobilized mucins (*e.g.*, mucins from porcine stomach), and measuring the number of microbial cells (*e.g.*, bacterial cells) bound to the mucin, *e.g.*, by detecting light absorbance at an appropriate wavelength, *e.g.*, at 405 nm (OD<sub>405</sub>); or by staining the bound microbial cells (*e.g.*, bound bacterial cells) with a dye (*e.g.*, crystal violet) and subsequently detecting light absorbance at a wavelength appropriate for the employed dye. For example, if crystal violet is used to stain bacterial cells bound to the mucin, light absorbance may be measured at 595 nm (OD<sub>595</sub>).

[0035] In some examples according to any of the above embodiments, the microorganism (*e.g.*, bacterium) exhibits increased *in vitro* Caco-2 cell-binding capability when compared to a corresponding microorganism (*e.g.*, bacterium) without the described genetic modification,

*i.e.*, not comprising an exogenous nucleic acid encoding the fusion protein, or not comprising the fusion protein. In some examples, the *in vitro* Caco-2 binding capability is increased by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 80%, at least about 100% (to about 2×), at least about 200%, at least about 300%, or at least about 400% (about 5×). In other examples, the *in vitro* Caco-2 binding capability is increased from at least about 10 % to about 200%, from at least about 10% to about 300%, from at least about 10% to about 400%, or from at least about 10% to about 500%. Caco-2 cell binding capability can be measured in accordance with any art-recognized method, such as those disclosed herein. For example, Caco-2 binding capability is measured by: (i) contacting the microorganism (*e.g.*, bacterium), *e.g.*, a culture of the microorganism (*e.g.*, bacterium) with Caco-2 cells; (ii) washing the Caco-2 cells to remove unbound microbial (*e.g.*, bacterial cells); (iii) detaching the microbial cells (*e.g.*, bacterial cells) that are bound to the Caco-2 cells; and (iv) determining the number of detached bacterial cells (*i.e.*, titering the detached cells), *e.g.*, as described herein.

[0036] In some examples according to any of the above embodiments, the microorganism (*e.g.*, bacterium) exhibits increased adherence to intestinal mucosa when compared to a corresponding microorganism (*e.g.*, bacterium) without the described genetic modification, *i.e.*, not comprising an exogenous nucleic acid encoding the fusion protein or not comprising the fusion protein. In some examples, adherence of the microorganism (*e.g.*, bacterium) to intestinal mucosa is increased from at least about 10% to about 100% (to about 2×), from at least about 10% to about 200%, from at least about 10% to about 400%, or from at least about 10% to about 500%.

[0037] The present disclosure further provides a composition comprising a microorganism (*e.g.*, a bacterium) of the present disclosure, *e.g.*, a microorganism (*e.g.*, bacterium) as described in any of the above embodiments. For example, the present disclosure provides a composition comprising a microorganism (*e.g.*, a bacterium) comprising a fusion protein (*e.g.*, anchored in a cell-wall of the microorganism, *e.g.*, bacterium), wherein the fusion protein comprises a TFF polypeptide and a CmbA polypeptide. In some examples, the microorganism (*e.g.*, bacterium) includes an exogenous nucleic acid comprising a secretion leader sequence (*e.g.*, SSusp45), a sequence encoding the TFF polypeptide, and a sequence encoding the CmbA polypeptide. In some examples, the secretion leader sequence encodes a secretion signal peptide, which is cleaved from the

fusion protein, *e.g.*, when the fusion protein is passing the cytoplasmic membrane of the bacterium.

[0038] The present disclosure further provides a pharmaceutical composition comprising a microorganism (*e.g.*, a bacterium) of the present disclosure and a pharmaceutically acceptable carrier. For example, the pharmaceutical composition contains a microorganism (*e.g.*, a bacterium) as described in any of the above embodiments.

[0039] The present disclosure further provides a microorganism (*e.g.*, bacterium) of the present disclosure, or a composition (*e.g.*, a pharmaceutical composition) of the present disclosure for use in the treatment of a disease. The present disclosure further provides a microorganism (*e.g.*, a bacterium) or a composition (*e.g.*, a pharmaceutical composition) for use in the preparation of a medicament for the prevention or treatment of a disease. In some examples according to any of these embodiments, the disease is selected from an autoimmune disease, an allergy, a nutritional or metabolic disease, a gastro-intestinal disease, and a genetic disorder. Further diseases that can be treated using the microorganisms (*e.g.*, bacteria) and compositions of the present disclosure are described herein.

[0040] The present disclosure further provides an isolated nucleic acid encoding a fusion protein, said nucleic acid comprising: (i) a sequence encoding a mucin-binding polypeptide, such as a TFF polypeptide (*e.g.*, TFF1, TFF2, or TFF3) or a MucBP polypeptide; and (ii) a sequence encoding a cell-adherence polypeptide, such as a CmbA polypeptide, a Mub polypeptide, a MapA polypeptide, or a MbpL polypeptide. In some examples according to these embodiments, the cell-adherence polypeptide is a CmbA polypeptide. Accordingly, the present disclosure provides an isolated nucleic acid encoding a fusion protein, wherein the nucleic acid contains (i) a sequence encoding a TFF polypeptide, such as TFF1 (*e.g.*, human TFF1), TFF2 (*e.g.*, human TFF2), or TFF3 (*e.g.*, human TFF3), and (ii) a sequence encoding a CmbA polypeptide.

[0041] The present disclosure further provides a plasmid comprising the isolated nucleic acid of the present disclosure, *e.g.*, an isolated nucleic acid in accordance with any of the above embodiments.

[0042] The present disclosure further provides a microbial (*e.g.*, bacterial) host cell comprising an isolated nucleic acid of the present disclosure or a plasmid of the present disclosure.

[0043] The present disclosure further provides a kit comprising (1) a microorganism (*e.g.*, a bacterium), a composition, a pharmaceutical composition, or a unit dosage form of the present disclosure; and (2) instructions for administering the microorganism (*e.g.*, bacterium), the composition, the pharmaceutical composition, or the unit dosage form to a mammal, such as an animal or human subject or patient.

## Methods

[0044] The present disclosure further provides methods for the treatment of a disease in a subject in need thereof. Exemplary methods include: administering to the subject a therapeutically effective amount of a microorganism (*e.g.*, a bacterium), a composition, or a pharmaceutical composition of the present disclosure. In some examples according to these embodiments, the microorganism (*e.g.*, bacterium) comprises an exogenous nucleic acid encoding a therapeutic polypeptide as described herein. Exemplary diseases that can be treated using such microorganisms (*e.g.*, bacteria) and compositions of the present disclosure are described herein. In some examples, the disease is an autoimmune disease, an allergy, a nutritional or a metabolic disease, a gastro-intestinal disease, a genetic disorder, or any combinations thereof. In some examples according to any of the above embodiments, the disease is an autoimmune disease, such as type-1 diabetes (T1D). In other examples, the disease is a metabolic disease, such as phenylketonuria (PKU). In other examples, the disease is a gastro-intestinal disease, such as celiac disease, or inflammatory bowel disease (IBD), *e.g.*, Crohn's disease or ulcerative colitis. In further examples, the disease is growth retardation.

[0045] In some embodiments, the disease is phenylketonuria (PKU). In some examples according to this embodiment, the microorganism (*e.g.*, bacterium) comprises an exogenous nucleic acid encoding a polypeptide, *e.g.*, an enzyme that is capable of degrading phenylalanine (Phe), *e.g.*, in the GI tract, *e.g.*, prior to absorption of the Phe by the subject to which the microorganism is being administered. In some examples according to these embodiments, the microorganism (*e.g.*, bacterium) comprises an exogenous nucleic acid encoding a phenylalanine ammonia lyase (PAL), an enzyme that converts Phe to cinnamic acid. Thus, the present disclosure provides methods for the treatment of PKU in a subject in need thereof. Exemplary methods include: administering to the subject a therapeutically effective amount of a microorganism (*e.g.*, a bacterium), a composition, or a pharmaceutical composition of the present disclosure, wherein the microorganism (*e.g.*, bacterium) comprises

an exogenous nucleic acid encoding PAL. Inhibition of Phe absorption and treatment of PKU may be analyzed using a mouse PKU model, *e.g.*, utilizing (enu2/2) mice (*see, e.g.*, Sarkissian, C. N. *et al.*, *Proc. Natl. Acad. Sci. USA* 1999, 96: 2339-2344), or using a rat model (*see, e.g.*, Chang *et al.*, *Artif. Cells Blood Substit. Immobil. Biotechnol.* 1995, 23(1):1-21).

[0046] The present disclosure further provides methods for preparing a genetically modified microorganism (*e.g.*, bacterium). Exemplary methods include: contacting an exogenous nucleic acid encoding a fusion protein with a microorganism (*e.g.*, bacterium), wherein the exogenous nucleic acid encoding the fusion protein comprises a sequence encoding a cell-adherence polypeptide, such as a CmbA polypeptide, a Mub polypeptide, a MapA polypeptide, or a MbpL polypeptide. In some examples, the cell-adherence polypeptide is CmbA, *e.g.*, *Lactobacillus reuteri* CmbA. In some examples, carrying out the above method (*i.e.*, contacting the microorganism with the exogenous nucleic acid) results in a microorganism (*e.g.*, bacterium) containing the exogenous nucleic acid encoding the fusion protein. In other examples, contacting the microorganism (*e.g.*, bacterium) with the exogenous nucleic acid results in a microorganism (*e.g.*, bacterium) containing the exogenous nucleic acid encoding the fusion protein, and thereby expressing the fusion protein encoded by the exogenous nucleic acid. In some examples, the method further includes culturing the microorganism (*e.g.*, bacterium) and expressing the fusion protein in the microorganism (*e.g.*, bacterium). In some examples, the contacting occurs under conditions sufficient for said bacterium to internalize the exogenous nucleic acid. In some examples according to any of these embodiments, the exogenous nucleic acid is located on a plasmid. In some examples according to these embodiments, the exogenous nucleic acid is integrated into the chromosome of the bacterium.

[0047] In some examples according to any of the above embodiments, the exogenous nucleic acid encoding the fusion protein further comprises a sequence encoding a mucin-binding polypeptide, such as a trefoil factor (TFF) polypeptide or a MucBP polypeptide. In some examples, the exogenous nucleic acid encoding the fusion protein comprises a sequence encoding CmbA and a sequence encoding a TFF polypeptide. Accordingly, the present disclosure provides a method for preparing a genetically modified microorganism (*e.g.*, bacterium) comprising: contacting an exogenous nucleic acid encoding a fusion protein with a microorganism (*e.g.*, bacterium), wherein the exogenous nucleic acid encoding the fusion

protein comprises (i) a sequence encoding a TFF polypeptide (*e.g.*, TFF1, TFF2, or TFF3) and (ii) a sequence encoding CmbA. In some examples according to any of these embodiments, the exogenous nucleic acid encoding the fusion protein is chromosomally integrated (*e.g.*, integrated into the chromosome of a bacterium), *e.g.*, by using homologous recombination. In accordance with this embodiment, the method can further include forming a plasmid (*i.e.*, an integration plasmid) comprising the exogenous nucleic acid encoding the fusion protein.

[0048] In one example according to any of the above embodiments, the method further includes contacting the microorganism (*e.g.*, bacterium) with an exogenous nucleic acid encoding a therapeutic polypeptide, *e.g.*, prior to or subsequent to contacting the microorganism (*e.g.*, bacterium) with an exogenous nucleic acid encoding the fusion protein.

[0049] In some examples according to any of the above embodiments, the genetically modified microorganism (*e.g.*, bacterium) prepared by the above methods exhibits increased muco- and/or cell-adhesive properties as described herein when compared to a corresponding microorganism (*e.g.*, bacterium) not modified according to the instant method, *i.e.*, not comprising an exogenous nucleic acid encoding the fusion protein.

[0050] In related embodiments, the adhesion to mucus and/or cells is specific to types of mucus and/or cells. As a result of preferential binding to specific receptors found in specific cells or mucus, the bacterium may be localized to specific sites. In such a way, it is possible to ensure localization of the bacterium to the site where delivery of specific molecules is most effective. In some embodiments that location may be the mucosae (intestine, oral cavity, eye, ear, urogenital). In some embodiments that location may be the small bowel, in some embodiments that location may be the upper small bowel.

[0051] In some examples, the method further includes combining a culture of the genetically modified bacterium with at least one cryopreserving agent to form a bacterial mixture. The method may further include drying (*e.g.*, freeze-drying or spray drying) the bacterial mixture to form a dried (*e.g.*, freeze-dried) composition. The method can further include combining the genetically modified bacterium, or the dried composition (*e.g.*, freeze-dried composition) with a pharmaceutically acceptable carrier to form a pharmaceutical composition. The method may further include formulating the genetically modified bacterium, the dried (*e.g.*, freeze-dried) composition, or the pharmaceutical composition into a pharmaceutical unit dosage form, such as a tablet, capsule, or granule.

[0052] The current disclosure further provides a unit dosage form comprising at least one microorganism (*e.g.*, a bacterium), a composition, or a pharmaceutical composition of the present disclosure. In some examples, such unit dosage form is an oral dosage form. In other examples according to these embodiments, the unit dosage form is a capsule (*e.g.*, a capsule  
5 containing a powder or containing micro-pellets or micro-granules), a tablet, a granule, a sachet, or a packaged liquid, *e.g.*, suspension. In other embodiments, the unit dosage form is a metered aerosol dose, or a suppository.

[0053] In some embodiments, the microorganism (*e.g.*, the non-pathogenic Gram-positive bacterium) contained in the dosage form is in a dry-powder form or a compacted version  
10 thereof.

[0054] The current disclosure further provides a unit dosage form comprising from about  $1 \times 10^4$  to about  $1 \times 10^{12}$  colony-forming units (cfu) of a microorganism of the present disclosure, *e.g.*, a non-pathogenic microorganism (*e.g.*, a non-pathogenic Gram-positive bacterium). In some embodiments, the unit dosage form comprises from about  $1 \times 10^6$  to  
15 about  $1 \times 10^{12}$  colony-forming units (cfu) of the microorganism (*e.g.*, the non-pathogenic Gram-positive bacterium). In other embodiments, the unit dosage form comprises from about  $1 \times 10^9$  to about  $1 \times 10^{12}$  colony-forming units (cfu) of the microorganism (*e.g.*, the non-pathogenic Gram-positive bacterium).

[0055] In some embodiments in any of the above methods, the microorganism (*e.g.*,  
20 bacterium) is administered to a subject orally. For example, the microorganism (*e.g.*, bacterium) is administered to the subject in the form of a pharmaceutical composition for oral administration (*e.g.*, a capsule, tablet, granule, suspension or liquid) comprising the microorganism (*e.g.*, bacterium) and a pharmaceutically acceptable carrier. In other examples, the microorganism (*e.g.*, bacterium) is administered to the subject in the form of a  
25 food product, or is added to a food (*e.g.*, a drink). In other examples, the microorganism (*e.g.*, bacterium) is administered to the subject in the form of a dietary supplement. In yet other examples, the microorganism (*e.g.*, bacterium) is administered to the subject in the form of a suppository product. In some examples, the compositions of the present disclosure are adapted for mucosal delivery of the polypeptides, which are expressed by the microorganism  
30 (*e.g.*, bacterium). For example, compositions may be formulated for efficient release of a therapeutic polypeptide in the intestinal tract of the subject.

[0056] The current disclosure further provides a genetically modified bacterium, a composition, a pharmaceutical composition, or a unit dosage form prepared by a method in accordance with any of the above embodiments.

[0057] The present disclosure further provides methods for enhancing growth in a mammal. Exemplary methods include administering to the mammal an effective amount of a microorganism (*e.g.*, bacterium), a composition, a pharmaceutical composition, or a unit dosage form of the present disclosure. In some examples, the mammal is a human, a farm animal (*e.g.*, a pig, a cow, a goat, or a sheep), a dog, a cat, or other domestic animal. In some examples, the microorganism (*e.g.*, bacterium), the composition, pharmaceutical composition, or the unit dosage form is formulated for administration to the mammal, *e.g.*, is formulated for oral administration. In some examples, the microorganism (*e.g.*, bacterium) employed in this method, contains an exogenous nucleic acid encoding a growth factor or growth hormone. In some examples according to this embodiment, the growth factor or growth hormone is constitutively expressed in the microorganism (*e.g.*, bacterium). In some examples, the growth factor is EGF. In other examples, the mammal is a pig and the EGF is porcine EGF.

[0058] The present disclosure further provides a method for increasing binding of a microorganism (*e.g.*, a bacterium) to intestinal mucosa (*e.g.*, as measured by *in vitro* binding to a mucin preparation). Exemplary methods include contacting the microorganism (*e.g.*, bacterium) with an exogenous nucleic acid encoding a fusion protein, wherein the exogenous nucleic acid encoding a fusion protein comprises a sequence encoding a CmbA polypeptide; and expressing the exogenous nucleic acid encoding a fusion protein in the microorganism (*e.g.*, bacterium). In some examples, the exogenous nucleic acid encoding a fusion protein further comprises a sequence encoding a mucin-binding polypeptide, such as a TFF polypeptide. In some examples, expression of the exogenous nucleic acid encoding a fusion protein by the microorganism (*e.g.*, bacterium) produces a fusion protein comprising the TFF polypeptide and the CmbA polypeptide.

[0059] In some examples according to any of the above described compositions and methods, the microorganism is a non-pathogenic microorganism, *e.g.*, any microorganism safe for consumption by a mammalian subject. In some embodiments, the microorganism in the above compositions and methods is yeast. The yeast may be selected from *Saccharomyces* species, *Hansenula* species, *Kluyveromyces* species, *Schizosaccharomyces*

species, *Zygosaccharomyces* species, *Pichia* species, *Monascus* species, *Geothchum* species, and *Yarrowia* species. In some examples, the yeast is *Saccharomyces cerevisiae*.

[0060] In other embodiments in the above compositions and methods, the non-pathogenic microorganism is a non-pathogenic bacterium. In some examples according to this embodiment, the non-pathogenic bacterium is a Gram-positive bacterium. In other examples, the Gram-positive bacterium is a lactic acid fermenting bacterium (LAB), e.g., is selected from *Lactococcus* species (e.g., *Lactococcus lactis*), *Lactobacillus* species, and *Bifidobacterium* species. In other examples, the non-pathogenic bacterium is a *Streptococcus* species or an *Enterococcus* species. Additional bacterial species are disclosed herein.

#### 10 BRIEF DESCRIPTION OF THE DRAWINGS

[0061] FIG. 1 is a plasmid map of *PthyA*>>SSusp45-htff3-CmbA expression/*thyA* integration vector pAGX2005. Abbreviations: *thyA*5', 5' flanking region of the thymidine synthase A (*thyA*) gene of MG1363; *PthyA*, *thyA* promoter of MG1363; SSups45, gene that encodes for the secretion signal of MG1363 protein usp45; hTFF3, gene that encodes for human trefoil factor 3 with optimized codon usage for *L. lactis*; CmbA, gene that encodes for *Lactobacillus reuteri* ATCC PTA6474 cell and mucus-binding protein A; *thyA*3', 3' flanking region of the *thyA* gene of MG1363; ORI, origin of replication of plasmid pORI19; Em, erythromycin resistance marker.

[0062] FIGS. 2A-B are graphs demonstrating enhanced binding of bacterial (*Lactococcus lactis*) cells to mucins and wherein mucin binding is measured using OD<sub>405</sub> (FIG. 2A) or crystal violet staining (FIG. 2B). pAGX1417: *L. lactis* strain LL108 that harbors an empty plasmid; pAGX1894: *L. lactis* strain LL108 that harbors a plasmid for the expression of hTFF1-SpaX on the bacterial surface; pAGX2005: *L. lactis* strain LL108 that harbors a plasmid for the expression of hTFF3-CmbA on the bacterial surface. Mucin type II = mucin from porcine stomach, bound sialic acid, ~1%; Mucin type III = mucin from porcine stomach, bound sialic acid (0.5-1.5%).

[0063] FIG. 3 demonstrates enhanced adherence of bacterial (*Lactococcus lactis*) cells to Caco-2 cells. pAGX1417: *L. lactis* strain LL108 that harbors an empty plasmid; pAGX1893: *L. lactis* strain LL108 that harbors a plasmid for expressing CmbA on the bacterial surface; pAGX1894: *L. lactis* strain LL108 that harbors a plasmid for expressing hTFF1-spaX on the

bacterial surface; pAGX2005: *L. lactis* strain LL108 that harbors a plasmid for expressing hTFF3-CmbA on the bacterial surface.

[0064] FIG. 4 illustrates an exemplary *SSusp45-hdff3* construct (amino acid and nucleic acid sequences: SEQ ID NO: 8 and SEQ ID NO: 9, respectively).

5 [0065] FIGs. 5A-D are collectively an illustration of an exemplary *SSusp45-hTFF3-CmbA* construct (amino acid and nucleic acid sequences: SEQ ID NO: 10 and SEQ ID NO: 11, respectively).

[0066] FIG. 6 is a graph illustrating enhanced adherence of bacterial (*Lactococcus lactis*) cells to Caco-2 cells, wherein the cells either episomally or constitutively express an exemplary cell adherence polypeptide (CmbA) or an exemplary fusion protein (hTFF3-CmbA). % recovery = % of *L. lactis* cells that were recovered from the mucin coated well after washing compared to the total applied *L. lactis* cells, as determined by colony forming units count. pAGX1893: *L. lactis* strain LL108 that harbors a plasmid for expressing CmbA on the bacterial surface; pAGX2005: *L. lactis* strain LL108 that harbors a plasmid for expressing hTFF3-CmbA on the bacterial surface; sAGX0618: *L. lactis* strain MG1363 constitutively expressing an exemplary therapeutic polypeptide PAL (*PgapB*>>*pal*) and constitutively expressing CmbA on the bacterial surface (*thyA*; *PthyA*>>*cmbA*); sAGX644: *L. lactis* strain MG1363 constitutively expressing an exemplary therapeutic polypeptide PAL (*PgapB*>>*pal*) and constitutively expressing fusion protein hTFF3-CmbA on the bacterial surface, wherein an exogenous nucleic acid encoding the fusion protein is transcriptionally regulated by an endogenous *thyA* promoter at the *thyA* locus (*thyA*; *PthyA*>>*SSusp45-hdff3-mbA*); sAGX660: *L. lactis* strain MG1363 constitutively expressing an exemplary therapeutic polypeptide PAL (*PgapB*>>*pal*) and constitutively expressing the fusion protein hTFF3-CmbA on the bacterial surface, wherein an exogenous nucleic acid encoding the fusion protein is transcriptionally regulated by an *hllA* promoter at the *thyA* locus (*thyA*; *PhllA*>>*SSusp45-hdff3-cmbA*).

[0067] FIG. 7 is a graph illustrating enhanced binding of bacterial (*Lactococcus lactis*) cells to mucins, wherein the cells either episomally or constitutively express an exemplary cell adherence polypeptide (CmbA) or an exemplary fusion protein (hTFF3-CmbA), and wherein mucin binding is measured using OD<sub>405</sub> (FIG. 7A) or crystal violet staining (FIG. 7B) as described herein, e.g., in Example 6. pAGX1893: *L. lactis* strain LL108 that harbors a plasmid for expressing CmbA on the bacterial surface; pAGX2005: *L. lactis* strain LL108

that harbors a plasmid for expressing hTFF3-CmbA on the bacterial surface; sAGX0618: *L. lactis* strain MG1363 constitutively expressing an exemplary therapeutic polypeptide PAL (PgapB>>pal) and constitutively expressing CmbA on the bacterial surface (thyA<sup>-</sup>; PthyA>>cmbA); sAGX644: *L. lactis* strain MG1363 constitutively expressing an exemplary therapeutic polypeptide PAL (PgapB>>pal) and constitutively expressing fusion protein hTFF3-CmbA on the bacterial surface, wherein an exogenous nucleic acid encoding the fusion protein is transcriptionally regulated by an endogenous thyA promoter at the thyA locus (thyA<sup>-</sup>; PthyA>>SSusp45-htff3-cmbA); sAGX660: *L. lactis* strain MG1363 constitutively expressing an exemplary therapeutic polypeptide PAL (PgapB>>pal) and constitutively expressing the fusion protein htff3-CmbA on the bacterial surface, wherein an exogenous nucleic acid encoding the fusion protein is transcriptionally regulated by an hllA promoter at the thyA locus (thyA<sup>-</sup>; PhllA>>SSusp45-htff3-cmbA).

[0068] FIG. 8 is a graph illustrating production of an exemplary therapeutic polypeptide (PAL) by bacterial cells either not expressing a mucoadhesive polypeptide (sAGX0599), expressing the exemplary cell adherence polypeptide CmbA (sAGX0618) on the bacterial surface, or the exemplary fusion protein hTFF3-CmbA on the bacterial surface (sAGX0644 (thyA<sup>-</sup>; PthyA>>SSusp45-htff3-cmbA) and sAGX0660 (thyA<sup>-</sup>; PhllA>>SSusp45-htff3-cmbA)). PAL production in µg per 10<sup>9</sup> cells was found comparable across PAL producing strains.

[0069] FIGs. 9A-D show that oral administration of *L. lactis* expressing PAL lowers blood concentrations of phenylalanine. Negative CTR = no lactis, bolus only; Positive CTR = *L. lactis* strain NZ9000[pAGX1886], nisin induced PAL (AGX No 3151); *L. lactis*-PAL = sAGX0599 (AGX No 2947), secreting PAL; *L. lactis*-PAL + TFF3-CmbA = sAGX0645 mucoadherent strain (AGX No 3290), secreting PAL and with surface TFF3-CmbA, which is cell and mucoadherent. FIGs. 9A-B show the blood levels of Phe and Tyr measured over time following the administration of a bolus of radiolabeled Phe and FIGs. 9C-D show the corresponding AUC. The new constructs are associated with lower levels of blood Phe. The final level of Phe was lowest in mice administered *L. lactis* expressing PAL and the TFF3-CmbA fusion protein.

#### DETAILED DESCRIPTION

[0070] The current disclosure provides microorganisms (e.g., lactic acid bacteria, such as *Lactococcus lactis*) exhibiting enhanced *in vitro* binding to mucins and enhanced *in vitro*

binding to cells, *e.g.*, adherence to Caco-2 cells. In some embodiments, such microorganisms have an increased GI transit time. For example, such microorganism may additionally express a therapeutic polypeptide. In some examples, upon oral administration of such bacteria to a mammalian subject, residence time of the bacteria in the different parts of the GI-tract is increased, and the subject is exposed to the therapeutic polypeptide in the GI tract for a longer period of time. For example, once the bacteria are released, *e.g.* from protective coated capsules in the duodenum, residence time of the bacteria in the jejunum and ileum is extended by surface display/expression of muco and cell-adhesive proteins that bind or interact with the intestinal mucosa. Consequently, bacterial doses may be reduced, microorganisms with lower expression profiles become acceptable for administering therapeutically effective doses, smaller unit dosage forms can be developed, and regimens with less frequent administration can be employed (*e.g.*, increasing patient compliance).

[0071] For example, the present disclosure provides microorganisms containing an exogenous nucleic acid encoding a fusion protein of human trefoil factor 3 (hTFF3) with cell and mucus binding protein A (CmbA) of *Lactobacillus reuteri* at the surface of *Lactococcus lactis*. An exemplary fusion protein is composed of the secretion signal of *Lactococcus lactis* protein usp45 (*see, e.g.*, Van Asseldonk *et al.*, *Mol. Gen. Genet.* 1993, 240:428-434), fused to hTFF3 (*see, e.g.*, Tomasetto *et al.*, *Gastroenterology* 2000, 118(1):70-80) and *Lactobacillus reuteri* CmbA (*e.g.*, without its secretion signal). The SSusp45-hTFF3-CmbA fusion protein is secreted by way of the usp45 secretion signal and the secretion signal peptide is cleaved when the hTFF3-CmbA fusion protein passes the *Lactococcus lactis* cytoplasmic membrane. The external part of CmbA can bind intestinal epithelial cells. By fusing hTFF3 to the CmbA protein, an additional mucus binding unit is added. Expression and surface display of hTFF3-CmbA enabled increased adherence to the intestinal mucosa and resulted in a slower GI-transit time of the modified *Lactococcus lactis* cells.

### Definitions

[0072] As used in the specification and claims, the singular forms “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a cell” includes a plurality of cells, including mixtures thereof. Similarly, use of “a compound” for treatment or preparation of medicaments as described herein contemplates using one or more compounds of this invention for such treatment or preparation unless the context clearly dictates otherwise.

- [0073] The term “about” in relation to a reference numerical value, and its grammatical equivalents as used herein, can include the reference numerical value itself and a range of values plus or minus 10% from that reference numerical value. For example, the term “about 10” includes 10 and any amounts from and including 9 to 11. In some cases, the term “about” in relation to a reference numerical value can also include a range of values plus or minus 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% from that reference numerical value. In some embodiments, “about” in connection with a number or range measured by a particular method indicates that the given numerical value includes values determined by the variability of that method.
- [0074] As used herein, the term “comprising” is intended to mean that the compositions and methods include the recited elements, but not excluding others. “Consisting essentially of” when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. “Consisting of” shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.
- [0075] The percentage identity of polypeptide sequences can be calculated using commercially available algorithms which compare a reference sequence with a query sequence. In some embodiments, polypeptides are 70%, at least 70%, 75%, at least 75%, 80%, at least 80%, 85%, at least 85%, 90%, at least 90%, 92%, at least 92%, 95%, at least 95%, 97%, at least 97%, 98%, at least 98%, 99%, or at least 99% or 100% identical to a reference polypeptide, or a fragment thereof (*e.g.*, as measured by BLASTP or CLUSTAL, or other alignment software) using default parameters. Similarly, nucleic acids can also be described with reference to a starting nucleic acid, *e.g.*, they can be 50%, at least 50%, 60%, at least 60%, 70%, at least 70%, 75%, at least 75%, 80%, at least 80%, 85%, at least 85%, 90%, at least 90%, 95%, at least 95%, 97%, at least 97%, 98%, at least 98%, 99%, at least 99%, or 100% identical to a reference nucleic acid or a fragment thereof (*e.g.*, as measured by BLASTN or CLUSTAL, or other alignment software using default parameters). When one molecule is said to have a certain percentage of sequence identity with a larger molecule, it

means that when the two molecules are optimally aligned, the percentage of residues in the smaller molecule finds a match residue in the larger molecule in accordance with the order by which the two molecules are optimally aligned, and the “%” (percent) identity is calculated in accord with the length of the smaller molecule.

- 5 [0076] It is to be understood that the expression of a foreign protein in a bacterium typically requires modifications. These include modification of the nucleic acid to remove of introns and other eukaryotic nucleic acid motifs that are not recognized by bacteria, and to optimize codon usage to the host.

- [0077] Likewise, proteins are modified to remove motifs that are necessary for proper  
10 processing in the natural host but are not recognized by the bacterial host, such as secretion signals from eukaryotes or other species of bacteria. Thus, when reference is made to a foreign protein being expressed in bacteria, the person of ordinary skill would understand that to refer to the mature form. For example, human IL-10 is translated in a human cell with a secretion leader sequence that is not present in the mature IL-10 secreted from the cell. The  
15 eukaryotic secretion leader sequence is nonfunctional in bacteria. Accordingly, *L. lactis* further comprises nucleic acid expressing “IL-10”, it contains the mature IL-10 protein.

- [0078] As used herein, the term “expressing” a gene or polypeptide or “producing” a polypeptide (*e.g.*, PAL, or an IL-2 polypeptide or T1D-specific antigen polypeptide) is meant to include “capable of expressing” and “capable of producing,” respectively. For example, a  
20 microorganism, which contains an exogenous nucleic acid can, under sufficient conditions (*e.g.*, sufficient hydration and/or in the presence of nutrients), produce a polypeptide encoded by the exogenous nucleic acid). However, the microorganism may not always actively produce the encoded polypeptide. The microorganism (*e.g.*, bacterium) may be dried (*e.g.*, freeze-dried), and in that state can be considered dormant (*i.e.*, is not actively producing  
25 polypeptide). However, once the microorganism is subjected to sufficient conditions, *e.g.*, is administered to a subject and is released (*e.g.*, in the gastro-intestinal tract of the subject) it may begin producing polypeptide. Thus, a microorganism “expressing” a gene or polypeptide or “producing” a polypeptide of the current disclosure includes the microorganism in its “dormant” state.

- 30 [0079] As used herein, the term “constitutive” in the context of a promoter (or by extension relating to gene expression or secretion of a polypeptide) refers to a promoter that allows for continual transcription of its associated gene.

[0080] The term “chromosomally integrated” or “integrated into a chromosome” or any variation thereof means that a nucleic acid sequence (*e.g.*, gene; open reading frame; exogenous nucleic acid encoding a polypeptide; promoter; expression cassette; and the like) is located on (integrated into) a microbial (*e.g.*, bacterial) chromosome, *i.e.*, is not located on  
5 an episomal vector, such as a plasmid. In some embodiments, in which the nucleic acid sequence is chromosomally integrated, the polypeptide encoded by such chromosomally integrated nucleic acid is constitutively expressed.

[0081] The terms “secretion leader sequence,” “secretion leader,” and “secretion signal sequence” are used interchangeably herein. The terms are used in accordance with their art  
10 recognized meaning, and generally refer to a nucleic acid sequence, which encodes a “signal peptide” or “secretion signal peptide” causes a polypeptide being expressed by a microorganism and comprising the signal peptide to be secreted by the microorganism, *i.e.*, causes the polypeptide to leave the intracellular space, *e.g.*, be secreted into the surrounding medium, or be anchored in the cell wall with at least a portion of the polypeptide be exposed  
15 to the surrounding medium, *e.g.* on the surface of the microorganism.

#### *Therapeutic Polypeptide*

[0082] The term “therapeutic polypeptide” includes any polypeptide that has a therapeutic, prophylactic, or other biological activity (*e.g.*, in a mammalian subject), or has the potential for eliciting a biological activity. Examples include known biologics (approved  
20 and investigational), and any signal polypeptides, such as hormones and cytokines, and their receptors, agonists and antagonists. A “therapeutic polypeptide” may be modified from a corresponding wild-type polypeptide. In some examples, the therapeutic polypeptide is a cytokine, *e.g.*, an interleukin (IL), such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21 or IL-22.

[0083] In other examples, the therapeutic polypeptide is an antigen. In some examples according to this embodiment, the antigen is an “auto-antigen” or self-antigen. The terms “self-antigen” or “auto-antigen” are used interchangeably herein. The terms are used herein in accordance with the art recognized meaning of self-antigen or auto-antigen, and generally  
25 refer to a polypeptide/protein originating from within a subjects own body (produced by the subject’s own body), wherein the antigen is recognized by the subject’s own immune system, and typically produces antibodies against such antigen. Autoimmune diseases are generally  
30

associated with certain disease-specific self-antigens. For example, in T1D a subject's immune system may produce antibodies against at least one antigen associated with the beta-cell destruction process. In some examples, the auto-antigen is a T1D-specific antigen. Exemplary T1D-specific antigens include proinsulin (PINS), glutamic acid decarboxylase (GAD65), insulinoma-associated protein 2 (IA-2), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), zinc transporter 8 (ZnT8), and any combinations thereof. Clinical T1D may further be associated with additional candidate target molecules expressed by beta-cells such as chromogranin A, (prepro) islet amyloid polypeptide (pIAPP), peripherin, and citrullinated glucose-regulated protein (GRP), and any combinations thereof. Exemplary amino acid sequences and nucleic acid sequences for the above T1D-specific antigens are disclosed, *e.g.*, in provisional patent application 62/350,472 (filed June 15, 2016), the disclosure of which is incorporated herein by reference in its entirety. In some examples, the T1D-specific antigen is PINS, such as wild-type human PINS. *See, e.g.*, CDS contained in accession number NM\_000207.2, or a sequence that is at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity with such wild-type human PINS. Additional exemplary PINS nucleotide sequences are represented by the coding sequences of NCBI accession numbers AY899304 (complete CDS, alternatively spliced); NM\_000207 (transcript variant 1); NM\_001185097 (transcript variant 2); NM\_001185098 (transcript variant 3); NM\_001291897 (transcript variant 4), and partial sequences thereof. Exemplary PINS amino acid sequences include those encoded by any one of the above PINS nucleic acid sequences.

[0084] In other examples, the antigen is an allergen, such as a tree pollen allergen, a weed pollen allergen, a grass pollen allergen, a food allergen, a dust-mite allergen, a mold allergen, an animal dander allergen, or a combination thereof. In some examples, the allergen is a weed pollen allergen, *e.g.*, a ragweed pollen allergen. In other examples, the allergen is a tree pollen allergen, such as a birch pollen allergen or a Japanese cedar pollen allergen. In yet other examples, the allergen is a food allergen, such as a peanut allergen, a milk allergen, an egg allergen, a gluten allergen (gliadin epitope), or a combination thereof. In other examples the therapeutic polypeptide is an antigen and an interleukin, such as IL-2, IL-10, or IL-22.

[0085] In further examples, the therapeutic polypeptide is an antibody or a fragment thereof. For example, the antibody is a single-domain antibody or a nanobody. Exemplary

antibodies include cytokine neutralizing antibodies such as antibodies to IL-4, antibodies to IL-5, antibodies to IL-7, antibodies to IL-13, antibodies to IL-15, as well as anti TNF $\alpha$  antibodies, antibodies to immunoglobulin E (IgE), anti-P40, and any fragments thereof.

[0086] In yet other examples, the therapeutic polypeptide is an enzyme or a fragment (e.g., functional fragment) thereof, e.g., a phenylalanine ammonia lyase (PAL), an amino acid decarboxylase, or a combination thereof. In one example, the therapeutic polypeptide is PAL, or a functional fragment thereof. Exemplary PAL sequences useful for this embodiment are disclosed, e.g., in International Patent Application Publication WO 2014/066945, the disclosure of which is incorporated herein by reference in its entirety. PAL metabolizes phenylalanine and thereby can reduce the level of Phe absorbed from the gut into the blood, and therefore can be used to treat phenylketonuria. Other enzymes may also be used to degrade Phe, such as the aromatic amino acid decarboxylases, such as phenylalanine decarboxylases. In some embodiments, the subject is administered bacteria that expresses and secretes PAL and second phenylalanine degrading enzyme. In another embodiment the subject is administered a bacteria that expresses and secretes PAL, and another bacteria that expresses and secretes a second phenylalanine degrading enzyme.

[0087] In a further example, a bacteria is engineered to enhance Phe uptake and utilization within the cell. Administration of such bacteria can further reduce the amount of Phe absorbed by the patient.

[0088] In further examples, the therapeutic polypeptide is a glucagon-like peptide 1 (GLP-1), glucagon-like peptide 2 (GLP-2), glucagon, exendin-4, or any combination thereof. In other examples, the therapeutic polypeptide is a growth factor, such as an epidermal growth factor (EGF), e.g., human EGF or porcine EGF. In yet other examples, the therapeutic polypeptide is a TFF, such as TFF1, TFF2, TFF3, or a combination thereof.

[0089] In some examples, the therapeutic polypeptide is an immuno-modulating compound. The terms “immuno-modulating compound” or immuno-modulator” are used herein in accordance with their art-recognized meaning. The immuno-modulating compound can be any immuno-modulating compound known to a person skilled in the art. In some embodiments, the immuno-modulating compound is a tolerance inducing compound. Tolerance induction can be obtained, e.g., by inducing regulatory T-cells, or in an indirect way, e.g., by activation of immature dendritic cells to tolerizing dendritic cells and/or inhibiting Th2 immune response inducing expression of “co-stimulation” factors on mature

dendritic cells. Immuno-modulating and immuno-suppressing compounds are known to the person skilled in the art and include, but are not limited to, bacterial metabolites such as spargualin, fungal and streptomycal metabolites such as tacrolimus or ciclosporin, immuno-suppressing cytokines such as IL-4, IL-10, IFN $\alpha$ , TGF $\beta$  (as selective adjuvant for regulatory T-cells) Flt3L, TSLP and Rank-L (as selective tolerogenic DC inducers), antibodies and/or antagonist (*e.g.*, antibodies) such as anti-CD40L, anti-CD25, anti-CD20, anti-IgE, anti-CD3, and proteins, peptides or fusion proteins such as the CTL-41 g or CTLA-4 agonist fusion protein. In some embodiments, the immuno-modulating compound is an immuno-suppressing compound. In other embodiments, the immuno-suppressing compound is an immuno-suppressing cytokine or antibody. In other embodiments, the immuno-suppressing cytokine is a tolerance-enhancing cytokine or antibody. It will be appreciated by the person skilled in the art that the term "immuno-modulating compound" also includes functional homologues thereof. A functional homologue is a molecule having essentially the same or similar function for the intended purposes, but can differ structurally. In some examples, the immuno-modulating compound is an anti-CD3 antibody, or a functional homologue thereof.

[0090] A microorganism of the present disclosure may express more than one, or at least one therapeutic polypeptide. The therapeutic polypeptide may be a combination of any of the above recited therapeutic polypeptides.

#### *Diseases*

[0091] The microorganisms (*e.g.*, bacteria), compositions and methods of the present disclosure can be used to treat or prevent any disease, *e.g.*, those which can be treated by a bioactive polypeptide that is active at the site of the mucosa, *e.g.*, gastro-intestinal mucosa. Exemplary diseases that can be treated or prevented using the methods of the present disclosure include autoimmune disease, allergies, nutritional or metabolic diseases, gastro-intestinal diseases, and genetic disorders, or any combinations thereof.

[0092] The term "nutritional disease" includes any disease that is associated with an insufficiency to process food or nutrients, and may result, *e.g.*, in malnutrition, low weight, or other secondary conditions (such as bloating). A "nutritional disease" may be associated with insufficient production of certain enzymes that process food or food components, such as lipids and carbohydrates (such as lipases, proteases, or sugar degrading enzymes). The term "nutritional disease" includes any metabolic process in an organism that can be enhanced

even if no defined condition or disease is present (“metabolic enhancement”), *e.g.*, certain farm animals, such as pigs, cows, birds, or sheep can be treated to grow faster or accumulate higher weights. In some examples, the “nutritional disease” is an intolerance to certain foods or food components based on insufficient or abnormal metabolism of such food or food component, such as lactose intolerance. The term “nutritional disease” is related to “metabolic disease” or “metabolic disorder” used interchangeably herein. The term “metabolic disease” is used herein in accordance with its art-recognized meaning, and generally refers to any condition, in which abnormal chemical reactions in the body alter a normal metabolic process. In some examples, the metabolic disease is caused by a genetic defect, and may be inherited. Examples of metabolic disorder include acid-base imbalances, metabolic brain diseases, calcium metabolism disorders, DNA repair-deficiency disorders, glucose metabolism disorders, hyperlactatemia, iron metabolism disorders, and lipid metabolism disorders.

[0093] Other examples of nutritional or metabolic diseases include glucose and/or galactose malabsorption, Lesch-Nyhan syndrome, Menkes syndrome, obesity, pancreatic cancer, Prader-Willi syndrome, porphyria, Refsum disease, Tangier disease, Wilson’s disease, Hurler syndrome (*e.g.*, characterized by abnormal bone structure and developmental delay), Niemann-Pick disease (*e.g.*, in which babies develop liver enlargement, difficulty feeding, and nerve damage), Tay-Sachs disease (*e.g.*, characterized by progressive weakness in a young child, progressing to severe nerve damage), Gaucher disease (*e.g.*, characterized by bone pain, enlarged liver, and low platelet counts); Fabry disease (*e.g.*, characterized by pain in the extremities in childhood, with kidney and heart disease and strokes in adulthood), Krabbe disease (*e.g.*, characterized by progressive nerve damage, developmental delay in young children); galactosemia (*e.g.*, characterized by impaired breakdown of the sugar galactose, can lead to jaundice, vomiting, and liver enlargement after breast or formula feeding by a newborn); maple syrup urine disease (*e.g.*, characterized by deficiency of the enzyme BCKD, causes buildup of amino acids in the body); phenylketonuria (PKU), glycogen storage diseases (*e.g.*, characterized by low blood sugar levels, muscle pain, and weakness); mitochondrial disorders, Friedreich ataxia (*e.g.*, characterized by problems related to the protein frataxin, which may cause nerve damage, heart problems, inability to walk), and peroxisomal disorders (*e.g.*, characterized by poor enzyme function inside peroxisomes, which may lead to buildup of toxic metabolites). Exemplary peroxisomal disorders include, *e.g.*, Zellweger syndrome (*e.g.*, characterized by abnormal facial features, enlarged liver, and

nerve damage in infants), and adrenoleukodystrophy (*e.g.*, characterized by symptoms of nerve damage in childhood or early adulthood). Other nutritional or metabolic disorders include metal metabolism disorders (*e.g.*, characterized by protein malfunction and toxic accumulation of metal in the body). Examples include, *e.g.*, Wilson disease (*e.g.*, characterized by accumulation of toxic copper levels in the liver, brain, and other organs), and hemochromatosis (*e.g.*, hereditary hemochromatosis), *e.g.*, in which the intestines absorb excessive iron, which builds up in the liver, pancreas, joints, and heart, causing damage. Further examples of nutritional or metabolic disorders include organic acidemias (such as methylmalonic acidemia and propionic acidemia), urea cycle disorders (such as ornithine transcarbamylase deficiency and citrullinemia). In some example, the nutritional or metabolic disease is phenylketonuria (PKU). In other examples, the nutritional or metabolic disease is a metabolic disorder related to energy dysregulation (*e.g.*, nonalcoholic steatohepatitis).

[0094] In some examples, the disease is an autoimmune disease. Exemplary autoimmune diseases include myocarditis, postmyocardial infarction syndrome, postpericardiotomy syndrome, subacute bacterial endocarditis (SBE), anti-glomerular basement membrane nephritis, interstitial cystitis, lupus nephritis, autoimmune hepatitis, primary biliary cirrhosis (PBC), primary sclerosing cholangitis, antisynthetase syndrome, alopecia areata, autoimmune angioedema, autoimmune progesterone dermatitis, autoimmune urticarial, bullous pemphigoid, cicatricial pemphigoid, dermatitis herpetiformis, discoid lupus erythematosus, epidermolysis bullosa acquisita, erythema nodosum, gestational pemphigoid, hidradenitis suppurativa, lichen planus, lichen sclerosus, linear IgA disease (LAD), morphea, pemphigus vulgaris, pityriasis lichenoides et varioliformis acuta, Mucha-Habermann disease, psoriasis, systemic scleroderma, vitiligo, Addison's disease, autoimmune polyendocrine syndrome (APS) type 1, autoimmune polyendocrine syndrome (APS) type 2, autoimmune polyendocrine syndrome (APS) type 3, autoimmune pancreatitis (AIP), diabetes mellitus type 1, autoimmune thyroiditis, Ord's thyroiditis, Graves' disease, autoimmune oophoritis, endometriosis, autoimmune orchitis, Sjogren's syndrome, autoimmune enteropathy, Coeliac disease, Crohn's disease, microscopic colitis, ulcerative colitis, antiphospholipid syndrome (APS, APLS), aplastic anemia, autoimmune hemolytic anemia, autoimmune lymphoproliferative syndrome, autoimmune neutropenia, autoimmune thrombocytopenic purpura, cold agglutinin disease, essential mixed cryoglobulinemia, Evans syndrome, paroxysmal nocturnal, hemoglobinuria, pernicious anemia, pure red cell aplasia,

thrombocytopenia, adiposis dolorosa, adult-onset Still's disease, ankylosing spondylitis, CREST syndrome, drug-induced lupus, enthesitis-related arthritis, eosinophilic fasciitis, Felty syndrome, IgG4-related disease, juvenile arthritis, Lyme disease (chronic), mixed connective tissue disease (MCTD), palindromic rheumatism, Parry Romberg syndrome, Parsonage-Turner syndrome, psoriatic arthritis, reactive arthritis, relapsing polychondritis, retroperitoneal fibrosis, rheumatic fever, rheumatoid arthritis, sarcoidosis, Schnitzler syndrome, systemic lupus erythematosus (SLE), undifferentiated connective tissue disease (UCTD), dermatomyositis, fibromyalgia, inclusion body myositis, myositis, myasthenia gravis, neuromyotonia, paraneoplastic cerebellar degeneration, polymyositis, acute disseminated encephalomyelitis (ADEM), acute motor axonal neuropathy, anti-N-methyl-D-aspartate (anti-NMDA) receptor encephalitis, balo concentric sclerosis, Bickerstaff's encephalitis, chronic inflammatory demyelinating polyneuropathy, Guillain-Barré syndrome, Hashimoto's encephalopathy, idiopathic inflammatory demyelinating diseases, Lambert-Eaton myasthenic syndrome, multiple sclerosis (MS), pattern II, Oshtoran Syndrome, pediatric autoimmune neuropsychiatric disorder associated with streptococcus (PANDAS), progressive inflammatory neuropathy, restless leg syndrome, stiff person syndrome, Sydenham chorea, transverse myelitis, autoimmune retinopathy, autoimmune uveitis, Cogan syndrome, Graves ophthalmopathy, intermediate uveitis, ligneous conjunctivitis, Mooren's ulcer, neuromyelitis optica, opsoclonus myoclonus syndrome, optic neuritis, scleritis, Susac's syndrome, sympathetic ophthalmia, Tolosa-Hunt syndrome, autoimmune inner ear disease (AIED), Ménière's disease, Behçet's disease, eosinophilic granulomatosis with polyangiitis (EGPA), giant cell arteritis, granulomatosis with polyangiitis (GPA), IgA vasculitis (IgAV), Kawasaki's disease, leukocytoclastic vasculitis, lupus vasculitis, rheumatoid vasculitis, microscopic polyangiitis (MPA), polyarteritis nodosa (PAN), polymyalgia rheumatic, urticarial vasculitis, vasculitis, and primary immune deficiency. In some examples, the autoimmune disease is type-1 diabetes (T1D).

**[0095]** In other examples, the disease is a gastro-intestinal disease, such as short bowel syndrome, celiac disease, or inflammatory bowel disease (IBD), *e.g.*, Crohn's disease or ulcerative colitis.

**[0096]** In some examples, the disease is an inflammatory disease (*e.g.*, Th2 and/or IgE driven inflammation). Exemplary inflammatory diseases include acne vulgaris, asthma, auto-inflammatory diseases, chronic prostatitis, glomerulonephritis, hypersensitivities,

inflammatory bowel diseases (IBD), pelvic inflammatory disease, reperfusion injury, rheumatic fever, rheumatoid arthritis, sarcoidosis, transplant rejection, graft-versus-host disease, vasculitis, hydradenitis suppurativa, diverticulitis, interstitial cystitis. Examples of autoinflammatory diseases include familial Mediterranean fever (FMF),  
5 hyperimmunoglobulinemia D with recurrent fever (HIDS), mevalonic aciduria, mevalonate kinase deficiency, TNF receptor associated periodic syndrome (TRAPS), Muckle-Wells syndrome (urticaria deafness amyloidosis), familial cold urticarial, neonatal onset multisystem inflammatory disease (NOMID), periodic fever, aphthous stomatitis, pharyngitis and adenitis (PFAPA syndrome), Blau syndrome, pyogenic sterile arthritis, pyoderma  
10 gangrenosum, acne (PAPA), deficiency of the interleukin-1-receptor antagonist (DIRA).

[0097] In further examples, the disease is growth retardation. In other examples, the disease is type-2 diabetes (T2D), obesity, or pain (*e.g.*, neuropathic pain).

[0098] In other examples, the disease is an allergy, *e.g.*, an allergy to an allergen selected from a tree pollen allergen, a weed pollen allergen, a grass pollen allergen, a food allergen, a  
15 dust-mite allergen, a mold allergen, an animal dander allergen, or a combination thereof. In some examples, the disease is an allergy to a weed pollen allergen, *e.g.*, a ragweed pollen allergen. In other examples, the disease is an allergy to a tree pollen allergen, such as a birch pollen allergen or a Japanese cedar pollen allergen. In yet other examples, the disease is an allergy to a food allergen, such as a peanut allergen, a milk allergen, an egg allergen, a gluten  
20 allergen (gliadin epitope), or a combination thereof.

### ***Phenylketonuria***

[0099] In some examples, the present disclosure provides methods for the treatment of phenylketonuria (PKU). The term “phenylketonuria” is used herein in accordance with its art-recognized meaning. Phenylketonuria (PKU) is one of the most prevalent disorders of  
25 amino acid metabolism. Genetic defects (deficiency of the enzyme PAH) result in high levels of blood phenylalanine (Phe), which can lead to severe mental retardation if not recognized, and treated early in life. Even with dietary compliance, PKU patients risk cognitive impairment from adolescence onward.

### ***Promoter***

30 [00100] By “promoter” is meant generally a region on a nucleic acid molecule, for example DNA molecule, to which an RNA polymerase binds and initiates transcription. A

- promoter is for example, positioned upstream, *i.e.*, 5', of the sequence the transcription of which it controls. The skilled person will appreciate that the promoter may be associated with additional native regulatory sequences or regions, *e.g.* operators. The precise nature of the regulatory regions needed for expression may vary from organism to organism, but shall
- 5 in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal the initiation of protein synthesis. Such regions will normally include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the Pribnow-box (*cf.* TATA-box), Shine-Dalgarno sequence, and the like.
- 10 [00101] The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences. For example, a promoter is said to be operably linked to a gene, open reading
- 15 frame or coding sequence, if the linkage or connection allows or effects transcription of said gene. In a further example, a 5' and a 3' gene, cistron, open reading frame or coding sequence are said to be operably linked in a polycistronic expression unit, if the linkage or connection allows or effects translation of at least the 3' gene. For example, DNA sequences, such as, *e.g.*, a promoter and an open reading frame, are said to be operably linked if the
- 20 nature of the linkage between the sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter to direct the transcription of the open reading frame, or 25 (3) interfere with the ability of the open reading frame to be transcribed by the promoter region sequence.

#### ***Expression Cassette***

- 25 [00102] The term "expression cassette" or "expression unit" is used in accordance with its generally accepted meaning in the art, and refers to a nucleic acid containing one or more genes and sequences controlling the expression of the one or more genes. Exemplary expression cassettes contain at least one promoter sequence and at least one open reading frame.

*Polycistronic Expression Cassette*

- [00103] The terms “polycistronic expression cassette,” “polycistronic expression unit,” or “polycistronic expression system” are used herein interchangeably and in accordance with their generally accepted meaning in the art. They refer to a nucleic acid sequence wherein the expression of two or more genes is regulated by common regulatory mechanisms, such as promoters, operators, and the like. The term polycistronic expression unit as used herein is synonymous with multicistronic expression unit. Examples of polycistronic expression units are without limitation bicistronic, tricistronic, tetracistronic expression units. Any mRNA comprising two or more, such as 3, 4, 5, 6, 7, 8, 9, 10, or more, open reading frames or coding regions encoding individual expression products such as proteins, polypeptides and/or peptides is encompassed within the term polycistronic. A polycistronic expression cassette includes at least one promoter, and at least two open reading frames controlled by the promoter, wherein an intergenic region is optionally placed between the two open reading frames.
- [00104] In some example, the “polycistronic expression cassette” includes one or more endogenous genes and one or more exogenous genes that are transcriptionally controlled by a promoter which is endogenous to the microorganism (*e.g.*, LAB). In another embodiment, the polycistronic expression unit or system as described herein is transcriptionally controlled by a promoter which is exogenous to the microorganism (*e.g.*, LAB). In a further embodiment, the translationally or transcriptionally coupled one or more endogenous genes and one or more exogenous genes as described herein are transcriptionally controlled by the native promoter of (one of) said one or more endogenous genes. In another embodiment, the polycistronic expression unit is transcriptionally controlled by the native promoter of (one of) said one or more endogenous genes comprised in said polycistronic expression unit. In another embodiment, the polycistronic expression unit is operably linked to a Gram-positive endogenous promoter. In an exemplary embodiment, the promoter may be positioned upstream of, *i.e.*, 5' of the open reading frame(s) to which it is operably linked. In a further embodiment, the promoter is the native promoter of the 5' most, *i.e.*, most upstream, endogenous gene in the polycistronic expression unit. Accordingly, in some examples, the polycistronic expression unit contains an endogenous gene and one or more exogenous genes transcriptionally coupled to the 3' end of said one or more endogenous gene, for example

wherein said one or more exogenous gene(s) is (are) the most 3' gene(s) of the polycistronic expression unit.

[00105] As used herein, the term “translationally coupled” is synonymous with “translationally linked” or “translationally connected”. These terms in essence relate to polycistronic expression cassettes or units. Two or more genes, open reading frames or coding sequences are said to be translationally coupled when common regulatory element(s) such as in particular a common promoter effects the transcription of said two or more genes as one mRNA encoding said two or more genes, open reading frames or coding sequences, which can be subsequently translated into two or more individual polypeptide sequences. The skilled person will appreciate that bacterial operons are naturally occurring polycistronic expression systems or units in which two or more genes are translationally or transcriptionally coupled.

#### *Intergenic Region*

[00106] As used herein, the term “intergenic region” is synonymous with “intergenic linker” or “intergenic spacer”. An intergenic region is defined as a polynucleic acid sequence between adjacent (*i.e.*, located on the same polynucleic acid sequence) genes, open reading frames, cistrons or coding sequences. By extension, the intergenic region can include the stop codon of the 5' gene and/or the start codon of the 3' gene which are linked by said intergenic region. As defined herein, the term intergenic region specifically relates to intergenic regions between adjacent genes in a polycistronic expression unit. For example, an intergenic region as defined herein can be found between adjacent genes in an operon. Accordingly, in an embodiment, the intergenic region as defined herein is an operon intergenic region.

[00107] In some examples, the intergenic region, linker or spacer is selected from intergenic regions preceding, *i.e.*, 5' to, more particularly immediately 5' to, *rplW*, *rpl P*, *rpmD*, *rplB*, *rpsG*, *rpsE* or *rplN* of a Gram-positive bacterium. In some embodiments, the Gram-positive bacterium is a lactic acid bacterium, for example a *Lactococcus* species, *e.g.*, *Lactococcus lactis*, and any subspecies or strain thereof. In an embodiment, said intergenic region encompasses the start codon of *rplW*, *rpl P*, *rpmD*, *rplB*, *rpsG*, *rpsE* or *rplN* and/or the stop codon of the preceding, *i.e.* 5', gene. In some embodiments, the invention relates to a Gram-positive bacterium or a recombinant nucleic acid as described herein, wherein the endogenous gene and the one or more exogenous genes are transcriptionally coupled by

intergenic region or regions active in the Gram-positive bacterium, for example wherein the intergenic region or regions is endogenous to said Gram-positive bacterium, for example, wherein the endogenous intergenic region is selected from intergenic regions preceding *rplW*, *rplP*, *rpmD*, *rplB*, *rpsG*, *rpsE* or *rplN*, *rplM*, *rplE*, and *rplF*.

- 5 [00108] The skilled person will appreciate that if the intergenic region encompasses a 5' stop codon and/or a 3' start codon, these respective codons in some cases are not present in the genes which are linked by said intergenic regions, in order to avoid double start and/or stop codons, which may affect correct translation initiation and/or termination. Methods for identifying intergenic regions are known in the art. By means of further guidance, intergenic regions can for instance be identified based on prediction of operons, and associated promoters and open reading frames, for which software is known and available in the art. Exemplary intergenic regions are described in international patent application publication WO2012/164083, the disclosure of which is incorporated herein by reference in its entirety.

#### *Subject*

- 15 [00109] A "subject" is an organism, which may benefit from being administered a composition of the present disclosure, *e.g.*, according to methods of the present disclosure. The subject may be a mammal ("mammalian subject"). Exemplary mammalian subjects include humans, farm animals (such as cows, pigs, horses, sheep, goats), pets (such as a dogs, cats, and rabbits), and other animals, such as mice, rats, and primates. In some examples, the mammalian subject is a human patient.

#### *Mucosa*

- [00110] The term "mucosa" or "mucous membrane" is used herein in accordance with its art recognized meaning. The "mucosa" can be any mucosa found in the body, such as oral mucosa, rectal mucosa, gastric mucosa, intestinal mucosa, urethral mucosa, vaginal mucosa, 25 ocular mucosa, buccal mucosa, bronchial or pulmonary mucosa, and nasal or olfactory mucosa. Mucosa may also refer to surface mucosa, *e.g.*, those found in fish and amphibia.

- [00111] The term "mucosal delivery" as used herein is used in accordance with its art recognized meaning, *i.e.*, delivery to the mucosa, *e.g.*, via contacting a composition of the present disclosure with a mucosa. Oral mucosal delivery includes buccal, sublingual and gingival routes of delivery. Accordingly, in some embodiments, "mucosal delivery" includes 30 gastric delivery, intestinal delivery, rectal delivery, buccal delivery, pulmonary delivery,

ocular delivery, nasal delivery, vaginal delivery and oral delivery. The person of ordinary skill will understand that oral delivery can affect delivery to distal portions of the gastrointestinal tract.

[00112] The term “mucosal tolerance” refers to the inhibition of specific immune responsiveness to an antigen in a mammalian subject (*e.g.*, a human patient), after the subject has been exposed to the antigen via the mucosal route. In some cases the mucosal tolerance is systemic tolerance. Low dose oral tolerance is oral tolerance induced by low doses of antigens, and is characterized by active immune suppression, mediated by cyclophosphamide sensitive regulatory T-cells that can transfer tolerance to naive hosts. High dose oral tolerance is oral tolerance induced by high doses of antigens, is insensitive to cyclophosphamide treatment, and proceeds to induction of T cell hyporesponsiveness via anergy and/or deletion of antigen specific T-cells. The difference in sensitivity to cyclophosphamide can be used to make a distinction between low dose and high dose tolerance (Strobel *et al.*, 1983). In some cases, the oral tolerance is low dose oral tolerance as described by Mayer and Shao (2004).

### *Mucin*

[00113] The term “mucin” is used herein in accordance with its art-recognized meaning. Mucins are a family of high molecular weight, glycosylated proteins (glycoconjugates) produced by epithelial tissues in humans and animals. Mucins have the ability to form gels, and are a key component of gel-like secretions. Some mucins are membrane-bound due to the presence of a hydrophobic membrane-spanning domain. Most mucins are secreted as principal components of mucus by mucous membranes or are secreted to become a component of saliva. Mucin genes include *MUC1*, *MUC2*, *MUC3A*, *MUC3B*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC6*, *MUC7*, *MUC8*, *MUC12*, *MUC13*, *MUC15*, *MUC16*, *MUC17*, *MUC19*, *MUC20*, and *MUC21*. *MUC2* is secreted mostly in the intestine but also in the airway. Mature mucins are composed of two distinct regions: amino- and carboxy-terminal regions are lightly glycosylated, but rich in cysteines. The cysteine residues participate in establishing disulfide linkages within and among mucin monomers. A large central region formed of multiple tandem repeats of 10 to 80 residue sequences in which up to half of the amino acids are serine or threonine. This area becomes saturated with mostly O-linked oligosaccharides. Overexpression of mucin proteins (*e.g.*, *MUC1*) is associated with many

types of cancer. In the context of this disclosure “mucin” may also mean “mucin preparation” or “mucous or other preparation containing mucins.”

### *Treating*

[00114] The terms “treatment”, “treating”, and the like, as used herein means ameliorating  
5 or alleviating characteristic symptoms or manifestations of a disease or condition, *e.g.*, PKU  
or T1D. For example, treatment of T1D can result in the restoration or induction of antigen-  
specific immune tolerance in the subject. In other examples, treatment means arresting auto-  
immune diabetes, or reversing autoimmune diabetes. As used herein these terms also  
10 encompass, preventing or delaying the onset of a disease or condition or of symptoms  
associated with a disease or condition, including reducing the severity of a disease or  
condition or symptoms associated therewith prior to affliction with said disease or condition.  
Such prevention or reduction prior to affliction refers to administration of the microorganism  
(*e.g.*, bacterium) or composition of the present disclosure to a patient that is not at the time of  
administration afflicted with the disease or condition. “Preventing” also encompasses  
15 preventing the recurrence or relapse-prevention of a disease or condition or of symptoms  
associated therewith, for instance after a period of improvement.

[00115] Treatment of a subject “in need thereof” conveys that the subject has a diseases or  
condition, and the therapeutic method of the invention is performed with the intentional  
purpose of treating the specific disease or condition.

### 20 *Therapeutically Effective Amount*

[00116] As used herein, the term “therapeutically effective amount” refers to an amount of  
a non-pathogenic microorganism or a composition of the present disclosure that will elicit a  
desired therapeutic effect or response when administered according to the desired treatment  
regimen. In some examples, the compounds or compositions are provided in a unit dosage  
25 form, for example a tablet or capsule, which contains an amount of the active component  
equivalent with the therapeutically effective amount when administered once, or multiple  
times per day.

[00117] A person of ordinary skill in the art will appreciate that a therapeutically effective  
amount of a recombinant microorganism, which is required to achieve a desired therapeutic  
30 effect (*e.g.*, for the effective treatment of T1D), will vary, *e.g.*, depending on the nature of the

polypeptide expressed by the microorganism, the route of administration, and the age, weight, and other characteristics of the recipient.

[00118] The amount of secreted polypeptide can be determined based on cfu, determined by state of the art methods such as Q-PCR, or by using ELISA. For example, a particular  
5 microorganism may secrete at least about 1 ng to about 1 µg of active polypeptide per 10<sup>9</sup> cfu. Based thereon, the skilled person can calculate the range of antigen polypeptide secreted at other cfu doses.

[00119] Therapeutically effective amounts may be administered in connection with any dosing regimen as described herein. The daily dose of active polypeptide may be  
10 administered in 1, 2, 3, 4, 5, or 6 portions throughout the day. Further the daily doses may be administered for any number of days, with any number of rest periods between administration periods. For example, a dose of the active agent (*e.g.* interleukin) of from about 0.01 to about 3.0 M IU/day/subject may be administered every other day for a total of 6 weeks. In other examples, PAL is administered at doses ranging from 0.1 to 1000 mg per day, such as doses  
15 of 1-100 mg at each meal.

#### *T1D-Specific Antigen*

[00120] In some embodiments, in any of the above compositions and methods, the T1D-specific antigen is selected from known auto-antigens implemented in T1D, and include proinsulin (PINS); insulin (INS); glutamic acid decarboxylase (GAD) (*e.g.*, GAD65, GAD67,  
20 or GAD2); insulinoma-associated protein 2 (islet antigen-2; IA-2) (also referred to as protein tyrosine phosphatase, receptor type, N (PTPRN), tyrosine phosphatase-like protein, or ICA512), (*see, e.g.*, Long *et al.*, *Diabetes* 2013, 62 (6), 2067-2071); islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), zinc transporter 8 (ZnT8), chromogranin A, (prepro) islet amyloid polypeptide (ppIAPP), peripherin, citrullinated  
25 glucose-regulated protein (*e.g.*, GRP78); *see, e.g.*, Rondas *et al.*, *Diabetes* 2015; 64(2):573-586; and Ye *et al.*, *Diabetes* 2010, 59(1):6-16), and combinations of two or more of these antigens. In other embodiments, in the above compositions and methods, the T1D-specific antigen is PINS, GAD65, or IA-2. In other embodiments, in the above compositions and methods, the T1D-specific antigen is PINS. In various embodiments, the T1D-specific  
30 antigen is encoded by a variant nucleic acid sequence shorter than a full length (*e.g.*, wild-type) gene, as such "trimmed" versions are often more efficiently expressed and/or secreted by the microorganisms used (*e.g.*, *Lactococcus lactis*). While secretion is more efficient,

many “trimmed” versions retain all (or a substantial portion) of their biological activity, *e.g.*, retain sufficient Treg stimulating and/or tolerance-inducing capacities.

### ***Microorganism***

[00121] In some examples according to any of the embodiments presented herein, the  
5 microorganism is a non-pathogenic microorganism, *e.g.*, a non-pathogenic and non-invasive  
bacterium. In other embodiments, the microorganism is a non-pathogenic and non-invasive  
yeast.

[00122] In some embodiments, the microorganism is a yeast strain selected from the group  
consisting of *Saccharomyces* sp., *Hansenula* sp., *Kluyveromyces* sp., *Schizosaccharomyces*  
10 sp., *Zygosaccharomyces* sp., *Pichia* sp., *Monascus* sp., *Geothchum* sp. and *Yarrowia* sp. In  
some embodiments, the yeast is *Saccharomyces cerevisiae*. In other embodiments, the *S.*  
*cerevisiae* is of the subspecies *boulardii*. In one embodiment of the present invention, the  
recombinant yeast host-vector system is a biologically contained system. Biological  
containment is known to the person skilled in the art and can be realized by the introduction  
15 of an auxotrophic mutation, *e.g.*, a suicidal auxotrophic mutation such as the *thyA* mutation,  
or its equivalents. Alternatively, the biological containment can be realized at the level of the  
plasmid carrying the gene encoding the polypeptide, such as, for example, by using an  
unstable episomal construct, which is lost after a few generations. Several levels of  
containment, such as plasmid instability and auxotrophy, can be combined to ensure a high  
20 level of containment, if desired.

[00123] In other embodiments of the present invention, the microorganism is a bacterium,  
such as a non-pathogenic bacterium, *e.g.*, a food grade bacterial strain. In some examples,  
the non-pathogenic bacterium is a Gram-positive bacterium, *e.g.*, a Gram-positive food-grade  
bacterial strain. In some embodiments, the Gram-positive food-grade bacterial strain is a  
25 lactic acid fermenting bacterial strain (*i.e.*, a lactic acid bacterium (LAB)) or a  
*Bifidobacterium*.

[00124] In some embodiments, the lactic acid fermenting bacterial strain is a *Lactococcus*,  
*Lactobacillus* or *Bifidobacterium* species. As used herein, *Lactococcus* or *Lactobacillus* is  
not limited to a particular species or subspecies, but meant to include any of the *Lactococcus*  
30 or *Lactobacillus* species or subspecies. Exemplary *Lactococcus* species include *Lactococcus*  
*garvieae*, *Lactococcus lactis*, *Lactococcus piscium*, *Lactococcus plantarum*, and *Lactococcus*

*raffinolactis*. In some examples, the *Lactococcus lactis* is *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *hordniae*, or *Lactococcus lactis* subsp. *lactis*.

[00125] Exemplary *Lactobacillus* species include *Lactobacillus acetotolerans*, *Lactobacillus acidophilus*, *Lactobacillus agilis*, *Lactobacillus algidus*, *Lactobacillus alimentarius*, *Lactobacillus amylolyticus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus aviarius*, *Lactobacillus aviarius* subsp. *araffinosus*, *Lactobacillus aviarius* subsp. *aviarius*, *Lactobacillus bavaricus*, *Lactobacillus bifermentans*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus bulgaricus*, *Lactobacillus carnis*, *Lactobacillus casei*, *Lactobacillus casei* subsp. *alactosus*, *Lactobacillus casei* subsp. *casei*, *Lactobacillus casei* subsp. *pseudopiantarum*, *Lactobacillus casei* subsp. *rhamnosus*, *Lactobacillus casei* subsp. *tolerans*, *Lactobacillus catenaformis*, *Lactobacillus cellobiosus*, *Lactobacillus collinoides*, *Lactobacillus confusus*, *Lactobacillus coryniformis*, *Lactobacillus coryniformis* subsp. *coryniformis*, *Lactobacillus coryniformis* subsp. *torquens*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus curvatus* subsp. *curvatus*, *Lactobacillus curvatus* subsp. *melibiosus*, *Lactobacillus delbrueckii*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus divergens*, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus fornicalis*, *Lactobacillus fructivorans*, *Lactobacillus fructosus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus graminis*, *Lactobacillus halotolerans*, *Lactobacillus hamsteri*, *Lactobacillus helveticus*, *Lactobacillus heterohiochii*, *Lactobacillus hilgardii*, *Lactobacillus homohiochii*, *Lactobacillus iners*, *Lactobacillus intestinalis*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*, *Lactobacillus kandleri*, *Lactobacillus kefir*, *Lactobacillus kefiranoferiens*, *Lactobacillus kefirgranum*, *Lactobacillus kunkeei*, *Lactobacillus lactis*, *Lactobacillus leichmannii*, *Lactobacillus lindneri*, *Lactobacillus malefermentans*, *Lactobacillus mali*, *Lactobacillus maltaromicus*, *Lactobacillus manihotivorans*, *Lactobacillus minor*, *Lactobacillus minutus*, *Lactobacillus mucosae*, *Lactobacillus murinus*, *Lactobacillus nagelii*, *Lactobacillus oris*, *Lactobacillus panis*, *Lactobacillus parabuchneri*, *Lactobacillus paracasei*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus paracasei* subsp. *tolerans*, *Lactobacillus parakefir*, *Lactobacillus paralimentarius*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus perolens*, *Lactobacillus piscicola*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus rimae*, *Lactobacillus rogosa*, *Lactobacillus ruminis*, *Lactobacillus sakei*, *Lactobacillus sakei* subsp. *camosus*,

*Lactobacillus sakei* subsp. *sakei*, *Lactobacillus salivarius*, *Lactobacillus salivarius* subsp. *salicinius*, *Lactobacillus salivarius* subsp. *salivarius*, *Lactobacillus sanfranciscensis*, *Lactobacillus sharpeae*, *Lactobacillus suebicus*, *Lactobacillus trichodes*, *Lactobacillus uli*, *Lactobacillus vaccinostercus*, *Lactobacillus vaginalis*, *Lactobacillus viridescens*,  
 5 *Lactobacillus vitulinus*, *Lactobacillus xylosus*, *Lactobacillus yamanashiensis*, *Lactobacillus yamanashiensis* subsp. *mali*, *Lactobacillus yamanashiensis* subsp. *Yamanashiensis*, *Lactobacillus zeae*, *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium longum*, and *Bifidobacterium infantis*. In some examples, the LAB is  
 10 *Lactococcus lactis* (LL).

[00126] In further examples, the bacterium is selected from the group consisting of *Enterococcus alcedinis*, *Enterococcus aquimarinus*, *Enterococcus asini*, *Enterococcus avium*, *Enterococcus caccae*, *Enterococcus camelliae*, *Enterococcus canintestini*, *Enterococcus canis*, *Enterococcus casseliflavus*, *Enterococcus cecorum*, *Enterococcus columbae*,  
 15 *Enterococcus devriesei*, *Enterococcus diestrammenae*, *Enterococcus dispar*, *Enterococcus durans*, *Enterococcus eurekaensis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Enterococcus gilvus*, *Enterococcus haemoperoxidus*, *Enterococcus hermanni*, *Enterococcus hirae*, *Enterococcus italicus*, *Enterococcus lactis*, *Enterococcus lemanii*, *Enterococcus malodoratus*, *Enterococcus moraviensis*, *Enterococcus mundtii*,  
 20 *Enterococcus olivae*, *Enterococcus pallens*, *Enterococcus phoeniculicola*, *Enterococcus plantarum*, *Enterococcus pseudoavium*, *Enterococcus quebecensis*, *Enterococcus raffinosus*, *Enterococcus ratti*, *Enterococcus rivorum*, *Enterococcus rotai*, *Enterococcus saccharolyticus*, *Enterococcus silesiacus*, *Enterococcus solitarius*, *Enterococcus sulfureus*, *Enterococcus termitis*, *Enterococcus thailandicus*, *Enterococcus ureasiticus*, *Enterococcus ureilyticus*,  
 25 *Enterococcus viikkiensis*, *Enterococcus villorum*, and *Enterococcus xiangfangensis*,

[00127] In further examples, the bacterium is selected from the group consisting of *Streptococcus agalactiae*, *Streptococcus anginosus*, *Streptococcus bovis*, *Streptococcus canis*, *Streptococcus constellatus*, *Streptococcus dysgalactiae*, *Streptococcus equinus*,  
 30 *Streptococcus iniae*, *Streptococcus intermedius*, *Streptococcus milleri*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus parasanguinis*, *Streptococcus peroris*, *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, *Streptococcus*

*pyogenes*, *Streptococcus ratti*, *Streptococcus salivarius*, *Streptococcus tigurinus*, *Streptococcus thermophilus*, *Streptococcus sanguinis*, *Streptococcus sobrinus*, *Streptococcus suis*, *Streptococcus uberis*, *Streptococcus vestibularis*, *Streptococcus viridans*, and *Streptococcus zooepidemicus*.

5 [00128] In a particular aspect of the present invention, the Gram-positive food grade bacterial strain is *Lactococcus lactis* or any of its subspecies, including *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *hordniae*, and *Lactococcus lactis* subsp. *lactis*. In another aspect of the present invention, the recombinant Gram-positive bacterial strains is a biologically contained system, such as the plasmid free *Lactococcus lactis* strain MG1363,  
10 that lost the ability of normal growth and acid production in milk (Gasson, M.J. (1983) *J. Bacteriol.* 154:1-9); or the threonine- and pyrimidine-auxotroph derivative *L. lactis* strains (Sorensen *et al.* (2000) *Appl. Environ. Microbiol.* 66:1253-1258; Glenting *et al.* (2002) 68:5051-5056).

[00129] In some examples according to any of the above embodiments, the bacterium is  
15 not *E. coli*.

[00130] In one embodiment of the present invention, the recombinant bacterial host-vector system is a biologically contained system. Biological containment is known to the person skilled in the art and can be realized by the introduction of an auxotrophic mutation, *e.g.*, a suicidal auxotrophic mutation such as the *thyA* mutation, or its equivalents. Alternatively, the  
20 biological containment can be realized at the level of the plasmid carrying the gene encoding a polypeptide, such as, for example, by using an unstable episomal construct, which is lost after a few generations. Several levels of containment, such as plasmid instability and auxotrophy, can be combined to ensure a high level of containment, if desired.

#### ***Binding and adherence molecules***

25 [00131] The terms “binding” and “adherence” are largely synonymous herein. Binding/adherence may be assessed through *in vitro* models with mammalian cells and/or biological surfaces such as mucus, fibronectin, collagen or other inanimate surfaces; and *in vivo* through, *e.g.*, measurements of colonization, persistence, or as implied by biological effect.

30 [00132] Such binding/adherence is typically specific to a target molecule, cell, or site. Organisms may produce a wide variety of molecules (often polypeptides, glycoproteins and

carbohydrates) that facilitate binding and adherence to other cells, inanimate objects, and cellular products such as mucus. Such molecules that facilitate binding and adherence have various degrees of specificity to a target molecule, typically another protein, glycoprotein or carbohydrate. The expression of different binding molecules leads to preferential binding to different biological surfaces. For example, the different cells of the GI tract express different surface molecules, and the frequency of specific surface molecules can differ along the GI tract. Thus, a bacterial cell can preferentially bind to a specific host cell, or a specific region of the GI tract. Binding may be associated with increased colonization, delivery of target proteins at the target site, increased GI transit time and more. Conversely, binding to one site in the GI tract may be associated with reduced binding to another GI site.

[00133] The natural variation of bacterial binding proteins can be supplemented by mutation, and by recombination to express motifs from other binding molecules, including from other organisms. Such recombinant binding proteins may be expressed alone, or as a fusion proteins to provide multiple binding specificities in a single molecule.

[00134] For a protein to bind the bacterium to a biological surface, the binding polypeptide is typically exported from the cytoplasm and anchored to the surface of the bacterium. In gram positive bacteria, such as *Lactococcus*, adhesion molecules are typically expressed with (a) an N-terminal secretion signal to direct secretion through the cytoplasmic membrane and (b) a C-terminal anchoring domain that anchors the polypeptide to the cell wall (*i.e.*, a “cell wall anchoring domain”). Without an anchoring domain, the polypeptide is released in the extracellular milieu.

[00135] For example, the trefoil factors (TFF) are secreted by animal cells, bind to mucus, and have a number of biological effects, including healing the mucus membrane. A bacterium can be engineered to secrete TFF into the extracellular milieu to promote healing of the mucus membrane, by recombinantly adding a bacterial secretion signal to TFF. A bacterium can also be engineered to have mucus binding properties, by adding both a secretion and anchor signal to TFF.

#### *Cell-Adherence Polypeptide*

[00136] In some embodiments of the present disclosure, the microorganism (*e.g.*, bacterium) contains an exogenous nucleic acid encoding a fusion protein containing a cell-adherence polypeptide. Any polypeptide exhibiting cell-adherence properties, *e.g.*, binding

- to intestinal cells or cell-lines thereof (*e.g.*, Caco-2, IEC-18, or HT29-MTX cells) are useful in the context of the present disclosure. Cell-adherence capabilities may be measured using art-recognized methods, such as those disclosed herein. In some examples, the cell-adherence polypeptide is selected from cell and mucus-binding protein A (CmbA) (*see, e.g.*, Jensen *et al.*, *Microbiology* 2014, 160(4):671-681), mucus binding protein or mub domain proteins (Mub) (*see, e.g.*, Boekhorst *et al.*, *Microbiology* 2006, 152(1):273-280), mucus adhesion promoting protein (MapA) (*see, e.g.*, Miyoshi *et al.*, *Biosci. Biotechnol. Biochem.* 2006, 70(7):1622-8), lactococcal mucin binding protein (MpbL) (*see, e.g.*, Lukic' *et al.*, *Appl. Environ. Microbiol.* 2012, 78(22):7993-8000). In some examples, the fusion protein may include a cell-wall anchor peptide, such as *Staphylococcus aureus* protein A anchor fragment (SpaX) (*see, e.g.*, Steidler *et al.*, *Appl. Environ. Microbiol.* 1998, 64(1):342-5). All of the above disclosures are incorporated herein by reference in their entirety. In some examples, the cell-adherence polypeptide is a CmbA polypeptide, such as CmbA from *Lactobacillus reuteri*. *See, e.g.*, ATCC PTA6474, *e.g.*, as disclosed in Jensen *et al.*, *supra*.
- 15 [00137] In some examples according to any of the above embodiments, the cell-adherence polypeptide is a CmbA polypeptide having an amino acid sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 1. In other examples according to any of the above embodiments, the cell-adherence polypeptide is a CmbA polypeptide encoded by an exogenous nucleic acid
- 20 sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 2.

#### ***Mucin-Binding Polypeptide***

- [00138] In some embodiments of the present disclosure the microorganism (*e.g.*, bacterium) contains an exogenous nucleic acid encoding a fusion protein containing a mucin-binding polypeptide. Any polypeptide exhibiting mucin-binding properties, *e.g.*, binding to mucin preparations *in vitro*, are useful in the context of the present disclosure. Mucin-binding capabilities may be measured using art-recognized methods, such as those disclosed herein. Exemplary mucin-binding polypeptides include trefoil factor (TFF) polypeptides (*e.g.*, TFF1, TFF2, or TFF3) (*see, e.g.*, Caluwaerts, S. *et al.*, *Oral. Oncol.* 2010, 46:564-570) and MucBP polypeptides (*see, e.g.*, Lukic *et al.*, *Appl. Environ. Microbiol.* 2012, 78(22):7993-8000). In some examples, the current disclosure provides a microorganism (*e.g.*, a bacterium) comprising an exogenous nucleic acid encoding a fusion protein, wherein
- 30

the fusion protein contains a cell-adherence polypeptide (*e.g.*, a CmbA polypeptide) and a mucin-binding polypeptide (*e.g.*, a TFF polypeptide). In some examples, the TFF polypeptide is a human TFF polypeptide (*e.g.*, hTFF1, hTFF2, or hTFF3). In other examples according to any of the above embodiments, the mucin-binding polypeptide is a human TFF3 polypeptide having an amino acid sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 3. In other examples according to any of the above embodiments, the mucin-binding polypeptide is a human TFF3 polypeptide encoded by an exogenous nucleic acid sequence that is at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 4. In some examples, the mucin-binding polypeptide is a fish TFF polypeptide, or an amphibian TFF polypeptide. In yet other examples, the mucin-binding polypeptide includes (or consists of) a trefoil-like domain, such as those disclosed in Fujita *et al.*, *Mol. Reprod. Dev.* 2006, 75(7):1217-1228.

#### Constructs

[00139] In some embodiments the microorganism (*e.g.*, bacterium, such as *Lactococcus lactis*) comprises an expression vector capable of expressing the fusion protein and optionally a therapeutic polypeptide. For example, the fusion protein is exposed on the cell surface under conditions present at the mucosa, *e.g.*, in the gastrointestinal tract. The microorganism (*e.g.*, bacterium) can comprise expression vectors capable of expressing the fusion protein, such that the fusion protein is exposed on the cell surface to a degree sufficient to provide the desired GI retention. One of skill in the art may adjust the amount of microorganisms (*e.g.*, bacterium) provided to the subject to deliver the desired amount of therapeutic polypeptide.

[00140] Usually, the expression system will comprise a genetic construct comprising at least one nucleotide sequence encoding at least one fusion protein, *e.g.*, operably linked to a promoter capable of directing expression of the sequence(s) in the hosting microorganism. Suitably the fusion protein to be expressed can be encoded by a nucleic acid sequence that is adapted to the preferred codon usage of the host. The construct may further contain (all) other suitable element(s), including enhancers, transcription initiation sequences, signal sequences, reporter genes, transcription termination sequences, etc., operable in the selected host, as is known to the person skilled in the art.

[00141] In some examples, the construct is in a form suitable for transformation of the host and/or in a form that can be stably maintained in the host, such as a vector, plasmid or mini-chromosome. Suitable vectors comprising nucleic acid for introduction into microorganisms (*e.g.*, bacteria) can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral (*e.g.*, phage or phagemid), as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press, the relevant disclosure of which is incorporated herein by reference.

[00142] Many known techniques and protocols for the manipulation of nucleic acids, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel *et al.*, eds., John Wiley & Sons, 1992, the relevant disclosure of which is incorporated herein by reference. In one embodiment, the coding sequence for the fusion protein is contained in an operon, *i.e.*, a nucleic acid construct for poly-cistronic expression. In an operon, transcription from the promoter results in a mRNA which comprises more than one coding sequence, each with its own suitably positioned ribosome binding site upstream. Thus, more than one polypeptide can be translated from a single mRNA. Use of an operon enables expression of the fusion protein and a therapeutic polypeptide to be coordinated. Polycistronic expression systems in bacterial host cells are described, *e.g.*, in U.S. Patent Application No. 2014/0105863 to Vanden-Broucke *et al.*, which is incorporated herein by reference in its entirety.

[00143] In an embodiment the present invention relates to stably transfected microorganisms (*e.g.*, bacteria). In some examples, the present disclosure provides microorganisms (*e.g.*, bacteria), in which the exogenous nucleic acid encoding the fusion protein has been integrated into the host cell's chromosome. Techniques for establishing stably transfected microorganisms are known in the art. For instance, the nucleic acid encoding the fusion protein may be cloned into the host's chromosome via homologous recombination. In some examples, an essential gene of the host is disrupted by the homologous recombination event, such as deletion of the gene, one or more amino acid substitutions leading to an inactive form of the protein encoded by the essential gene, or to a frameshift mutation resulting in a truncated form of the protein encoded by the essential gene.

In an embodiment, the essential gene is the *thyA* gene. An exemplary technique is described, *e.g.*, in WO 02/090551, which is incorporated herein by reference in its entirety. The transforming plasmid can be any plasmid, as long as it cannot complement the disrupted essential gene, *e.g.*, *thyA* gene. The plasmid may be self-replicating, may carry one or more  
5 genes of interest, and may carry one or more resistance markers. In some examples, the plasmid is an integrative plasmid (*i.e.*, integration plasmid). Such integrative plasmid may be used to disrupt the essential gene, by causing integration at the locus of the essential gene, *e.g.*, *thyA* site, because of which the function of the essential gene, *e.g.*, the *thyA* gene, is disrupted. In some examples, the essential gene, such as the *thyA* gene, is replaced by double  
10 homologous recombination by a cassette comprising the gene or genes of interest, flanked by targeting sequences that target the insertion to the essential gene, such as the *thyA* target site. It will be appreciated that that these targeting sequences are sufficiently long and sufficiently homologous to enable integration of the gene of interest into the target site.

[00144] The genetic construct encoding the fusion protein may be present in the host cell  
15 extra-chromosomally, *e.g.*, autonomously replicating using an own origin of replication, or may be integrated into the microbial genomic DNA, *e.g.*, bacterial or yeast chromosome, *e.g.*, *Lactococcus* chromosome. In the latter case, a single copy or multiple copies of the nucleic acid may be integrated; the integration may occur at a random site of the chromosome or, as described above, at a predetermined site thereof, such as in the *thyA* locus of *Lactococcus*,  
20 *e.g.*, *Lactococcus lactis*.

[00145] Hence, in some embodiments, the genetic construct encoding the fusion protein may further comprises sequences configured to effect insertion of the genetic construct into the chromosome of a host cell. In some examples, insertion of the genetic construct into particular sites within a microbial genome, *e.g.*, chromosome of a host cell may be facilitated  
25 by homologous recombination. For instance, a genetic construct of the present disclosure may comprise one or more regions of homology to the site of integration within the chromosome, of the host cell. The sequence at the chromosome site may be natural, *i.e.*, as occurring in nature, or may be an exogenous sequence introduced by previous genetic engineering.

30 [00146] In some examples, the region(s) of homology may be at least 50 base pairs (bp), 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1000 bp, or more.

[00147] In one example, two regions of homology may be included, one flanking each side of the relevant expression units present in the genetic construct. Such configuration may advantageously insert the relevant sequences into host cells. Methods for homologous recombination, especially in bacterial hosts, and selecting for recombinants, are generally known in the art.

[00148] Transformation methods of microorganisms are known to the person skilled in the art, such as for instance protoplast transformation and electroporation.

[00149] High degrees of expression can be achieved by using homologous expression and/or secretion signals on the expression vectors present in the microorganism, *e.g.*, *Lactococcus lactis*. Expression signals will be apparent to the person skilled in the art. The expression vector can be optimized for expression depending on the microorganism it is incorporated into. For instance, specific expression vectors that gave sufficient levels of expression in *Lactococcus*, *Lactobacillus lactis*, *casei* and *plantarum* are known. Moreover, systems are known which have been developed for the expression of heterologous antigens in the non-pathogenic, non-colonizing, non-invasive food-grade bacterium *Lactococcus lactis* (see UK patent GB2278358B, which is incorporated herein by reference). In some examples, construct of the present disclosure comprises the multi-copy expression vector described in PCT/NL95/00135 (WO-A-96/32487), in which the nucleotide sequence encoding the fusion protein has been incorporated. Such a construct is particularly suitable for expression of a desired polypeptide in a lactic acid bacterium, in particular in a *Lactobacillus*, at a high level of expression, and also can be used to direct the expressed product to the surface of the bacterial cell. The constructs (*e.g.*, of PCT/NL95/00135) may be characterized in that the nucleic acid sequence encoding the fusion protein is preceded by a 5' non-translated nucleic acid sequence comprising at least the minimal sequence required for ribosome recognition and RNA stabilization. This can be followed by a translation initiation codon which may be (immediately) followed by a fragment of at least 5 codons of the 5' terminal part of the translated nucleic acid sequence of a gene of a lactic acid bacterium or a structural or functional equivalent of the fragment. The fragment may also be controlled by the promoter. The contents of PCT/NL95/00135, including the differing embodiments disclosed therein, and all other documents mentioned in this specification, are incorporated herein by reference. One aspect of the present invention provides a method which permits the high level regulated expression of heterologous genes in the host and the coupling of expression to secretion. In

another embodiment, the T7 bacteriophage RNA polymerase and its cognate promoter are used to develop a powerful expression system according to WO 93/17117, which is incorporated herein by reference. In one embodiment, the expression plasmid is derived from pTI NX.

- 5 [00150] In some embodiments, a promoter employed in accordance with the present disclosure is expressed constitutively in the bacterium. The use of a constitutive promoter avoids the need to supply an inducer or other regulatory signal for expression to take place. For example, the promoter directs expression at a level at which the bacterial host cell remains viable, *i.e.*, retains some metabolic activity, even if growth is not maintained.
- 10 Advantageously, such expression may be at a low level. For example, where the expression product accumulates intracellularly, the level of expression may lead to accumulation of the expression product at less than about 10% of cellular protein, or about or less than about 5%, for example about 1-3%. The promoter may be homologous to the bacterium employed, *i.e.*, one found in that bacterium in nature. For example, a Lactococcal promoter may be used in a
- 15 *Lactococcus*. An exemplary promoter for use in *Lactococcus lactis* (or other *Lactococci*) is "P1" derived from the chromosome of *Lactococcus lactis* (Waterfield, N R, Lepage, R W F, Wilson, P W, *et al.* (1995). "The isolation of lactococcal promoters and their use in investigating bacterial luciferase synthesis in *Lactococcus lactis*" *Gene* 165(1):9-15). Another example of a promoter is the *usp45* promoter. Other useful promoters are described
- 20 in U.S. Patent 8,759,088 to Steidler *et al.*, and in U.S. Patent Application No. 2014/0105863 to Vandembroucke *et al.*, the disclosures of which are incorporated herein by reference in their entirety.

- [00151] The nucleic acid construct or constructs may comprise a secretory signal sequence. Thus, in some embodiments the nucleic acid encoding the fusion protein may
- 25 provide for secretion of the polypeptides, *e.g.*, by appropriately coupling a nucleic acid sequence encoding a signal sequence to the nucleic acid sequence encoding the polypeptide). Ability of a bacterium harboring the nucleic acid to secrete the antigen may be tested *in vitro* in culture conditions which maintain viability of the organism. Exemplary secretory signal sequences include any of those with activity in Gram-positive organisms such as *Bacillus*,
- 30 *Clostridium* and *Lactobacillus*. Such sequences may include the  $\alpha$ -amylase secretion leader of *Bacillus amyloliquefaciens* or the secretion leader of the *Staphylokinase* enzyme secreted by some strains of *Staphylococcus*, which is known to function in both Gram-positive and

Gram-negative hosts (*see* “Gene Expression Using *Bacillus*,” Rapoport (1990) Current Opinion in Biotechnology 1:21-27), or leader sequences from numerous other *Bacillus* enzymes or S-layer proteins (*see* pp 341-344 of Harwood and Cutting, “Molecular Biological Methods for *Bacillus*,” John Wiley & Co. 1990). In one embodiment, said secretion signal is  
5 derived from *usp45* (Van Asseldonk *et al.*, *Mol. Gen. Genet.* 1993, 240:428-434). In some embodiments, the fusion protein is constitutively secreted.

### Formulations and Regimens

[00152] In the methods of the present disclosure, multiple therapeutic polypeptides may be expressed by the same or different microorganisms. For example, (a) PAL and amino acid  
10 decarboxylase; (b) a T1D specific antigen such as PINS and a Treg activating cytokine such as IL-2 or IL-10 (c) a gluten antigen and IL-2 or IL-10, and the like. If expressed in separate organisms, one or preferably both bacteria will express mucin and/or cell-binding factors. When the two polypeptides are expressed by different microorganisms, those may be administered to the subject in the same (*e.g.*, combined) formulation or may be administered  
15 in separate (*e.g.*, different) formulations. Separate formulations may be administered at the same time or at different time points. For example, the use of first and second therapeutic polypeptide producing microorganisms in their respective formulations can be administered to the subject simultaneously or may be administered sequentially, *e.g.*, with a rest period between administrations.

20 [00153] In some embodiments, the first and second therapeutic polypeptide producing microorganisms are administered simultaneously. In some examples, according to these embodiments, the first therapeutic polypeptide microorganism, and the second therapeutic polypeptide microorganism are comprised in the same pharmaceutical formulation, or in more than one pharmaceutical formulation taken at the same time. In some embodiments, the  
25 two bioactive polypeptides are delivered to the subject using a microorganism producing both the IL-2 and the T1D-specific antigen.

[00154] In some embodiments, the microorganism will be administered, once, twice, three, four, five, or six times daily, *e.g.*, using an oral formulation. In some embodiments, the microorganisms are administered every day, every other day, once per week, twice per week,  
30 three times per week, or four times per week. In other embodiments, treatment occurs once

every two weeks. In other embodiments, treatment occurs once every three weeks. In other embodiments, treatment occurs once per month.

[00155] The duration of a treatment cycle for the method is, for example, 7 days to the subject's lifetime, as needed to treat or reverse disease, or prevent relapse. In some  
5 embodiments, a treatment cycle lasts for about 30 days to about 2 years. In other  
embodiments, the subject will have a treatment cycle that lasts from 30 days to 1.5 years. In  
other embodiments, the subject will have a treatment cycle that lasts from 30 days to 1 year.  
In other embodiments, the subject will have a treatment cycle that lasts from 30 days to 11  
10 months. In other embodiments, the subject will have a treatment cycle that lasts from  
30 days to 10 months. In other embodiments, the subject will have a treatment cycle that lasts from  
30 days to 9 months. In other embodiments, the subject will have a treatment cycle that lasts  
from 30 days to 8 months. In other embodiments, the subject will have a treatment cycle that  
lasts from 30 days to 7 months. In other embodiments, the subject will have a treatment  
15 cycle that lasts from 30 days to 6 months. In other embodiments, the subject will have a  
treatment cycle that lasts from 30 days to 5 months. In other embodiments, the subject will  
have a treatment cycle that lasts from 30 days to 4 months. In other embodiments, the subject  
will have a treatment cycle that lasts from 30 days to 3 months. In other embodiments, the  
subject will have a treatment cycle that lasts from 30 days to 2 months.

[00156] Daily maintenance doses can be given for a period clinically desirable in the  
20 subject, for example from 1 day up to several years (*e.g.* for the subject's entire remaining  
life); for example from about (2, 3 or 5 days, 1 or 2 weeks, or 1 month) upwards and/or for  
example up to about (5 years, 1 year, 6 months, 1 month, 1 week, or 3 or 5 days).  
Administration of the daily maintenance dose for about 3 to about 5 days or for about 1 week  
to about 1 year is typical. Unit doses can be administered from twice daily to once every two  
25 weeks until a therapeutic effect is observed.

[00157] The microorganisms producing the first and second therapeutic polypeptide may  
be delivered in mono- or combination therapy for the treatment of the disease. In some  
embodiments, the compositions of the present disclosure include additional therapeutically  
active agents. In some embodiments, the compositions of the present disclosure, and  
30 treatment of the subject, does not involve other active components, *e.g.*, does not involve  
additional immune-modulating substances, such as antibodies (*e.g.*, anti-CD3 for treatment of  
T1D). Thus, in some examples, the pharmaceutical compositions of the present disclosure

consist essentially of the microorganism as described herein (expressing the therapeutic IL-2 and antigen polypeptides), and a pharmaceutically acceptable carrier.

#### Pharmaceutical Compositions and Carriers

5 [00158] Microorganisms (*e.g.*, bacteria or yeast as described herein) may be administered in pure form, combined with other active ingredients, and/or combined with pharmaceutically acceptable (*i.e.*, nontoxic) excipients or carriers. The term “pharmaceutically acceptable” is used herein in accordance with its art-recognized meaning and refers to carriers that are compatible with the other ingredients of a pharmaceutical composition, and are not deleterious to the recipient thereof.

10 [00159] The compositions of the present invention can be prepared in any known or otherwise effective dosage or product form suitable for use in providing systemic delivery of the microorganism (*e.g.*, bacteria) to the mucosa, which would include pharmaceutical compositions and dosage forms as well as nutritional product forms.

15 [00160] In some embodiments, the formulation is an oral formulation or pharmaceutical composition. In some examples according to this embodiment, the formulation or pharmaceutical composition comprises the non-pathogenic microorganism in a dry-powder form (*e.g.*, freeze-dried form) or in compacted form thereof, optionally in combination with other dry carriers. Oral formulations will generally include an inert diluent carrier or an edible carrier.

20 [00161] In some examples, the oral formulation comprises a coating or utilizes an encapsulation strategy, which facilitates the delivery of the formulation into the intestinal tract, and/or allows the microorganism be released and hydrated in the intestinal tract (*e.g.*, the ileum, small intestine, or the colon). Once the microorganism is released from the formulation and sufficiently hydrated, it begins expressing the bioactive polypeptide, which  
25 is subsequently released into the surroundings, or expressed on the surface of the microorganism. Such coating and encapsulation strategies (*i.e.*, delayed-release strategies) are known to those of skill in the art. *See, e.g.*, U.S. 5,972,685; WO 2000/18377; and WO 2000/22909, the disclosures of which are incorporated herein by reference in their entirety.

30 [00162] In some embodiments, the disclosure provides a pharmaceutical composition comprising the microorganism (*e.g.*, the non-pathogenic bacteria) in a lyophilized or freeze dried form, optionally in conjunction with other components, such as dextrans, sodium

glutamate, and polyols. Exemplary freeze dried compositions are described, *e.g.*, in U.S. Patent Application No. 2012/0039853 to Corveleyn *et al.*, the disclosure of which is incorporated herein by reference in its entirety. Exemplary formulations comprise freeze-dried bacteria (*e.g.*, a therapeutically effective amount of the bacteria) and a pharmaceutically acceptable carrier. Freeze-dried bacteria may be prepared in the form of capsules, tablets, granulates and powders, each of which may be administered orally. Alternatively, freeze-dried bacteria may be prepared as aqueous or oily suspensions in suitable media, or lyophilized bacteria may be suspended in a suitable medium, such as a drink, just prior to use.

[00163] For oral administration, the formulation may be a gastro-resistant oral dosage form. For example, the oral dosage form (*e.g.*, capsules, tablets, pellets, micro-pellets, granulates, and the like) may be coated with a thin layer of excipient (usually polymers, cellulosic derivatives and/or lipophilic materials) that resists dissolution or disruption in the stomach, but not in the intestine, thereby allowing transit through the stomach in favor of disintegration, dissolution and absorption in the intestine (*e.g.*, the small intestine, or the colon). In some examples, oral formulations may include compounds providing controlled release, sustained release, or prolonged release of the microorganism, and thereby provide controlled release of the desired protein encoded therein. These dosage forms (*e.g.*, tablets or capsules) typically contain conventional and well known excipients, such as lipophilic, polymeric, cellulosic, insoluble, swellable excipients. Controlled release formulations may also be used for any other delivery sites including intestinal, colon, bioadhesion or sublingual delivery (*i.e.*, dental mucosal delivery) and bronchial delivery. When the compositions of the invention are to be administered rectally or vaginally, pharmaceutical formulations may include suppositories and creams. In this instance, the host cells are suspended in a mixture of common excipients also including lipids. Each of the aforementioned formulations are well known in the art and are described, for example, in the following references: Hansel *et al.* (1990, Pharmaceutical dosage forms and drug delivery systems, 5th edition, William and Wilkins); Chien 1992, (Novel drug delivery system, 2nd edition, M. Dekker); Prescott *et al.* (1989, Novel drug delivery, J. Wiley & Sons); Gazzaniga *et al.*, (1994, Oral delayed release system for colonic specific delivery, *Int. J. Pharm.* 108:77-83).

[00164] In some embodiments, the oral formulation includes compounds that can enhance mucosal delivery and/or mucosal uptake of the bioactive polypeptides expressed by the

microorganism. In other examples, the formulation includes compounds, which enhance the viability of the microorganism within the formulation, and/or once released.

[00165] The bacteria of the invention can be suspended in a pharmaceutical formulation for administration to the human or animal having the disease to be treated. Such pharmaceutical formulations include but are not limited to live Gram-positive bacteria and a medium suitable for administration. The bacteria may be lyophilized in the presence of common excipients such as lactose, other sugars, alkaline and/or alkali earth stearate, carbonate and/or sulphate (*e.g.*, magnesium stearate, sodium carbonate and sodium sulphate), kaolin, silica, flavorants and aromas. Bacteria so-lyophilized may be prepared in the form of capsules, tablets, granulates and powders (*e.g.*, a mouth rinse powder), each of which may be administered by the oral route. Alternatively, some Gram-positive bacteria may be prepared as aqueous suspensions in suitable media, or lyophilized bacteria may be suspended in a suitable medium just prior to use, such medium including the excipients referred to herein and other excipients such as glucose, glycine and sodium saccharinate.

[00166] In some examples, the microorganism is locally delivered to the gastrointestinal tract of the subject using any suitable method. For example, microsphere delivery systems could be employed to enhance delivery to the gut. Microsphere delivery systems include microparticles having a coating that provides localized release into the gastrointestinal tract of the subject (*e.g.*, controlled release formulations such as enteric-coated formulations and colonic formulations).

[00167] For oral administration, gastroresistant oral dosage forms may be formulated, which dosage forms may also include compounds providing controlled release of the Gram-positive bacteria and thereby provide controlled release of the desired protein encoded therein (*e.g.*, IL-2). For example, the oral dosage form (including capsules, tablets, pellets, granulates, powders) may be coated with a thin layer of excipient (*e.g.*, polymers, cellulosic derivatives and/or lipophilic materials) that resists dissolution or disruption in the stomach, but not in the intestine, thereby allowing transit through the stomach in favor of disintegration, dissolution and absorption in the intestine.

[00168] The oral dosage form may be designed to allow slow release of the Gram-positive bacteria and of the produced exogenous proteins, for instance as controlled release, sustained release, prolonged release, sustained action tablets or capsules. These dosage forms usually contain conventional and well-known excipients, such as lipophilic, polymeric, cellulosic,

insoluble, swellable excipients. Such formulations are well-known in the art and are described, for example, in the following references: Hansel *et al.*, Pharmaceutical dosage forms and drug delivery systems, 5th edition, William and Wilkins, 1990; Chien 1992, Novel drug delivery system, 2nd edition, M. Dekker; Prescott *et al.*, Novel drug delivery, J.Wiley & Sons, 1989; and Gazzaniga *et al.*, *Int. J. Pharm.* 108:77-83 (1994).

[00169] The pharmaceutical dosage form (*e.g.* capsule) is coated with pH-dependent Eudragit polymers to obtain gastric juice resistance and for the intended delivery at the terminal ileum and colon, where the polymers dissolve at pH 6.5. By using other Eudragit polymers or a different ratio between the polymers, the delayed release profile could be adjusted, to release the bacteria for example in the duodenum or jejunum.

[00170] Pharmaceutical compositions contain at least one pharmaceutically acceptable carrier. Non-limiting examples of suitable excipients, diluents, and carriers include preservatives, inorganic salts, acids, bases, buffers, nutrients, vitamins, fillers and extenders such as starch, sugars, mannitol, and silicic derivatives; binding agents such as carboxymethyl cellulose and other cellulose derivatives, alginates, gelatin, and polyvinyl pyrrolidone; moisturizing agents such as glycerol/ disintegrating agents such as calcium carbonate and sodium bicarbonate; agents for retarding dissolution such as paraffin; resorption accelerators such as quaternary ammonium compounds; surface active agents such as acetyl alcohol, glycerol monostearate; adsorptive carriers such as kaolin and bentonite ; carriers such as propylene glycol and ethyl alcohol, and lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols.

[00171] Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. Tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a dispersing agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials that modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or enteric agents. Further, a syrup may contain, in addition to the active

compounds, sucrose as a sweetening agent and certain preservatives, dyes, colorings, and flavorings. It will be appreciated that the form and character of the pharmaceutically acceptable carrier is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables. The carrier(s) must be  
5 “acceptable” in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

[00172] Alternative preparations for administration include sterile aqueous or nonaqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are dimethylsulfoxide, alcohols, propylene glycol, polyethylene glycol, vegetable oils such as  
10 olive oil and injectable organic esters such as ethyl oleate. Aqueous carriers include mixtures of alcohols and water, buffered media, and saline. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer’s dextrose, and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, inert gases, and the like. Various liquid  
15 formulations are possible for these delivery methods, including saline, alcohol, DMSO, and water based solutions.

[00173] Oral aqueous formulations include excipients, such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and/or the like. These compositions take the form of solutions such as  
20 mouthwashes and mouth rinses, further comprising an aqueous carrier such as for example water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer’s dextrose, and the like.

[00174] Aqueous mouthwash formulations are well-known to those skilled in the art. Formulations pertaining to mouthwashes and oral rinses are discussed in detail, for example,  
25 in U.S. Patent 6,387,352, U.S. Patent 6,348,187, U.S. Patent 6,171,611, U.S. Patent 6,165,494, U.S. Patent 6,117,417, U.S. Patent 5,993,785, U.S. Patent 5,695,746, U.S. Patent 5,470,561, U.S. Patent 4,919,918, U.S. Patent Appl. No. 2004/0076590, U.S. Patent Appl. No. 2003/0152530, and U.S. Patent Appl. No. 2002/0044910, each of which is herein specifically incorporated by reference into this section of the specification and all other  
30 sections of the specification .

[00175] Other additives may be present in the formulations of the present disclosure, such as flavoring, sweetening or coloring agents, or preservatives. Mint, such as from peppermint

or spearmint, cinnamon, eucalyptus, citrus, cassia, anise and menthol are examples of suitable flavoring agents. Flavoring agents can be present in the oral compositions in an amount in the range of from 0 to 3%, *e.g.*, up to 2%, such as up to 0.5%, *e.g.*, around 0.2%, in the case of liquid compositions.

- 5 [00176] Sweeteners include artificial or natural sweetening agents, such as sodium saccharin, sucrose, glucose, saccharin, dextrose, levulose, lactose, mannitol, sorbitol, fructose, maltose, xylitol, thaumatin, aspartame, D-tryptophan, dihydrochalcones, acesulfame, and any combinations thereof, which may be present in an amount in the range of from about 0 to 2%, *e.g.*, up to 1% w/w, such as 0.05 to 0.3% w/w of the oral composition.
- 10 [00177] Coloring agents are suitable natural or synthetic colors, such as titanium dioxide or CI 42090, or mixtures thereof. Coloring agents can be present in the compositions in an amount in the range of from 0 to 3%; *e.g.*, up to 0.1%, such as up to 0.05%, *e.g.*, about 0.005-0.0005%, in the case of liquid compositions. In some examples, sodium benzoate is added as a preservative, *e.g.*, in concentrations insufficient substantially to alter the pH of the
- 15 composition, otherwise the amount of buffering agent may need to be adjusted to arrive at the desired pH.

- [00178] Other optional ingredients include humectants, surfactants (non-ionic, cationic or amphoteric), thickeners, gums and binding agents. A humectant adds body to the formulation and retains moisture in a dentifrice composition. In addition, a humectant helps to prevent
- 20 microbial deterioration during storage of the formulation. It also assists in maintaining phase stability and provides a way to formulate a transparent or translucent dentifrice.

- [00179] Suitable humectants include glycerin, xylitol, glycerol and glycols such as propylene glycol, which may be present in an amount of up to 50% w/w each, but total humectant is *e.g.*, not more than about 60-80% w/w of the composition. For example, liquid
- 25 compositions may comprise up to about 30% glycerin plus up to about 5%, or about 2% w/w xylitol. In some examples, surfactants are not anionic and may include polysorbate 20 or cocoamidobetaine or the like in an amount up to about 6%, or about 1.5 to 3%, w/w of the composition.

- [00180] In some examples, when the oral compositions of the invention is in a liquid form,
- 30 it a film-forming agent that may be added up to about 3% w/w of the oral composition, such as in the range of from 0 to 0.1%, or about 0.001 to 0.01%, such as about 0.005% w/w of the

oral composition. Suitable film-formers include (in addition to sodium hyaluronate) those sold under the tradename Gantrez.

[00181] Liquid nutritional formulations for oral or enteral administration may comprise one or more nutrients such as fats, carbohydrates, proteins, vitamins, and minerals. Many  
5 different sources and types of carbohydrates, lipids, proteins, minerals and vitamins are known and can be used in the nutritional liquid embodiments of the present invention, provided that such nutrients are compatible with the added ingredients in the selected formulation, are safe and effective for their intended use, and do not otherwise unduly impair product performance.

10 [00182] These nutritional liquids can be formulated with sufficient viscosity, flow, or other physical or chemical characteristics to provide a more effective and soothing coating of the mucosa while drinking or administering the nutritional liquid. These nutritional embodiments may also represent a balanced nutritional source suitable for meeting the sole, primary, or supplemental nutrition needs of the individual.

15 [00183] Non-limiting examples of suitable nutritional liquids are described in U. S. Patent 5,700,782 (Hwang *et al.*); U. S. Patent 5,869,118 (Morris *et al.*); and U. S. Patent 5,223,285 (DeMichele *et al.*), which descriptions are incorporated herein by reference.

[00184] Nutritional proteins suitable for use herein can be hydrolyzed, partially hydrolyzed or non-hydrolyzed, and can be derived from any known or otherwise suitable  
20 source such as milk (*e.g.*, casein, whey), animal (*e.g.*, meat, fish), cereal (*e.g.*, rice, corn), vegetable (*e.g.*, soy), or combinations thereof.

[00185] Fats or lipids suitable for use in the nutritional liquids include, but are not limited to, coconut oil, soy oil, corn oil, olive oil, safflower oil, high oleic safflower oil, MCT oil (medium chain triglycerides), sunflower oil, high oleic sunflower oil, structured triglycerides,  
25 palm and palm kernel oils, palm olein, canola oil, marine oils, cottonseed oils, and combinations thereof. Carbohydrates suitable for use in the nutritional liquids may be simple or complex, lactose- containing or lactose-free, or combinations thereof. Non-limiting examples of suitable carbohydrates include hydrolyzed corn starch, maltodextrin, glucose polymers, sucrose, corn syrup, corn syrup solids, rice-derived carbohydrate, glucose,  
30 fructose, lactose, high fructose corn syrup and indigestible oligosaccharides such as fructo-oligosaccharides (FOS), and combinations thereof.

[00186] The nutritional liquids may further comprise any of a variety of vitamins, non-limiting examples of which include vitamin A, vitamin D, vitamin E, vitamin K, thiamine, riboflavin, pyridoxine, vitamin B12, niacin, folic acid, pantothenic acid, biotin, vitamin C, choline, inositol, salts and derivatives thereof, and combinations thereof.

- 5 [00187] The nutritional liquids may further comprise any of a variety of minerals known or otherwise suitable for use in patients at risk of or suffering from T1D, non-limiting examples of which include calcium, phosphorus, magnesium iron, selenium, manganese, copper, iodine, sodium, potassium, chloride, and combinations thereof.

- [00188] The microorganisms and in particular the yeast and bacteria of the present  
10 invention can also be formulated as elixirs or solutions for convenient oral or rectal administration or as solutions appropriate for parenteral administration, for instance by intramuscular, subcutaneous or intravenous routes. Additionally, the nucleoside derivatives are also well suited for formulation as a sustained or prolonged release dosage forms, including dosage forms that release active ingredient only or in a particular part of the  
15 intestinal tract, *e.g.*, over an extended or prolonged period of time to further enhance effectiveness. The coatings, envelopes, and protective matrices in such dosage forms may be made, for example, from polymeric substances or waxes well known in the pharmaceutical arts.

- [00189] The compositions of the present invention include pharmaceutical dosage forms  
20 such as lozenges, troches or pastilles. These are typically discoid-shaped solids containing the active ingredient in a suitably flavored base. The base may be a hard sugar candy, glycerinated gelatin, or the combination of sugar with sufficient mucilage to give it form. Troches are placed in the mouth where they slowly dissolve, liberating the active ingredient for direct contact with the mucosa.

- 25 [00190] The troche embodiments of the present invention can be prepared, for example, by adding water slowly to a mixture of the powdered active, powdered sugar, and a gum until a pliable mass is formed. A 7% acacia powder can be used to provide sufficient adhesiveness to the mass. The mass is rolled out and the troche pieces cut from the flattened mass, or the mass can be rolled into a cylinder and divided. Each cut or divided piece is shaped and  
30 allowed to dry, to thus form the troche dosage form.

[00191] If the active ingredient is heat labile, it may be made into a lozenge preparation by compression. For example, the granulation step in the preparation is performed in a manner similar to that used for any compressed tablet. The lozenge is made using heavy compression equipment to give a tablet that is harder than usual as it is desirable for the dosage form to  
5 dissolve or disintegrate slowly in the mouth. In some examples, ingredients are selected to promote slow-dissolving characteristics.

[00192] In a particular formulation of the present invention, the microorganisms will be incorporated in a bioadhesive carrier containing pregelatinized starch and cross-linked poly (acrylic acid) to form a bioadhesive tablet and a bioadhesive gel suitable for buccal  
10 application (*i.e.*, having prolonged bioadhesion and sustained drug delivery). For example, a powder mixture of non-pathogenic and non-invasive bacterium according to the invention), bioadhesive polymers (pregelatinized starch and cross-linked poly (acrylic acid) coprocessed via spray drying), sodium stearyl fumarate (lubricant) and silicon dioxide (glidant) is processed into tablets (weight: 100 mg; diameter: 7 mm). The methods for the production of  
15 these tablets are well known to the person skilled in the art and has been described before for the successful development of bioadhesive tablets containing various drugs (miconazol, testosterone, fluoride, ciprofloxacin) (Bruschi M. L. and de Freitas O., *Drug Development and Industrial Pharmacy*, 2005 31:293-310). All excipient materials are commercially available in pharmaceutical grades.

[00193] To optimize the formulation, the drug load in the tablets and the ratio between starch and poly (acrylic acid) will be varied. Based on previous research, the maximum drug load in the coprocessed bioadhesive carrier is about 60% (w/w) and the starch/poly (acrylic acid) ratio can be varied between 75/25 and 95/5 (w/w). During the optimization study the bioadhesive properties of the tablets and the drug release from the tablets are the main  
20 evaluation parameters, with the standard tablet properties (hardness, friability) as secondary evaluation criteria.

[00194] The bacteria are incorporated into an aqueous dispersion of pregelatinized starch and cross-linked poly (acrylic acid). This polymer dispersion is prepared via a standard procedure using a high shear mixer.

[00195] Similar to the tablet, the drug load of the gel and the starch/poly (acrylic acid) ratio need to be optimized in order to obtain a gel having optimal adherence to the esophageal  
30

mucosa. For a gel, the concentration of the polymers in the dispersion is an additional variable as it determines the viscosity of the gel, hence its muco-adhesive properties.

[00196] A model to screen the bioadhesive properties of polymer dispersions to the mucosa of esophagus has been described in detail by Batchelor *et al.* (*Int. J. Pharm.*, 5 238:123- 132, 2002).

[00197] Other routes and forms of administration include food preparations containing the live microorganisms. In some examples, the bioactive polypeptide-expressing microorganism can be included into a dairy product.

[00198] The pharmaceutical compositions of the present invention can be prepared by any 10 known or otherwise effective method for formulating or manufacturing the selected dosage form. For example, the microorganisms can be formulated along with common, *e.g.*, pharmaceutically acceptable carriers, such as excipients and diluents, formed into oral tablets, capsules, sprays, lozenges, treated substrates (*e.g.*, oral or topical swabs, pads, or disposable, non-digestible substrate treated with the compositions of the present invention); oral liquids 15 (*e.g.*, suspensions, solutions, emulsions), powders, suppositories, or any other suitable dosage form. In some embodiments, the present disclosure provides a method for the manufacture of a pharmaceutical composition. Exemplary methods include: contacting the microorganism (*e.g.*, the non-pathogenic bacterium) with a pharmaceutically acceptable carrier, thereby forming the pharmaceutical composition. In some examples, the method further includes: 20 growing the microorganism in a medium. The method may further include drying (*e.g.*, freeze-drying) a liquid containing the microorganism, wherein the liquid optionally includes the pharmaceutically acceptable carrier.

#### Unit Dosage Forms

[00199] The current disclosure further provides unit dosage forms comprising a certain 25 amount of a microorganism (*e.g.*, bacterium) of the present disclosure optionally in combination with a food-grade or pharmaceutically acceptable carrier. Exemplary unit dosage forms contain from about  $1 \times 10^3$  to about  $1 \times 10^{14}$  colony-forming units (cfu) of a non-pathogenic microorganism (*e.g.*, a non-pathogenic Gram-positive bacterium). Other exemplary unit dosage forms contain from about  $1 \times 10^4$  to about  $1 \times 10^{13}$  colony-forming 30 units (cfu) of a non-pathogenic microorganism (*e.g.*, a non-pathogenic Gram-positive bacterium), or from about  $1 \times 10^4$  to about  $1 \times 10^{12}$  colony-forming units (cfu) of a non-

pathogenic microorganism (*e.g.*, a non-pathogenic Gram-positive bacterium). In other embodiments, the unit dosage form comprises from about  $1 \times 10^5$  to about  $1 \times 10^{12}$  colony-forming units (cfu), or from about  $1 \times 10^6$  to about  $1 \times 10^{12}$  colony-forming units (cfu) of the non-pathogenic microorganism (*e.g.*, the non-pathogenic Gram-positive bacterium). In other  
5 embodiments, the unit dosage form comprises from about  $1 \times 10^8$  to about  $1 \times 10^{12}$  colony-forming units (cfu), or from about  $1 \times 10^9$  to about  $1 \times 10^{12}$  colony-forming units (cfu) of the non-pathogenic microorganism (*e.g.*, the non-pathogenic Gram-positive bacterium). In yet other embodiments, the unit dosage form comprises from about  $1 \times 10^9$  to about  $1 \times 10^{11}$  colony-forming units (cfu), or from about  $1 \times 10^9$  to about  $1 \times 10^{10}$  colony-forming units  
10 (cfu) of the non-pathogenic microorganism (*e.g.*, the non-pathogenic Gram-positive bacterium). In yet other embodiments, the unit dosage form comprises from about  $1 \times 10^7$  to about  $1 \times 10^{11}$  colony-forming units (cfu), or from about  $1 \times 10^8$  to about  $1 \times 10^{10}$  colony-forming units (cfu) of the non-pathogenic microorganism (*e.g.*, the non-pathogenic Gram-positive bacterium).

15 [00200] In yet other embodiments, the unit dosage form comprises from about  $1 \times 10^9$  to about  $1 \times 10^{10}$  colony-forming units (cfu), or from about  $1 \times 10^9$  to about  $100 \times 10^9$  colony-forming units (cfu) of the non-pathogenic microorganism (*e.g.*, the non-pathogenic Gram-positive bacterium).

[00201] The unit dosage form can have any physical form or shape. In some embodiments,  
20 the unit dosage form is adapted for oral administration. In some examples according to these embodiments, the unit dosage form is in the form of a capsule, a tablet, or a granule. Exemplary capsules include capsules filled with micro-granules. In some embodiments, the non-pathogenic microorganism (*e.g.*, the non-pathogenic Gram-positive bacterium) contained in the dosage form is in a dry-powder form. For example, the microorganism is in a freeze-dried powder form, which is optionally compacted and coated.  
25

[00202] This invention will be better understood by reference to the Examples that follow, but those skilled in the art will readily appreciate that these are only illustrative of the invention as described more fully in the claims that follow thereafter. Additionally, throughout this application, various publications are cited. The disclosures of these  
30 publications are hereby incorporated by reference into this application in their entirety to describe more fully the state of the art to which this invention pertains.

## EXAMPLES

### Example 1

#### Generation of *Lactococcus lactis* Strains Expressing CmbA on the Bacterial Surface

##### (a) *Episomal Expression of CmbA*

5 [00203] The codon usage of the CmbA encoding gene (*see*, SEQ ID NO: 2 and coding region for CmbA in SEQ ID NO: 11, **FIGs. 5A-D**) was optimized to *L. lactis* MG1363. The resulting DNA sequence was made synthetically. Using overlay PCR assembly, *PthyA*>>*SSusp45* (*usp45* secretion signal) and *cmbA* were fused to form one PCR fragment. The *PthyA*>>*SSusp45-cmbA* expression unit was positioned between the 5'*thyA* and 3'*thyA* regions of *L. lactis* strain MG1363 by PCR. The resulting PCR fragment: 5'*thyA*>>*PthyA*>>*SSusp45-cmbA*>>3'*thyA* was subcloned into plasmid pORI19 (*see, e.g.,* Law *et al., J. Bacteriol.* 1995, 177(24):7011-7018) to generate plasmid pAGX1893. Plasmid pAGX1893 was transformed into *Lactococcus lactis* strain LL108 resulting in strain LL108[pAGX1893].

##### 15 (b) *Chromosomal Integration (Constitutive Expression of CmbA)*

[00204] Integration vector pAGX1893 was electroporated into two different *L. lactis* strains containing a bicistronic expression cassette containing a heterologous nucleic acid sequence encoding the therapeutic polypeptide PAL (*gapB*>>*rpmD*>>*pal*) (SEQ ID NOs: 24 and 25). These two strains were sAGX0599 and sAGX0585 (a trehalose accumulating strain). Insertion of *SSusp45-cmbA* by homologous recombination (double-crossover) in the *thyA* locus was confirmed by PCR and Sanger DNA sequencing. The resulting strains were termed sAGX0618 and sAGX0619, respectively.

### Example 2

#### Generation of *Lactococcus lactis* Strains Expressing MbpL on the Bacterial Surface

##### 25 (a) *Episomal Expression of MbpL (Using Native Signal Peptide)*

[00205] Construction of a [*PthyA*>>*mbpL*] *thyA* integration/expression vector pAGX1903: The codon usage of the MbpL encoding gene (with signal peptide; SSmbpL) was optimized to *L. lactis* MG1363. The resulting DNA sequence was made synthetically. Using overlay PCR assembly, *PthyA* and *mbpL* were fused to form one PCR fragment. The

30 *PthyA*>>*SSmbpL*>>*mbpL* expression unit (SEQ ID NO: 17) was positioned between the

5' *thyA* and 3' *thyA* regions of *L. lactis* strain MG1363 by PCR. The resulting PCR fragment: 5' *thyA*>>P*thyA*>>SS*mbpL*>>*mbpL*>>3' *thyA* was subcloned into plasmid pORI19 to generate plasmid pAGX1903, which was transformed into *L. lactis* strain LL108 resulting in strain LL108[pAGX1903].

5 (b) *Episomal Expression of MbpL (Using SSusp45)*

[00206] Another construct, which incorporates *mbpL* may include secretion leader sequence SSusp45 instead of MbpL's own signal peptide. A [P*thyA*>>SS*mbpL*] *thyA* integration/expression vector can be prepared in accordance with the procedure outlined in Example 1, wherein the nucleic acid sequence encoding *cmbA* can be replaced with a nucleic acid sequence encoding MbpL polypeptide without its own signal peptide. The MbpL encoding gene (without signal peptide) can be optimized to *L. lactis* MG1363. The resulting DNA sequence can be made synthetically. Using overlay PCR assembly, P*thyA*>>SS*mbpL* and *mbpL* can be fused to form one PCR fragment. The P*thyA*>>SS*mbpL* expression unit (SEQ ID NO: 15) can be positioned between the 5' *thyA* and 3' *thyA* regions of *L. lactis* strain MG1363 by PCR. The resulting PCR fragment: 5' *thyA*>>P*thyA*>>SS*mbpL*>>3' *thyA* can be subcloned, e.g., into plasmid pORI19 to generate another plasmid, which can be transformed into a *L. lactis*, e.g., strain LL108.

### Example 3

#### **Generation of *Lactococcus lactis* Strains Expressing MapA on the Bacterial Surface**

20 (a) *Episomal Expression of MapA (Using Native Signal peptide)*

[00207] Construction of a [P*thyA*>>*mapA*] *thyA* integration/expression vector pAGX1946: The *mapA* encoding gene (with signal peptide) was optimized to *L. lactis* MG1363. The resulting DNA sequence was made synthetically. Using overlay PCR assembly, P*thyA* and *mapA* were fused to form one PCR fragment. The P*thyA*>>SS*mapA*>>*mapA* expression unit (SEQ ID NO: 21) was positioned between the 5' *thyA* and 3' *thyA* regions of *L. lactis* strain MG1363 by PCR. The resulting PCR fragment: 5' *thyA*>>P*thyA*>>SS*mapA*>>*mapA*>>3' *thyA* was subcloned in plasmid pORI19 to generate plasmid pAGX1946, which was transformed into *L. lactis* strain LL108 resulting in strain LL108[pAGX1946].

(b) *Episomal Expression of MapA (Using SSusp45)*

30 [00208] Another construct, which incorporates *mapA* may include secretion leader sequence SSusp45 instead of MapA's own signal peptide. A [P*thyA*>>SS*mapA*] *thyA* integration/expression vector can be prepared in accordance with the procedure outlined in Example 1, wherein the nucleic acid sequence encoding *cmbA* can be replaced with a nucleic acid sequence encoding MapA polypeptide without its own signal peptide. The MapA encoding gene (without signal peptide) can be optimized to *L. lactis* MG1363. The resulting DNA sequence can be made synthetically. Using overlay PCR assembly, P*thyA*>>SS*mapA* and *mapA* can be fused to form one PCR fragment. The P*thyA*>>SS*mapA* expression unit (SEQ ID NO: 22) can be positioned between the 5' *thyA* and 3' *thyA* regions of *L. lactis* strain MG1363 by PCR. The resulting PCR fragment: 5' *thyA*>>P*thyA*>>SS*mapA*>>*mapA*>>3' *thyA* can be subcloned, e.g., into plasmid pORI19 to generate another plasmid, which can be transformed into a *L. lactis*, e.g., strain LL108.

*thyA* integration/expression vector can be prepared, *e.g.*, in accordance with the procedure in Example 1, wherein the nucleic acid sequence encoding *cmbA* can be replaced with a nucleic acid sequence encoding MapA polypeptide without its signal peptide. The *mapA* encoding gene can be optimized to *L. lactis* MG1363. The resulting DNA sequence can be made synthetically. Using overlay PCR assembly, *PthyA>>SSusp45* and *mapA* (without signal peptide) can be fused to form one PCR fragment. The *PthyA>>SSusp45-mapA* expression unit (SEQ ID NO: 19) can be positioned between the 5'*thyA* and 3'*thyA* regions of *L. lactis* strain MG1363 by PCR. The resulting PCR fragment: 5'*thyA>>PthyA>>SSusp45-mapA>>3'thyA* can be subcloned, *e.g.*, into plasmid pORI19 to generate another plasmid, which can be transformed into a *L. lactis* strain, *e.g.*, LL108.

#### **Example 4**

#### ***Lactococcus lactis* Strains Expressing an hTFF3-CmbA Fusion Protein on the Bacterial Surface**

##### ***(a) Expression of hTFF3***

[00209] **FIG. 4** illustrates an exemplary *SSusp45-htff3* construct (amino acid and nucleic acid sequences) which can be used as the N-terminal portion of a fusion protein, with a C-terminal adherence polypeptide. The C-terminal adherence polypeptide can be added through PCR fusion or more traditional ligation.

##### ***(b) Episomal Expression of hTFF3-CmbA***

[00209] A [*PthyA>>SSusp45-hTFF3-cmbA*] *thyA* integration/expression vector was constructed. The codon usage of the hTFF3 and CmbA (without signal peptide) encoding genes were optimized to *L. lactis* MG1363. The resulting *htff3* and *cmbA* DNA sequences were made synthetically. Using overlay PCR assembly, *PthyA>>SSusp45*, *hTFF3* and *cmbA* were fused to form one PCR fragment. The *PthyA>>SSusp45-hTFF3-cmbA* expression unit (*see* SEQ ID NO: 11 and **FIGs. 5A-D**) was positioned between the 5'*thyA* and 3'*thyA* regions of *L. lactis* strain MG1363 by PCR. The resulting PCR fragment: 5'*thyA>>PthyA>>SSusp45-hTFF3-cmbA>>3'thyA* was subcloned in plasmid pORI19 (Law *et al.*, 1995 *supra*) to generate plasmid pAGX2005 (*see* **FIG. 1**), which was transformed into *L. lactis* strain LL108 resulting in strain LL108[pAGX2005].

*(b) Chromosomal Integration (Constitutive Expression of hTFF3-cmbA; thyA Promoter)*

[00210] Integration vector pAGX2005 (*see above and FIG. 1*) was electroporated into two different *thyA*-wild type *Lactococcus lactis* strains, *L. lactis* sAGX0599 and sAGX0585 (trehalose accumulating strain) containing a bicistronic expression cassette encoding the therapeutic polypeptide PAL (*gapB>>rpmD>>pal*). Insertion of *SSusp45-hTFF3-CmbA* (*see, e.g., SEQ ID NO: 11 and FIGs. 5A-D*) by homologous recombination (double crossover) in the *thyA* locus was confirmed by PCR and Sanger DNA sequencing. The resulting strains were termed sAGX0644 and sAGX0645.

*(c) Chromosomal Integration (Constitutive Expression of hTFF3-CmbA; PhlIA Promoter)*

10 [00211] Promoter replacement vector pAGX2041 (*PthyA->PhlIA*) was electroporated into *L. lactis* strain sAGX0644 (*see above*) containing a bicistronic expression cassette encoding the therapeutic polypeptide PAL (*gapB>>rpmD>>pal*) and *PthyA>>SSusp45-hTFF3-cmbA*. Replacement of *PthyA* by *PhlIA* by homologous recombination (double crossover) was confirmed by PCR and Sanger DNA sequencing. The resulting strain was termed:  
15 sAGX0660.

**Example 5*****Lactococcus lactis* Strains Expressing hTFF1-SpaX on the Bacterial Surface***(a) Episomal expression of hTFF1-spaX*

[00212] Construction of a [*PthyA>>SSusp45-hTFF1-spaX*] (SEQ ID NOs: 22 and 23) *thyA*  
20 integration/expression vector pAGX1894: The codon usage of the hTFF1 encoding gene was optimized to *L. lactis* MG1363. The resulting DNA sequence was made synthetic. Using overlay PCR assembly, *PthyA>>SSusp45*, *hTFF1* and the gene encoding for the cell wall anchor of protein A of *Staphylococcus aureus* (SpaX, Steidler *et al.*, 1998) were fused to form one PCR fragment. The *PthyA>>SSusp45-hTFF1-spaX* expression unit was positioned  
25 between the 5' *thyA* and 3' *thyA* regions of *L. lactis* strain MG1363 by PCR. The resulting PCR fragment: 5' *thyA>>PthyA>>SSusp45-hTFF1-spaX>>3' thyA* was subcloned into plasmid pORI19 to generate plasmid pAGX1894, which was transformed into *L. lactis* strain LL108 resulting in strain LL108[pAGX1894].

**Example 6*****Lactococcus lactis* Strains Episomally Expressing Mucin-Binding and Cell-Binding Polypeptides**

[00213] The following plasmids and *Lactococcus lactis* strains were prepared as described herein and were tested for their mucin- and cell-binding (Caco-2, IEC-18, and HT29-MTX cells) capabilities. The plasmids listed in Table 1 below were electroporated into *L. lactis* strain LL108.

**Table 1**

10 ***Lactococcus lactis* Strains Episomally Expressing Mucin-Binding and Cell-Binding Polypeptides**

| Plasmid  | Strain          | BioAdhesion Construct                     | Mucin binding | Cell binding |
|----------|-----------------|-------------------------------------------|---------------|--------------|
| pAGX1417 | LL108[pAGX1417] | none (control)                            | -             | -            |
| pAGX1893 | LL108[pAGX1893] | <i>PthyA</i> >>cmbA                       | -             | +++          |
| pAGX1903 | LL108[pAGX1903] | <i>PthyA</i> >>mbpL                       | -             | +++          |
| pAGX1946 | LL108[pAGX1946] | <i>PthyA</i> >>mapA                       | -             | +            |
| pAGX1894 | LL108[pAGX1894] | <i>PthyA</i> >> <i>SSusp45-hTFF1-spaX</i> | +++           | -            |
| pAGX1986 | LL108[pAGX1986] | <i>PthyA</i> >> <i>SSusp45-hTFF3-spaX</i> | N/A           | N/A          |
| pAGX2005 | LL108[pAGX2005] | <i>PthyA</i> >> <i>SSusp45-hTFF3-cmbA</i> | +++           | +++          |

(+): ; (++): ; (+++): cell recovery between 0 and 12 % (indicates relative overall performance based on various experiments, using various kinds of cells (Caco-2, IEC-18, HT29-MTX) and substrates (mucins type II & III).

15 **(a) Binding of *Lactococcus Lactis* LL108[pAGX2005] to Mucins**

[00214] Experimental procedure: mucins (Sigma type II, cat#M2378-100G, mucins from porcine stomach and Sigma type III, cat#M1778-10G, mucin from porcine stomach, bound sialic acid (0.5-1.5%), partially purified) were coated at 500 µg/ml in 50 mM carbonate buffer on Nunc MaxiSorp® plates. Plates were washed 3 times with PBS, blocked with PBS + Tween20 and washed 3 times with PBS. Overnight saturated *L. lactis* cultures were diluted in PBS to OD<sub>600</sub> = 1. Cultures were washed with PBS+0.05% Tween20 and resuspended in 1 volume PBS+0.05% Tween20. 100 µl bacterial suspension was applied on each well. Plates were incubated for 16 hours at 4°C. Plates were washed 3 times with PBS+0.05% Tween20. Plates were dried for 1 hour at 55°C. (A) OD was measured at 405 nm. (B) 100 µl per well crystal violet (1 mg/ml) was added and incubated for 45 minutes at room temperature. OD was subsequently measured at 595 nm.

Result:

[00215] FIG. 2 shows that strain *LL108[pAGX1894]*, expressing human trefoil factor 1 (hTFF1) on its surface by use of the *Staphylococcus aureus* protein A anchor fragment SpaX (Steidler *et al.*, 1998, *supra*) and strain *LL108[pAGX2005]*, expressing hTFF3-CmbA on its surface bind to mucins with comparable strength (Sigma type II & type III) when measured using two different readout methods. In contrast, a control strain containing an empty control vector, *LL108[pAGX1417]* did not show any binding to mucins for the two readout methods.

*(b) Adherence of Lactococcus Lactis LL108[pAGX2005] to Caco-2 cells*

[00216] Experimental procedure: Caco-2 cells were seeded in a 12-well plate at  $5 \times 10^4$  cells/cm<sup>2</sup>. Cells were grown for 17 days to differentiate. Overnight saturated *L. lactis* cultures were diluted 1/1000 in DMEM and washed 3 times with DMEM. *L. lactis* cells were finally resuspended in DMEM. 1 ml of the bacterial suspensions was applied on the cells in the 12-well plate and incubated for 1 hour at 37°C. Cells were washed 3 times with DMEM. Cells were harvested by lysis with Triton-X100 (1 ml 0.1% Triton-X100 in PBS was applied on the cells, incubation for 5 to 10 minutes until total detachment of the cells). Harvested cell suspensions were appropriately diluted and plated out on GM17E plates. Bacterial counts on the plates were measured after 24 hours of incubation at 30°C. Percent recovery was determined as *L. lactis* cells that were recovered from the plating of the harvested cell suspensions divided with the number of cells that were applied initially to the Caco-2 cells.

[00217] FIG. 3 shows that strain *LL108[pAGX1893]* expressing CmbA on its surface and strain *LL108[pAGX2005]* expressing hTFF3-CmbA on its surface exhibit comparable adhesion to Caco-2 cells. In contrast, control strain *LL108[pAGX1417]* (control) and strain *LL108[pAGX1894]* expressing hTFF1-SpaX on its surface did not exhibit enhanced adherence to Caco-2 cells.

Conclusion:

[00218] *L. lactis* strains expressing an hTFF3-CmbA fusion polypeptide on their surface exhibit enhanced *in vitro* binding to mucins and enhanced adherence to Caco-2 cells when compared to corresponding strains not expressing an hTFF3-CmbA fusion polypeptide.

**Example 7****Construction of Integration Plasmids Containing Bicistronic Expression Units**

[00219] The integration plasmids listed in Table 2 below were prepared as described herein using a construct incorporating an intergenic region (e.g., the intergenic region preceding *rplN*) and can be tested for their mucin- and cell-binding capabilities as described herein. The plasmids listed in Table 2 can be electroporated into a host cell, such as *L. lactis* strain LL108 to generate bacteria expressing (e.g., constitutively expressing) polypeptides encoded by these constructs.

**Table 2****Integration Plasmids Containing Bicistronic Expression Units**

| Plasmid  | BioAdhesion Construct<br>(in indicated operon)                         |
|----------|------------------------------------------------------------------------|
| pAGX1935 | <i>PhlA</i> >> <i>hllA</i> >> <i>rplN</i> >> <i>cmbA</i>               |
| pAGX1938 | <i>Pusp45</i> >> <i>usp45</i> >> <i>rplN</i> >> <i>cmbA</i>            |
| pAGX1997 | <i>PhlA</i> >> <i>hllA</i> >> <i>rplN</i> >> <i>SSusp45-hTFF1-spaX</i> |
| pAGX1998 | <i>PhlA</i> >> <i>hllA</i> >> <i>rplN</i> >> <i>SSusp45-hTFF3-spaX</i> |
| pAGX2016 | <i>PhlA</i> >> <i>hllA</i> >> <i>rplN</i> >> <i>SSusp45-hTFF3-cmbA</i> |

**Example 8*****Lactococcus lactis* Strains Containing Chromosomally Integrated Expression Units**

[00220] The following plasmids and *Lactococcus lactis* strains were prepared as described herein and were tested for their mucin- and cell-binding capabilities as described in Example 6. Results are illustrated in **FIGs. 6 and 7**. The plasmids listed in Table 3 below were integrated into a *L. lactis* host strain containing a chromosomally integrated PAL expression unit (*PgapB*>>*gapB*>>*rpmD*>>*pal*). Some of the host strains further included genetic modifications leading to intracellular trehalose accumulation (\*). Exemplary trehalose accumulating strains (i) lack functional trehalose-6-phosphate phosphorylase (*ΔtrePP*) and lack functional cellobiose-specific PTS system IIc component (*ΔptcC*) genes; (ii) express an *E. coli* trehalose-6-phosphate phosphatase (*E. coli otsB*), and (iii) express trehalose transporter genes (PTS genes). See, e.g., U.S. Patent 9,200,249 and U.S. Patent Application Publication 2014/0234371, the disclosures of which are incorporated herein in their entireties.

[00221] Selected strains were also tested for PAL expression. Results (summarized in FIG. 8) indicate that expression of the cell adherence and mucoadhesive polypeptides did not have a significant influence on the amount of PAL expressed per cell ( $\mu\text{g}/10^9$  cells).

**Table 3**

5 ***Lactococcus lactis* Strains Constitutively Expressing Mucin-Binding and Cell-Binding Polypeptides**

| Strain   | Trehalose | BioAdhesion Construct<br>( <i>thyA</i> locus) | Mucin<br>binding | Cell binding |
|----------|-----------|-----------------------------------------------|------------------|--------------|
| sAGX0585 | (*)       | none (control)                                | -                | -            |
| sAGX0599 | wt        | none (control)                                | -                | -            |
| sAGX0618 | wt        | <i>PthyA</i> >> <i>cmbA</i>                   | -                | ++           |
| sAGX0619 | (*)       | <i>PthyA</i> >> <i>cmbA</i>                   | -                | ++           |
| sAGX0644 | wt        | <i>PthyA</i> >> <i>SSusp45-hTFF3-cmbA</i>     | ++               | ++           |
| sAGX0645 | (*)       | <i>PthyA</i> >> <i>SSusp45-hTFF3-cmbA</i>     | ++               | ++           |
| sAGX0660 | wt        | <i>PhlA</i> >> <i>SSusp45-hTFF3-cmbA</i>      | +++              | ++           |

(\*) *PhlA*>>PTS;  $\Delta$  *trePP*;  $\Delta$  *ptcC*; *usp45*>>*otsB*.

wt : wild-type (no trehalose accumulation)

10 (+); (++); (+++): cell recovery between 0 and 12 % (indicates relative overall performance based on various experiments, using various kinds of cells (Caco-2, IEC-18, HT29-MTX) and substrates (mucins type II & III)).

**Example 9**

**Construction of *Lactococcus lactis* Strains Containing Chromosomally Integrated Expression Units**

15 [00222] The *Lactococcus lactis* strains listed in Table 4 below can be prepared as described herein using appropriate constructs and can be tested for their mucin- and cell-binding capabilities as described herein

[00223] Each of these bacterial strains expressing mucin-binding and cell-adherence polypeptides on their surface (e.g., *Lactococcus lactis* strains expressing hTFF3-CmbA fusion proteins on their surface) can be evaluated for their gastro-intestinal (GI)-transit time, and can be compared with corresponding strains not expressing the mucin-binding and cell-adherence polypeptides (e.g., corresponding strains not expressing a hTFF3-CmbA fusion polypeptide).

25 [00224] For example, an equal number of bacterial cells (e.g., based on cfu) of the bacterial strains, which either express hTFF3-CmbA, or do not express the fusion protein, can be administered to an individual (human or animal). Non-episomal strains +/- hTFF3-CmbA

can be equipped with an antibiotic selection marker (*e.g.*, pTINX, conveying erythromycin resistance). Episomal strains can be further selected based on their Em resistance. GI transit times may be measured by one of the following methods:

**Table 4**

5 ***Lactococcus lactis* Strains Constitutively Expressing Mucin-Binding and Cell-Binding Polypeptides**

| Strain   | Trehalose | BioAdhesion Construct<br>( <i>thyA</i> locus) |
|----------|-----------|-----------------------------------------------|
| sAGX0620 | wt        | <i>PthyA&gt;&gt;SSmbpL&gt;&gt;mbpL</i>        |
| sAGX0621 | (a)       | <i>PthyA&gt;&gt; SSmbpL &gt;&gt; mbpL</i>     |
| sAGX0624 | wt        | <i>PthyA&gt;&gt;SSmapA&gt;&gt;mapA</i>        |
| sAGX0625 | (a)       | <i>PthyA&gt;&gt; SSmapA &gt;&gt; mapA</i>     |
| sAGX0661 | (a)       | <i>PhlIA&gt;&gt;SSusp45-hTFF3-cmbA</i>        |

(\*) *PhlIA>>PTS*;  $\Delta$  *trePP*;  $\Delta$  *ptcC*; *usp45>>otsB*.

wt : wild-type (no trehalose accumulation)

- 10 [00225] (1) At regular, (*e.g.*, 1h) intervals, total feces can be collected and the number of cfu recovered of can be determined for each strain, *e.g.*, by Q-PCR or dilutive plating on solid agar containing erythromycin. This will yield for every strain a kinetic of recovery, which can show a time point of collection where the number of cfu recovered is maximal. It is observed that at time points beyond this maximum, more cfus are recovered for hTFF3-  
15 CmbA+ strains.

[00226] (2) At regular (*e.g.*, 1h) intervals, test animals (*e.g.*, mice) can be sacrificed, and the number of bacterial cfu recovered from those animals can be determined by dilutive plating on solid agar containing erythromycin. This shows that at progressing time, more cfus are recovered for hTFF3-CmbA+ strains.

20

**Example 10**

**Treatment of PKU - Gastrointestinal Degradation of Phenylalanine Using PAL  
Expressed by Genetically Modified Bacteria of the Current Disclosure**

- [00227] The enzyme phenylalanine ammonia lyase (PAL), which converts Phe into the cinnamic acid and does not require a co-factor, has been used to treat phenylketonuria (PKU).  
25 See, *e.g.*, Sarkissian, C. N. *et al.*, *Proc. Natl. Acad. Sci. USA* 2008, 105: 20894-20899.

Because PAL is rapidly degraded in the gastrointestinal (GI) tract, oral delivery is difficult to accomplish. A proof-of-concept study showed that oral administration of PAL expressed by genetically engineered *E. coli* in PKU (enu2/2) mice resulted in a significant reduction in plasma Phe levels. See, e.g., Sarkissian, C. N. *et al.*, *Proc. Natl. Acad. Sci. USA* 1999, 96:2339-2344. However, safety concerns associated with the administration of *E. coli* prevents the use of this strategy in humans. A *Lactococcus lactis* strain expressing PAL has been generated and evaluated in a PKU mouse model. Oral administration of the recombinant bacteria resulted in a reduction of deuterated Phe absorption. See, e.g., International Patent Application Publication WO 2014066945.

10 [00228] To allow for the administration of genetically modified bacteria expressing PAL to human PKU patients, the inventors have prepared "clinical grade" strains, which constitutively express the PAL enzyme, and are further modified to exhibit increased GI transit times, greater stability under GI conditions, and self-containment. Chromosomal integration of the bioactive polypeptide is important because episomal expression is associated with safety concerns. For example, episomes can be readily transmitted to other bacteria in the gastro-intestinal system. Further, episomal maintenance can provide hurdles for manufacturing. Additionally, known PAL producing strains are rapidly destroyed in the proximal GI tract. Further, there is a concern that the known PAL producing bacteria could survive and propagate outside of the body.

20 [00229] Multiple *L. lactis* strains with one or more of the following improved features were generated, e.g., as described herein above in Example 8: 1) a PAL expression cassette that is integrated into the bacterial chromosome and driven by a constitutive promoter for improved safety and ease of manufacture; 2) modifications that promote trehalose accumulation, which improves *L. lactis* survival in the GI tract (see, e.g., Termont, S. *et al.*, *Appl. Environ. Microbiol.* 2006, 72: 7694-7700); 3) incorporation of an auxotrophic dependency on thymidine to prevent survival outside the human body (see, e.g., Steidler, L. *et al.*, *Nat. Biotechnol.* 2003, 21: 785-789); and 4) genetic modifications to express mucoadhesive surface proteins to improve retention in the proximal GI tract and prolong Phe degradation (see, e.g., Caluwaerts, S. *et al.*, *Oral. Oncol.* 2010, 46:564-570). The strains were evaluated for PAL expression and their respective cell adherence and mucoadhesive properties as described in Examples 6 and 8. Surface expression of the adhesion protein improved bacterial retention on Caco-2 monolayers by approximately 8-fold. Adhesion to

both Type II and Type III mucins was similarly enhanced. Importantly, the trehalose modification for improved survival in the GI tract and the expression of the adhesion molecule did not alter PAL levels expressed by the bacteria.

[00230] Selected bacterial strains, *e.g.*, *Lactococcus lactis* strains (*e.g.*, strains constitutively expressing PAL; *e.g.*, sAGX0599, sAGX0644, aAGX0585, and sAGX0645) can be tested for their efficacy in the enu2/2 “PKU” mouse model to identify strains suitable for human clinical trials.

[00231] In the first study, sAGX0599, and sAGX0645 were tested in the PKU mice that received a dose of deuterated Phe concurrently with  $10^9$  cfu of *L. lactis* via gavage. Blood was sampled at 15 minute intervals for one hour and assayed for deuterated Phe. Positive control animals received a known episomal recombinant strain, *e.g.*, as described in International Patent Application Publication WO 2014066945). Negative control animals received no bacteria. As shown in **FIG. 9**, the total Phe absorbed by mice was lowest in mice fed sAGX0599 (secreting PAL) and sAGX0645 (PAL and TFF3-CmbA), and was superior to the positive control. Interestingly, the final level of Phe was lowest in the mice fed sAGX0645 (PAL and TFF3-CmbA), but the Cmax of Phe was higher. This may reflect that sAGX0645 is moving slower down the small bowel towards the particular site of Phe uptake compared to *L. lactis* without the adhesion fusion protein. A lower Cmax and AUC may be obtained by administration of the Phe-degrading bacteria can prior to feeding. A lower Cmax and AUC may also be obtained by regular administration of the bacteria with a meal such that distal Phe-degrading bacteria to reduce Phe from one meal are already present from a previous meal.

[00232] It should be noted that feeding Phe is an artificial model of PKU. Single amino acids are very efficiently absorbed. In typical situations, proteins are broken down as they pass through the intestine such that free amino acids available for absorption occur primarily in more distal portions than the upper small intestine. Thus, a PAL-secreting bacterium located in the more distal portion of the intestine may be able to better block Phe uptake from food than as suggested by experiments administering Phe directly.

[00233] Moreover, *L. lactis* may be modified with different cell and mucus adhesion molecules to localize the delivery of PAL to the optimal section(s) of the intestine.

[00234] A second study may assess the pharmacodynamics of the selected bacterial strains, *e.g.*, *Lactococcus lactis* strains. Bacteria can be administered up to six hours before the deuterated Phe. Blood can be assayed as described above. The resulting data can inform the scheduling for subsequent studies and provide information relevant to clinical application.

5 For example, humans may take one unit dosage form (*e.g.*, capsule) containing the bacteria with each meal.

[00235] A third study may assess the long-term effectiveness of the bacteria in lowering Phe blood levels. Animals can be gavaged with a selected bacterial strain (*e.g.*, *Lactococcus lactis* strain), *e.g.*, optimal strain from the first study, and can then be fed a standard chow

10 diet. For example, two *L. lactis*-associated feedings each day with the duration for each feeding determined by the outcome from the second. Blood Phe levels can be assessed regularly, *e.g.*, semi-weekly for three weeks. Controls can be those employed in the first study, and can also include a low Phe diet cohort. Note: this study may not use deuterated Phe. A positive outcome can be a statistically significant improvement in Phe blood levels,

15 *e.g.*, sufficient to likely result in clinical efficacy. Other studies can combine bacterial therapy (*e.g.*, *L. lactis* therapy) with dietary intervention.

[00236] Each study can utilize cohorts of 5 mice to provide sufficient power for statistical analyses, and mice can be reused after a washout. Phe can be assayed by tandem mass spectrometry, which is both sensitive and specific and requires minimal amounts of blood.

20

### Example 11

#### **Treatment of oral mucositis**

[00237] Oral mucositis is a breakdown of the oral mucosa and is a common complication of cancer therapy, especially for treatment of head and neck cancer. TFF1 is secreted in the upper GI tract and is associated with protection and healing of mucosal surfaces. TFF1

25 shows promise as a treatment for oral mucositis. To increase delivery of TFF1 at the oral mucosa, *L. lactis* is engineered to express both an hTFF1-CmbA fusion along with “free” hTFF1, both on the chromosome. If this was done step wise, there would be risk that one *htff1* gene would recombine with another *htff1* gene on the chromosome. To minimize this risk, *hTFF1-cmbA* and *htff1* can be constructed polycistronically, and are therefore able to

30 transform and integrate into the chromosome at one step. Further, the third base in each codon can be changed in one *tff1* sequence so that its nucleotide sequence differs from the other

tffl sequence, but the translated hTFF1 is the same. For example, there are six alanine codons with the following frequency: UCU (18.6), UCC (4.0), UCA (20.6), UCG (3.9), AGU (16.7), and AGC (7.3). Therefore, UCC and UCG may be interchangeable. UCU, UCA, and to a lesser extent AGU, may be interchangeable.

- 5 [00238] After modifying the codon frequency, the *Phl1A*>>*SSusp45*>>*hTFF1*>>*rpmD*>>*SSusp45*>>*hTFF1-CmbA* is constructed through PCR, and is cloned between the 5' *thyA* and 3' *thyA* on a conditionally replicative carrier plasmid derived from pORI19. Transformation into *L. lactis* and selection, as described elsewhere herein, lead to integration into chromosomal *thyA* locus.
- 10 [00239] The resulting strain is shown to secrete TFF1 and express a TFF-CmbA fusion that mediates increased binding to oral mucus compared with a strain expressing TFF1 alone. The strain is tested in models of mucositis, and the presence of the TFF-CmbA increases persistence in the oral cavity and increases delivery of therapeutic TFF1, such that the frequency of dosing may be decreased.

15

### Example 12

#### **Treatment of diabetes**

- [00240] The *Lactococcus lactis* strain secreting hTFF3-CmbA, as described above, is modified to express human proinsulin PINS and human IL10. For example, a construct for hPINS expression may be hPINS with a SSUsp45 secretion leader, under the control of the strong *gapB* promoter and after the *gapB* gene and *rpmD* spacer (*i.e.*, *PgapB* >>*gapB* >> *rpmD* >> *usp45* >>PINS). For hIL-10, a construct may be *Phl1A*>>*SSusp45*>>*hil-10*.
- 20

- [00241] Because hTFF3-CmbA are already inserted into the *thyA* locus, PINS and IL-10 may be inserted into another genomic site, such as *ptcC* or *trePP*. *PtcC* and *trePP* mutations are associated with trehalose accumulation. The resulting strain is tested for expression of hIL-10, PINS and hTFF3-CmbA. The strain is then tested in NOD mice, an animal model of type 1 diabetes, against a strain lacking hTFF3-CmbA. The strain lacking hTFF3-CmbA has previously been shown to generate a Treg response to PINS, reverse the autoimmune response to beta cells of the pancreas, and thereby treat diabetes. The treatment is especially efficacious in recent onset disease. The presence of the hTFF-CmbA may increase gut
- 25
- 30 colonization and persistence, resulting in a stronger Treg response.

[00242] While some embodiments have been shown and described herein, such  
embodiments are provided by way of example only. Numerous variations, changes, and  
substitutions will now occur to those skilled in the art without departing from the invention.  
It should be understood that various alternatives to the embodiments of the invention  
5 described herein will be employed in practicing the invention.

### Exemplary embodiments

1. A bacterium comprising an exogenous nucleic acid encoding a fusion protein, wherein  
said fusion protein comprises a cell-adherence polypeptide.
- 10 2. The bacterium of embodiment 1, wherein said cell-adherence polypeptide is selected  
from the group consisting of cell and mucus-binding protein A (CmbA), mucus binding  
protein (Mub), mucus adhesion promoting protein (MapA), lactococcal mucin binding  
protein (MbpL), and any combination thereof.
3. The bacterium of embodiment 2, wherein said cell-adherence polypeptide is CmbA.
- 15 4. The bacterium of embodiment 3, wherein said CmbA is *Lactobacillus reuteri* CmbA.
5. The bacterium of any one of the preceding embodiments, wherein said fusion protein  
further comprises a mucin-binding polypeptide.
6. The bacterium of embodiment 5, wherein said mucin-binding polypeptide is a trefoil  
factor (TFF) polypeptide.
- 20 7. The bacterium according to embodiment 6, wherein said fusion protein comprises  
CmbA and a TFF.
8. The bacterium of any one of embodiments 1 to 7, wherein said exogenous nucleic acid  
encoding a fusion protein is integrated into the chromosome of said bacterium.
9. The bacterium of any one of embodiments 1 to 7, wherein said exogenous nucleic acid  
25 encoding a fusion protein is located on a plasmid.
10. The bacterium of any one of the preceding embodiments, wherein said fusion protein is  
expressed by said bacterium.
11. The bacterium of embodiment 10, wherein said fusion protein is anchored in a cell wall  
of said bacterium.

12. The bacterium of any one of the preceding embodiments, wherein said exogenous nucleic acid encoding a fusion protein further comprises a secretion leader sequence encoding a secretion signal peptide.
13. The bacterium of embodiment 12, wherein said secretion leader sequence is SSusp45.
- 5 14. The bacterium of embodiment 13, wherein said secretion leader sequence encodes a secretion signal peptide having an amino acid sequence that is at least 90% identical to SEQ ID NO: 5.
15. The bacterium of any one of embodiments 12 to 14, wherein said secretion signal peptide is bound to said mucin-binding polypeptide.
- 10 16. The bacterium of any one of embodiments 12 to 15, wherein said secretion signal peptide is bound to a linker.
17. The bacterium of any one of embodiments 12 to 16, wherein said secretion signal peptide is cleaved from said fusion protein.
18. The bacterium of embodiment 17, wherein said secretion signal peptide is cleaved from  
15 said fusion protein when said fusion protein is anchored in a cell wall of said bacterium.
19. The bacterium of any one of embodiments 1 to 18, wherein said exogenous nucleic acid encoding a fusion protein is transcriptionally regulated by a promoter selected from the group consisting of a *thyA* promoter (*PthyA*), an *hlla* promoter (*PhllA*), and a *gapB* promoter.
- 20 20. The bacterium of embodiment 19, wherein said exogenous nucleic acid encoding a fusion protein is transcriptionally regulated by *PthyA*.
21. The bacterium of embodiment 19, wherein said exogenous nucleic acid encoding a fusion protein is transcriptionally regulated by *PhllA*.
22. A bacterium comprising a fusion protein anchored in a cell-wall of said bacterium,  
25 wherein said fusion protein comprises a TFF polypeptide and a CmbA polypeptide.
23. The bacterium of any one of embodiments 1 to 22, wherein said bacterium is a Gram-positive bacterium.
24. The bacterium of embodiment 23, wherein said Gram-positive bacterium is non-pathogenic.

25. The bacterium of any one of embodiments 1 to 24, wherein said bacterium is a lactic acid bacterium (LAB).
26. The bacterium of embodiment 25, wherein said LAB is selected from the group consisting of a *Lactococcus* species (sp.) bacterium, a *Lactobacillus* sp. bacterium, a *Bifidobacterium* sp. bacterium, a *Streptococcus* sp. bacterium, and an *Enterococcus* sp. bacterium.
27. The bacterium of embodiment 25, wherein said LAB is selected from the group consisting of *Lactococcus garvieae*, *Lactococcus lactis*, *Lactococcus piscium*, *Lactococcus plantarum*, *Lactococcus raffinolactis*, *Lactobacillus acetotolerans*, *Lactobacillus acidophilus*, *Lactobacillus agilis*, *Lactobacillus algidus*, *Lactobacillus alimentarius*, *Lactobacillus amylolyticus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus aviarius*, *Lactobacillus aviarius* subsp. *araffinosus*, *Lactobacillus aviarius* subsp. *aviarius*, *Lactobacillus bavaricus*, *Lactobacillus bif fermentans*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus bulgaricus*, *Lactobacillus carnis*, *Lactobacillus casei*, *Lactobacillus casei* subsp. *alactosus*, *Lactobacillus casei* subsp. *casei*, *Lactobacillus casei* subsp. *pseudoplan tarum*, *Lactobacillus casei* subsp. *rhamnosus*, *Lactobacillus casei* subsp. *tolerans*, *Lactobacillus catenaformis*, *Lactobacillus cellobiosus*, *Lactobacillus collinoides*, *Lactobacillus confusus*, *Lactobacillus coryniformis*, *Lactobacillus coryniformis* subsp. *coryniformis*, *Lactobacillus coryniformis* subsp. *torquens*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus curvatus* subsp. *curvatus*, *Lactobacillus curvatus* subsp. *melibiosus*, *Lactobacillus delbrueckii*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus divergens*, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus fornicalis*, *Lactobacillus fructivorans*, *Lactobacillus fructosus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus graminis*, *Lactobacillus halotolerans*, *Lactobacillus hamsteri*, *Lactobacillus helveticus*, *Lactobacillus heterohiochii*, *Lactobacillus hilgardii*, *Lactobacillus homohiochii*, *Lactobacillus iners*, *Lactobacillus intestinalis*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*, *Lactobacillus kandleri*, *Lactobacillus kefir*, *Lactobacillus kefirano faciens*, *Lactobacillus kefirgranum*, *Lactobacillus kunkeei*, *Lactobacillus lactis*, *Lactobacillus leichmannii*, *Lactobacillus*

lindneri, *Lactobacillus malefermentans*, *Lactobacillus mali*, *Lactobacillus*  
*maltaromicus*, *Lactobacillus manihotivorans*, *Lactobacillus minor*, *Lactobacillus*  
*minutus*, *Lactobacillus mucosae*, *Lactobacillus murinus*, *Lactobacillus nagelii*,  
 5 *Lactobacillus oris*, *Lactobacillus panis*, *Lactobacillus parabuchneri*, *Lactobacillus*  
*paracasei*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus paracasei* subsp.  
*tolerans*, *Lactobacillus parakefiri*, *Lactobacillus paralimentarius*, *Lactobacillus*  
*paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus perolens*, *Lactobacillus*  
*piscicola*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus reuteri*,  
 10 *Lactobacillus rhamnosus*, *Lactobacillus rimae*, *Lactobacillus rogosae*, *Lactobacillus*  
*ruminis*, *Lactobacillus sakei*, *Lactobacillus sakei* subsp. *camosus*, *Lactobacillus sakei*  
 subsp. *sakei*, *Lactobacillus salivarius*, *Lactobacillus salivarius* subsp. *salicinius*,  
*Lactobacillus salivarius* subsp. *salivarius*, *Lactobacillus sanfranciscensis*,  
*Lactobacillus sharpeae*, *Lactobacillus suebicus*, *Lactobacillus trichodes*, *Lactobacillus*  
 15 *uli*, *Lactobacillus vaccinostercus*, *Lactobacillus vaginalis*, *Lactobacillus viridescens*,  
*Lactobacillus vitulinus*, *Lactobacillus xylosus*, *Lactobacillus yamanashiensis*,  
*Lactobacillus yamanashiensis* subsp. *mali*, *Lactobacillus yamanashiensis* subsp.  
*Yamanashiensis*, *Lactobacillus zae*, *Bifidobacterium adolescentis*, *Bifidobacterium*  
*angulatum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium*  
*catenulatum*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Enterococcus*  
 20 *alcedinis*, *Enterococcus aquimarinus*, *Enterococcus asini*, *Enterococcus avium*,  
*Enterococcus caccae*, *Enterococcus camelliae*, *Enterococcus canintestini*, *Enterococcus*  
*canis*, *Enterococcus casseliflavus*, *Enterococcus cecorum*, *Enterococcus columbae*,  
*Enterococcus devriesei*, *Enterococcus diestrammenae*, *Enterococcus dispar*,  
*Enterococcus durans*, *Enterococcus eurekensis*, *Enterococcus faecalis*, *Enterococcus*  
 25 *faecium*, *Enterococcus gallinarum*, *Enterococcus gilvus*, *Enterococcus*  
*haemoperoxidus*, *Enterococcus hermannienseis*, *Enterococcus hiraе*, *Enterococcus*  
*italicus*, *Enterococcus lactis*, *Enterococcus lemanii*, *Enterococcus malodoratus*,  
*Enterococcus moraviensis*, *Enterococcus mundtii*, *Enterococcus olivae*, *Enterococcus*  
 30 *pallens*, *Enterococcus phoeniculicola*, *Enterococcus plantarum*, *Enterococcus*  
*pseudoavium*, *Enterococcus quebecensis*, *Enterococcus raffinosus*, *Enterococcus ratti*,  
*Enterococcus rivorum*, *Enterococcus rotai*, *Enterococcus saccharolyticus*,  
*Enterococcus silesiacus*, *Enterococcus solitarius*, *Enterococcus sulfureus*, *Enterococcus*  
*termitis*, *Enterococcus thailandicus*, *Enterococcus ureasiticus*, *Enterococcus*

- ureilyticus, *Enterococcus viikkiensis*, *Enterococcus villorum*, *Enterococcus xiangfangensis*, *Streptococcus agalactiae*, *Streptococcus anginosus*, *Streptococcus bovis*, *Streptococcus canis*, *Streptococcus constellatus*, *Streptococcus dysgalactiae*, *Streptococcus equinus*, *Streptococcus iniae*, *Streptococcus intermedius*, *Streptococcus milleri*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus parasanguinis*, *Streptococcus peroris*, *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, *Streptococcus pyogenes*, *Streptococcus rattus*, *Streptococcus salivarius*, *Streptococcus tigurinus*, *Streptococcus thermophilus*, *Streptococcus sanguinis*, *Streptococcus sobrinus*, *Streptococcus suis*, *Streptococcus uberis*, *Streptococcus vestibularis*, *Streptococcus viridans*, and *Streptococcus zooepidemicus*.
28. The bacterium of embodiment 27, wherein said bacterium is *Lactococcus lactis*.
29. The bacterium of embodiment 28, wherein said *Lactococcus lactis* is selected from the group consisting of *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *hordniae*, and *Lactococcus lactis* subsp. *lactis*.
30. The bacterium of any one of embodiments 6 to 29, wherein said TFF polypeptide is selected from the group consisting of TFF1, TFF2, and TFF3.
31. The bacterium of any one of embodiments 6 to 29, wherein said TFF polypeptide is selected from the group consisting of human TFF, mouse TFF, pig TFF, dog TFF, cat TFF, cow TFF, sheep TFF, fish TFF, and amphibial TFF.
32. The bacterium of embodiment 31, wherein said TFF polypeptide is human TFF.
33. The bacterium of embodiment 32, wherein said human TFF is human TFF3.
34. The bacterium of any one of embodiment 6 to 29, wherein said TFF polypeptide has an amino acid sequence at least 80% identical to SEQ ID NO: 3.
35. The bacterium of any one of embodiments 6 to 29, wherein said TFF polypeptide is a TFF variant polypeptide.
36. The bacterium of embodiment 35, wherein said TFF variant polypeptide has enhanced mucin-binding capability compared to a corresponding wild-type TFF polypeptide.
37. The bacterium of any one of embodiments 1 to 36, wherein said CmbA polypeptide has an amino acid sequence at least 80% identical to SEQ ID NO: 1.

38. The bacterium of any one of embodiments 1 to 37, further comprising an exogenous nucleic acid encoding at least one therapeutic polypeptide.
39. The bacterium of embodiment 38, wherein said at least one therapeutic polypeptide is a cytokine.
- 5 40. The bacterium of embodiment 38 or 39, wherein said at least one therapeutic polypeptide is an interleukin (IL).
41. The bacterium of embodiment 40, wherein said interleukin is selected from the group consisting of IL-2, IL-10, IL-18, and any combinations thereof.
42. The bacterium of embodiment 38, wherein said at least one therapeutic polypeptide is an antigen.
- 10 43. The bacterium of embodiment 42, wherein said at least one therapeutic polypeptide is an antigen and an interleukin.
44. The bacterium of embodiment 43, wherein said interleukin is IL-2 or IL-10.
45. The bacterium of any one of embodiments 42 to 44, wherein said antigen is an autoantigen.
- 15 46. The bacterium of embodiment 45, wherein said autoantigen is a type-1 diabetes (T1D)-specific antigen.
47. The bacterium of embodiment 46, wherein said T1D-specific antigen is selected from the group consisting of proinsulin (PINS), glutamic acid decarboxylase (GAD65), insulinoma-associated protein 2 (IA-2), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), zinc transporter 8 (ZnT8), chromogranin A, (prepro) islet amyloid polypeptide (ppIAPP), peripherin, citrullinated glucose-regulated protein (GRP), and any combinations thereof.
- 20 48. The bacterium of embodiment 38, wherein said at least one therapeutic polypeptide is an antibody or a fragment thereof.
- 25 49. The bacterium of embodiment 48, wherein said antibody is a single-domain antibody or a nanobody.
50. The bacterium of embodiment 48 or 49, wherein said antibody is selected from the group consisting of an anti TNF $\alpha$  antibody, an antibody to IL-4, an antibody to IL-5, an

antibody to IL-13, an antibody to IL-15, an antibody to immunoglobulin E (IgE), and any combinations thereof.

51. The bacterium of embodiment 48 or 49, wherein said antibody is a fusion protein comprising a soluble TNF receptor and an Fc region of an antibody.
- 5 52. The bacterium of embodiment 38, wherein said at least one therapeutic polypeptide is an enzyme or fragment thereof.
53. The bacterium of embodiment 52, wherein said enzyme or fragment thereof is functional.
54. The bacterium of embodiment 52 or 53, wherein said enzyme or fragment thereof is  
10 selected from the group consisting of a phenylalanine ammonia lyase (PAL), an amino acid decarboxylase, and any combinations thereof.
55. The bacterium of embodiment 54, wherein said enzyme or fragment thereof is PAL.
56. The bacterium of embodiment 38, wherein said at least one therapeutic polypeptide is selected from the group consisting of glucagon-like peptide 1 (GLP-1), glucagon-like  
15 peptide 2 (GLP-2), glucagon, exendin-4, and any combinations thereof.
57. The bacterium of embodiment 38, wherein said at least one therapeutic polypeptide is a growth factor.
58. The bacterium of embodiment 57, wherein said growth factor is epidermal growth factor (EGF).
- 20 59. The bacterium of embodiment 58, wherein said EGF is human EGF or porcine EGF.
60. The bacterium of embodiment 38, wherein said at least one therapeutic polypeptide is a TFF.
61. The bacterium of embodiment 60, wherein said TFF is selected from the group consisting of TFF 1, TFF2, TFF3, and any combinations thereof.
- 25 62. The bacterium of any one of embodiments 38 to 61, wherein said therapeutic polypeptide is a human polypeptide.
63. The bacterium of any one of embodiments 42 to 44, wherein said antigen is an allergen.
64. The bacterium of embodiment 63, wherein said allergen is selected from the group consisting of a tree pollen allergen, a weed pollen allergen, a grass pollen allergen, a

food allergen, a dust-mite allergen, a mold allergen, an animal dander allergen, and any combinations thereof.

65. The bacterium of embodiment 64, wherein said allergen is a weed pollen allergen.
66. The bacterium of embodiment 65, wherein said weed pollen allergen is a ragweed  
5 pollen allergen.
67. The bacterium of embodiment 64, wherein said allergen is a tree pollen allergen.
68. The bacterium of embodiment 67, wherein said tree pollen allergen is a birch pollen allergen or a Japanese cedar pollen allergen.
69. The bacterium of embodiment 64, wherein said allergen is a food allergen.
- 10 70. The bacterium of embodiment 69, wherein said food allergen is selected from the group consisting of a peanut allergen, a milk allergen, an egg allergen, a gluten allergen (gliadin epitope), and any combinations thereof.
71. The bacterium of any one of embodiments 38 to 70, wherein said exogenous nucleic acid encoding at least one therapeutic polypeptide is transcriptionally regulated by a  
15 gapB promoter.
72. The bacterium of any one of embodiments 1 to 71, wherein said bacterium has an increased gastro-intestinal (GI) transit time when compared to a corresponding bacterium not comprising said exogenous nucleic acid or not comprising said fusion protein.
- 20 73. The bacterium of embodiment 72, wherein said GI transit time is increased by at least about 10 %.
74. The bacterium of embodiment 73, wherein said GI transit time is increased by at least about 30 %.
75. The bacterium of embodiment 72, wherein said GI transit time is increased from at least  
25 about 10 % to about 50 %.
76. The bacterium of any one of embodiments 1 to 75, wherein said bacterium has increased *in vitro* mucin-binding capability when compared to a corresponding bacterium not comprising said exogenous nucleic acid or not comprising said fusion protein.

77. The bacterium of embodiment 76, wherein said *in vitro* mucin-binding capability is increased by at least about 20 %.
78. The bacterium of embodiment 77, wherein said *in vitro* mucin-binding capability is increased by at least about 50 %.
- 5 79. The bacterium of embodiment 78, wherein said *in vitro* mucin-binding capability is increased by at least about 100 % (about 2×).
80. The bacterium of embodiment 76, wherein said *in vitro* mucin-binding capability is increased from at least about 20 % to about 500 %.
81. The bacterium of any one of embodiments 76 to 80, wherein said *in vitro* mucin-binding capability is measured by binding said bacterium to mucins from porcine stomach followed by:
- 10 (a) detecting light absorbance at 405 nm (OD<sub>405</sub>); or
- (b) staining said bacterium with crystal violet and subsequently detecting light absorbance at 595 nm (OD<sub>595</sub>).
- 15 82. The bacterium of any one of embodiments 1 to 81, wherein said bacterium has increased *in vitro* Caco-2 cell-binding capability when compared to a corresponding bacterium not comprising said exogenous nucleic acid or not comprising said fusion protein.
83. The bacterium of embodiment 82, wherein said *in vitro* Caco-2 binding capability is increased by at least about 10%.
- 20 84. The bacterium of embodiment 83, wherein said *in vitro* Caco-2 binding capability is increased by at least about 100% (about 2×).
85. The bacterium of embodiment 84, wherein said *in vitro* Caco-2 binding capability is increased by at least about 400% (about 5×).
- 25 86. The bacterium of embodiment 82, wherein said *in vitro* Caco-2 binding capability is increased from by at least about 10 % to about 500%.
87. The bacterium of any one of embodiments 82 to 86, wherein said *in vitro* Caco-2 binding capability is measured by:
- (a) contacting a culture of said bacterium with Caco-2 cells;

- (b) washing said Caco-2 cells to remove unbound bacterial cells;
- (c) detaching bacterial cells bound to said Caco-2 cells, thereby forming detached bacterial cells; and
- (d) titering said detached bacterial cells.

- 5
88. The bacterium of any one of embodiments 1 to 87, wherein said bacterium exhibits increased adherence to intestinal mucosa when compared to a corresponding bacterium not comprising said exogenous nucleic acid or not comprising said fusion protein.
- 10
89. The bacterium of embodiment 88, wherein said adherence to intestinal mucosa is increased from at least about 10% to about 500%.
90. A composition comprising the bacterium of any one of embodiments 1 to 89.
91. A pharmaceutical composition comprising the bacterium of any one of embodiments 1 to 89, and a pharmaceutically acceptable carrier.
- 15
92. The bacterium of any one of embodiments 1 to 89, the composition of embodiment 90, or the pharmaceutical composition of embodiment 91, for use in the treatment or prevention of a disease selected from the group consisting of an autoimmune disease, an allergy, a metabolic disease, and a gastro-intestinal disease.
- 20
93. The bacterium of any one of embodiments 1 to 89, the composition of embodiment 90, or the pharmaceutical composition of embodiment 91, for use in the preparation of a medicament for the treatment or prevention of a disease.
94. The bacterium, the composition, or pharmaceutical composition of embodiment 93, wherein said disease is selected from the group consisting of an autoimmune disease, an allergy, a metabolic disease, a gastro-intestinal disease, and a nutritional defect.
- 25
95. A method for the treatment of a disease in a subject in need thereof comprising administering to said subject a therapeutically effective amount of the bacterium of any one of embodiments 1 to 89, the composition of embodiment 90, or the pharmaceutical composition of embodiment 91.
- 30
96. The method of embodiment 95, wherein said disease is selected from the group consisting of an autoimmune disease, an allergy, a metabolic disease, a gastro-intestinal disease, and any combination thereof.
97. The method of embodiment 96, wherein said disease is an autoimmune disease.

98. The method of embodiment 97, wherein said autoimmune disease is type-1 diabetes (T1D).
99. The method of embodiment 96, wherein said disease is a metabolic disease.
100. The method of embodiment 99, wherein said metabolic disease is phenylketonuria  
5 (PKU).
101. The method of embodiment 96, wherein said disease is a gastro-intestinal disease.
102. The method of embodiment 101, wherein said gastro-intestinal disease is celiac disease.
103. The method of embodiment 101, wherein said gastro-intestinal disease is inflammatory bowel disease (IBD).
- 10 104. The method of embodiment 103, wherein said IBD is Crohn's disease or ulcerative colitis.
105. A method for preparing a genetically modified bacterium comprising contacting a bacterium with an exogenous nucleic acid encoding a fusion protein, wherein said exogenous nucleic acid encoding a fusion protein comprises a sequence encoding a cell-  
15 adherence polypeptide.
106. The method of embodiment 105, wherein said contacting occurs under conditions sufficient for said bacterium to internalize said exogenous nucleic acid.
107. The method of embodiment 105 or 106, wherein said exogenous nucleic acid is located on a plasmid.
- 20 108. The method of embodiment 106 or 107, wherein said exogenous nucleic acid is integrated into a chromosome of said bacterium.
109. The method of any one of embodiments 105 to 108, further comprising culturing said bacterium and expressing said fusion protein in said bacterium.
110. The method of any one of embodiments 105 to 109, wherein said cell-adherence  
25 polypeptide is selected from the group consisting of cell and mucus-binding protein A (CmbA), Mub, MapA, MbpL, and any combinations thereof.
111. The method of embodiment 110, wherein said cell-adherence polypeptide is CmbA.
112. The method of embodiment 111, wherein said CmbA is *Lactobacillus reuteri* CmbA.

113. The method of any one of embodiments 105 to 112, wherein said exogenous nucleic acid encoding a fusion protein further comprises a sequence encoding a mucin-binding polypeptide.
114. The method of embodiment 113, wherein said mucin-binding polypeptide is a trefoil factor (TFF) polypeptide.
- 5 115. The method of embodiment 114, wherein said exogenous nucleic acid encoding a fusion protein comprises a sequence encoding CmbA and a sequence encoding a TFF polypeptide.
116. The method of any one of embodiments 105 to 115, wherein said exogenous nucleic acid encoding a fusion protein is integrated into the chromosome of said bacterium.
- 10 117. The method of embodiment 116, wherein said exogenous nucleic acid is integrated into the chromosome of said bacterium using homologous recombination.
118. The method of any one of embodiments 105 to 117, further comprising contacting said bacterium with an exogenous nucleic acid encoding a therapeutic polypeptide.
- 15 119. The method of embodiment 118, wherein said contacting said bacterium with an exogenous nucleic acid encoding a therapeutic polypeptide occurs prior to said contacting said bacterium with an exogenous nucleic acid encoding a fusion protein.
120. The method of embodiment 118, wherein said contacting said bacterium with an exogenous nucleic acid encoding a therapeutic polypeptide occurs subsequent to said contacting said bacterium with an exogenous nucleic acid encoding a fusion protein.
- 20 121. The method of any one of embodiments 105 to 120, wherein said genetically modified bacterium exhibits increased muco- and cell-adhesive properties when compared to a corresponding bacterium not comprising said exogenous nucleic acid encoding a fusion protein.
- 25 122. The method of any one of embodiments 105 to 121, further comprising combining a culture of said genetically modified bacterium with at least one cryopreserving agent to form a bacterial mixture.
123. The method of embodiment 122 further comprising freeze-drying said bacterial mixture to form a freeze-dried composition.

124. The method of any one of embodiments 105 to 121 or embodiment 123 further comprising combining said genetically modified bacterium, or said freeze-dried composition with a pharmaceutically acceptable carrier to form a pharmaceutical composition.
- 5 125. The method of embodiment 123 or 124 further comprising formulating said freeze-dried composition or said pharmaceutical composition into a pharmaceutical dosage form.
126. A genetically modified bacterium prepared by the method of any one of embodiments 105 to 125.
127. A unit dosage form comprising the bacterium of any one of embodiments 1 to 89, the  
10 composition of embodiment 90, or the pharmaceutical composition of embodiment 91.
128. The unit dosage form of embodiment 127, wherein said unit dosage form is an oral dosage form.
129. The unit dosage form of embodiment 128, wherein said oral dosage form is selected from the group consisting of a tablet, a capsule, a sachet, and a packaged liquid.
- 15 130. A method for enhancing growth in a mammal comprising administering to said mammal an effective amount of the bacterium of any one of embodiments 1 to 89, the composition of embodiment 90, the pharmaceutical composition of embodiment 91, or the unit dosage form of any one of embodiments 127 to 129.
131. The method of embodiment 130, wherein said mammal is selected from the group  
20 consisting of a human, a pig, a cow, and a sheep.
132. The method of embodiment 130 or 131, wherein said bacterium, said composition, said pharmaceutical composition, or said unit dosage form is formulated for administration to said mammal.
133. The method of any one of embodiments 130 to 132, wherein said bacterium comprises  
25 an exogenous nucleic acid encoding a growth factor.
134. The method of embodiment 133, wherein said growth factor is constitutively expressed in said bacterium.
135. The method of embodiment 133 or 134, wherein said growth factor is EGF.

136. The method of embodiment 135, wherein said mammal is a pig and said EGF is porcine EGF.
137. A method of increasing binding of a bacterium to intestinal mucosa comprising:
- 5 (a) contacting said microorganism with an exogenous nucleic acid encoding a fusion protein, wherein said exogenous nucleic acid encoding a fusion protein comprises a sequence encoding a CmbA polypeptide; and
- (b) expressing said exogenous nucleic acid encoding a fusion protein in said bacterium.
138. The method of embodiment 137, wherein said exogenous nucleic acid encoding a fusion protein further comprises a sequence encoding a TFF polypeptide.
- 10 139. The method of embodiment 138, wherein expression of said exogenous nucleic acid encoding a fusion protein produces a fusion protein comprising said TFF and said CmbA.
140. A kit comprising (1) a bacterium according to any one of embodiments 1 to 89, a composition according to embodiment 90, a pharmaceutical composition of embodiment 91, or a unit dosage form of any one of embodiments 127 to 129, and (2)
- 15 instructions for administering said bacterium, said composition, said pharmaceutical composition, or said unit dosage form to a mammal.
141. The kit of embodiment 140, wherein said mammal is a human.
- 20 142. A nucleic acid encoding a fusion protein, said nucleic acid comprising:
- (i) a sequence encoding a cell-adherence polypeptide selected from the group consisting of cell and mucus-binding protein A (CmbA), Mub, MapA, MbpL, and any combinations thereof; and
- (ii) a sequence encoding a mucin-binding polypeptide selected from a TFF
- 25 polypeptide, MubBP, and a combination thereof.
143. The nucleic acid of embodiment 142, wherein said mucin-binding polypeptide is a TFF polypeptide.
144. The nucleic acid of embodiment 143, wherein said TFF polypeptide is TFF3.
145. The nucleic acid of any one of embodiments 142 to 144, wherein said cell-adherence
- 30 polypeptide is CmbA.
146. A plasmid comprising the nucleic acid of any one of embodiments 142 to 145.

147. A bacterial host cell comprising the plasmid of embodiment 146.