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Haught et al.

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(54) **DETECTION OF ORAL MICROBIAL
VIRULENCE FACTORS**

(2013.01); *G01N 2333/705* (2013.01); *G01N 2333/70596* (2013.01); *G01N 2400/50* (2013.01)

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(58) **Field of Classification Search**

CPC *G01N 33/566*
See application file for complete search history.

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(57) **ABSTRACT**

Methods for detecting and quantifying toxins present in the oral cavity. The methods may include providing a biological sample, providing reporter cells expressing one or more Toll like receptors, exposing the cells to the biological sample, measuring the EC50 value of the lipopolysaccharide on activation of a Toll like receptor, quantification of the lipopolysaccharide in the biological sample.

9 Claims, 31 Drawing Sheets

Specification includes a Sequence Listing.

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U.S.C. 154(b) by 0 days.

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(65) **Prior Publication Data**

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16, 2016, provisional application No. 62/157,659,
filed on May 6, 2015.

(51) **Int. Cl.**

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G01N 33/566	(2006.01)
G01N 33/50	(2006.01)
C12Q 1/6897	(2018.01)
G01N 33/579	(2006.01)
A61K 8/02	(2006.01)
A61K 8/21	(2006.01)
A61K 8/36	(2006.01)
A61K 8/39	(2006.01)
A61K 8/42	(2006.01)
A61K 8/49	(2006.01)
A61Q 11/00	(2006.01)

(52) **U.S. Cl.**

CPC *G01N 33/566* (2013.01); *A61K 8/0204*
(2013.01); *A61K 8/21* (2013.01); *A61K 8/361*
(2013.01); *A61K 8/39* (2013.01); *A61K 8/42*
(2013.01); *A61K 8/4926* (2013.01); *A61Q 11/00* (2013.01); *C12Q 1/6897* (2013.01);
G01N 33/5041 (2013.01); *G01N 33/5044*
(2013.01); *G01N 33/5091* (2013.01); *G01N 33/579* (2013.01); *A61K 2800/74* (2013.01);
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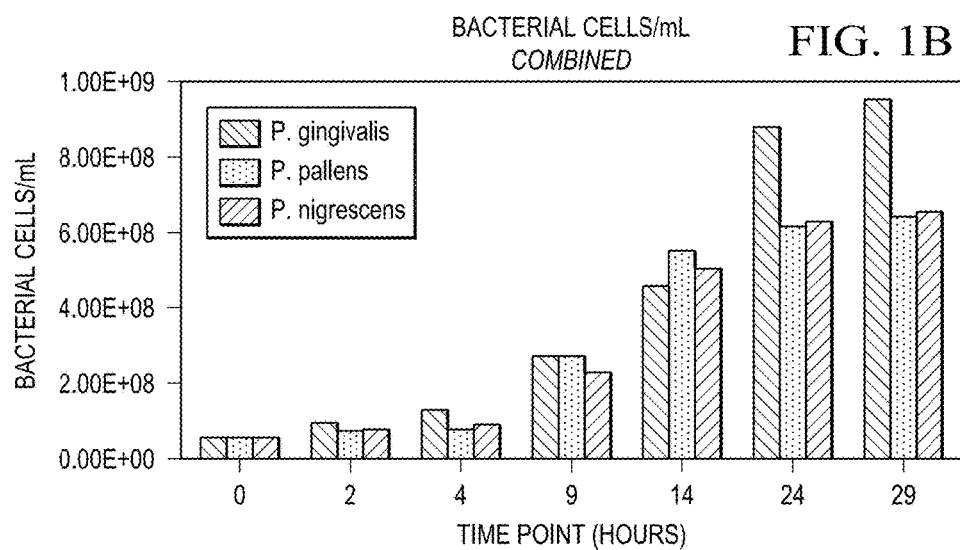
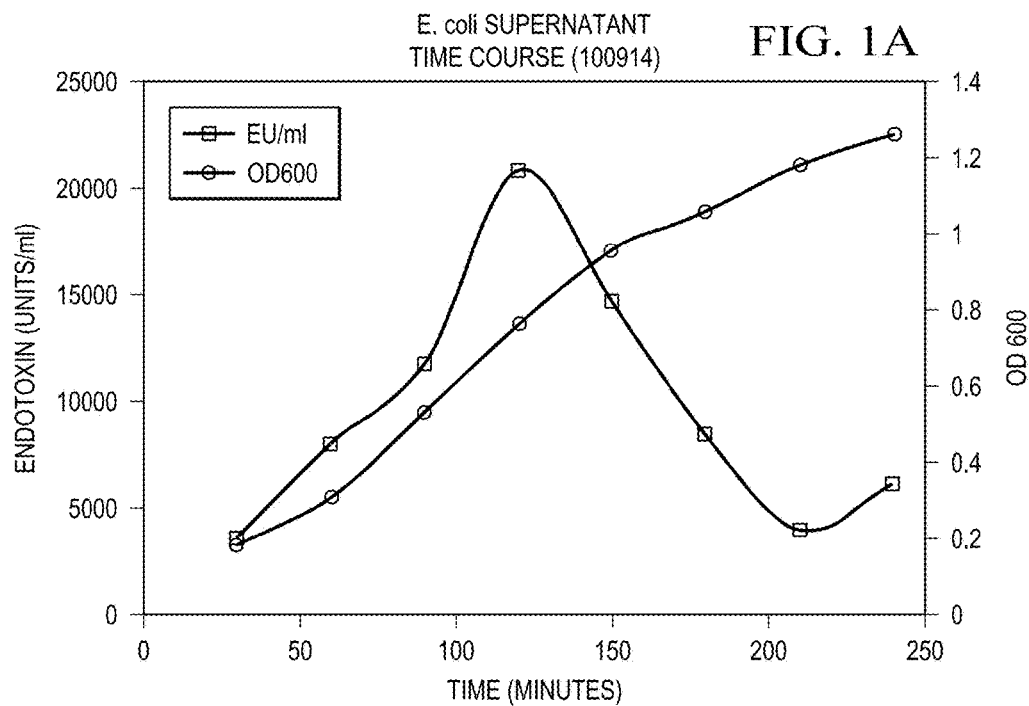
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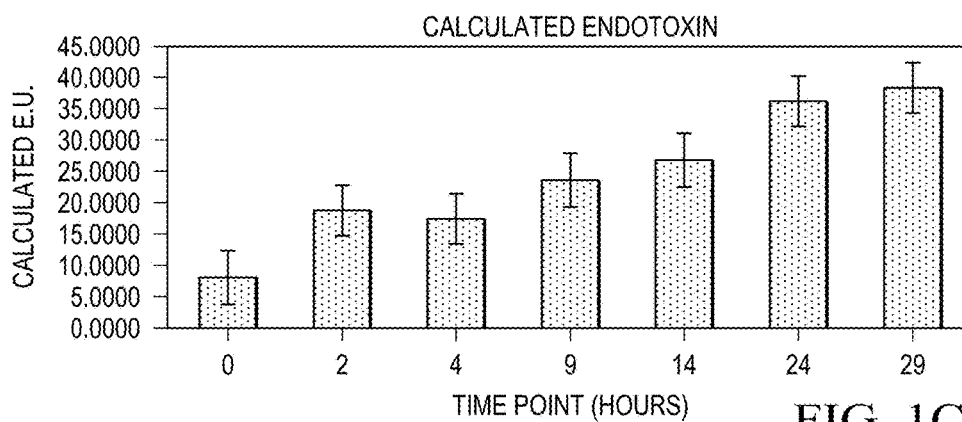


FIG. 1C

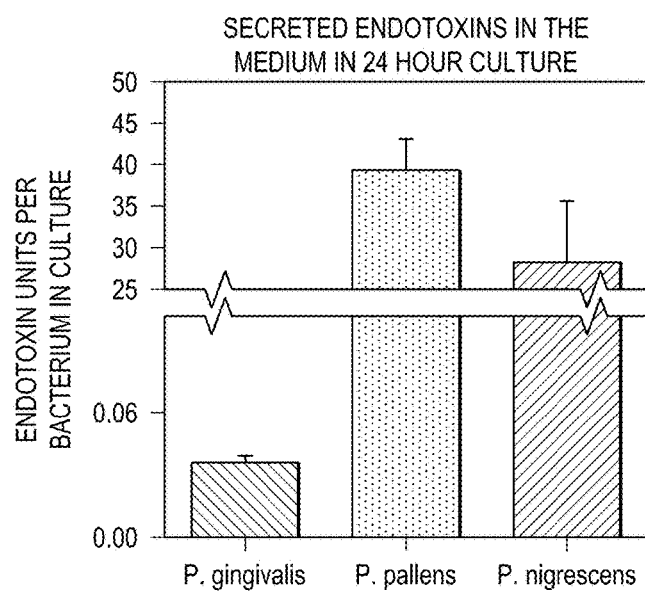


FIG. 1D

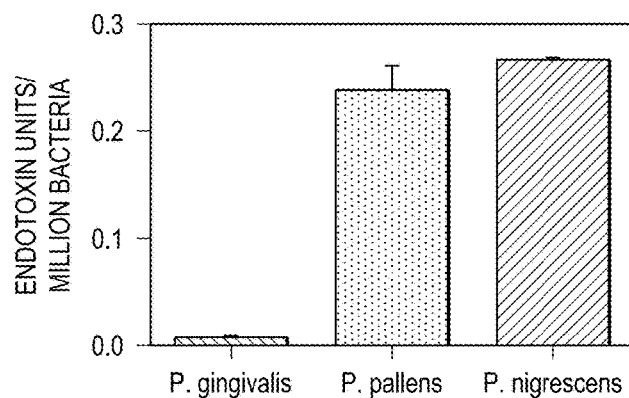
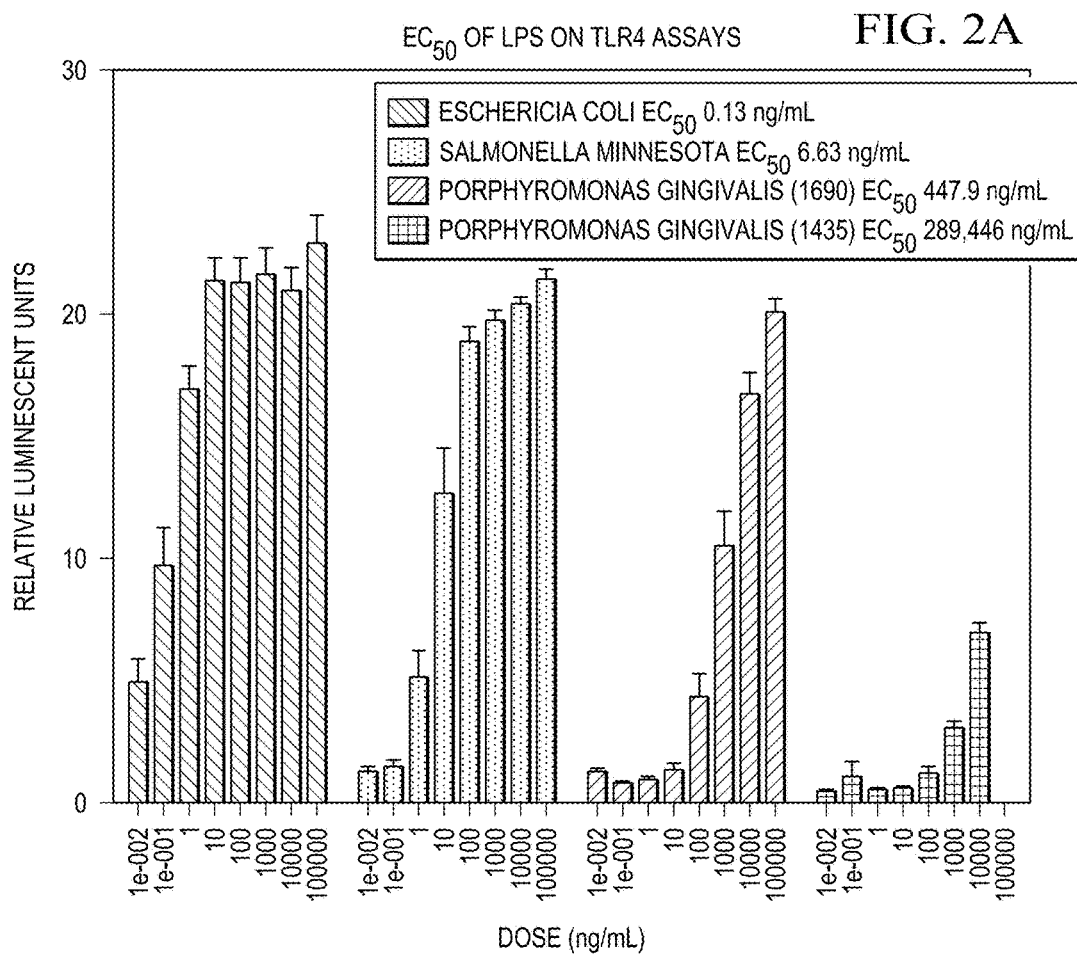
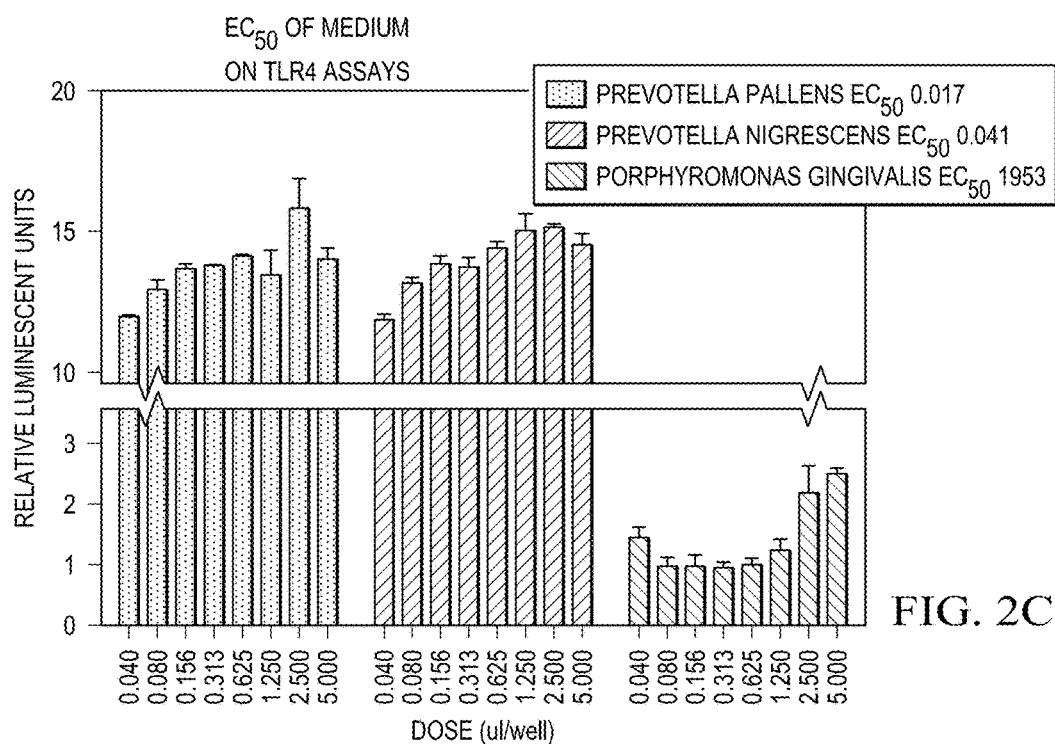
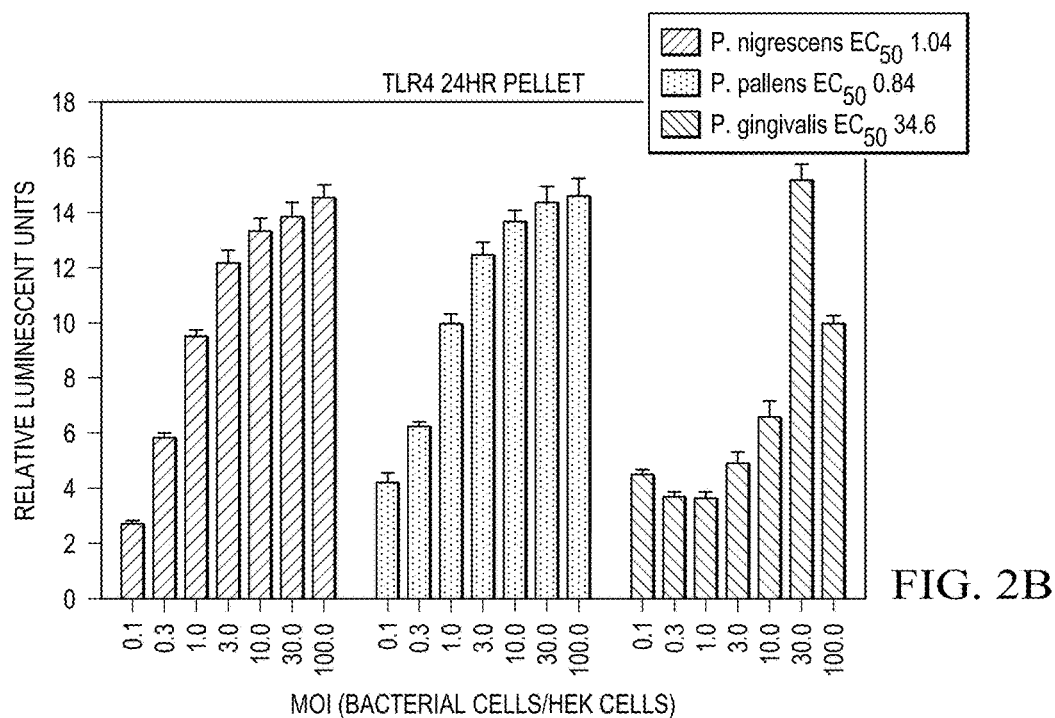
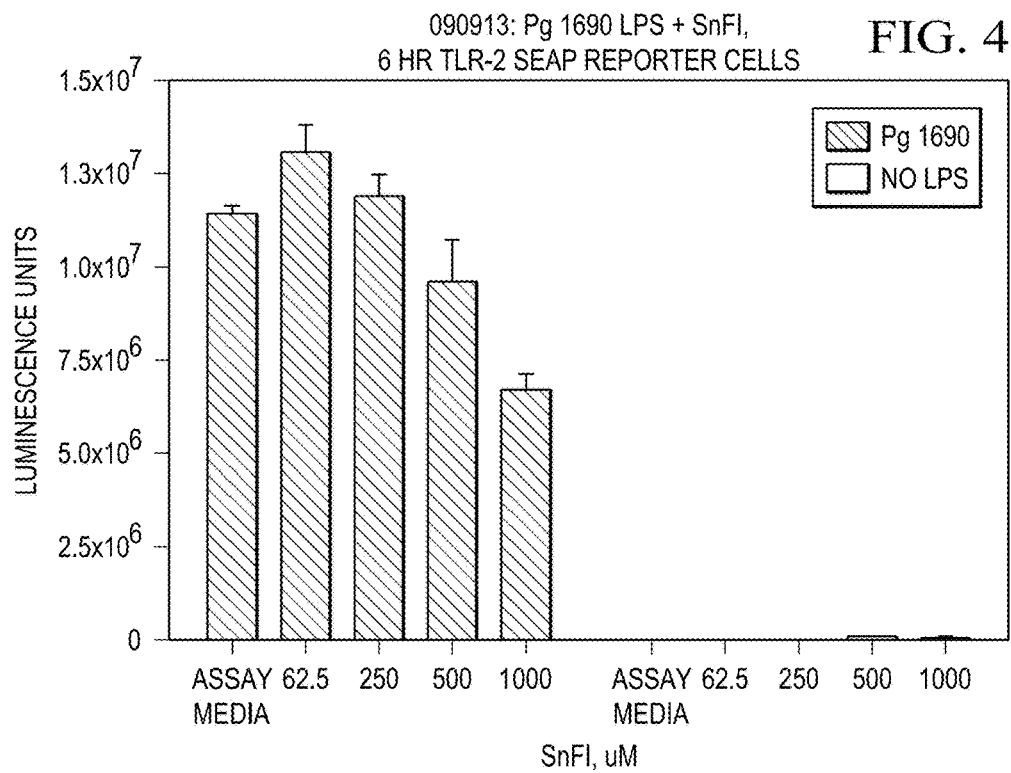
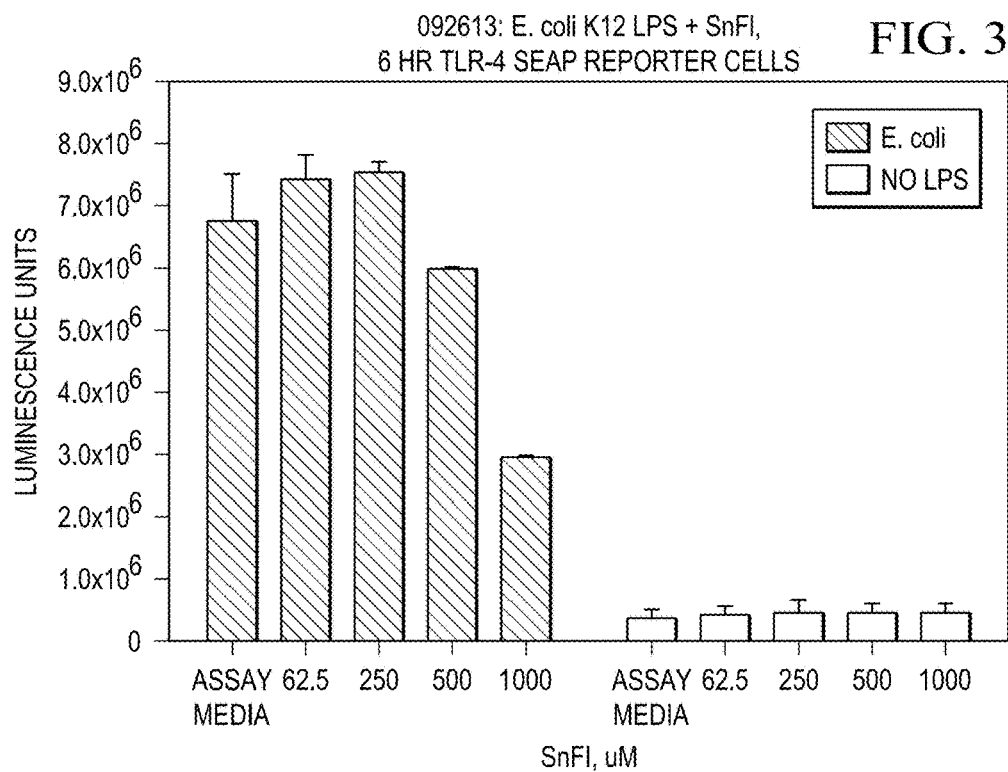


FIG. 1E

FIG. 1F







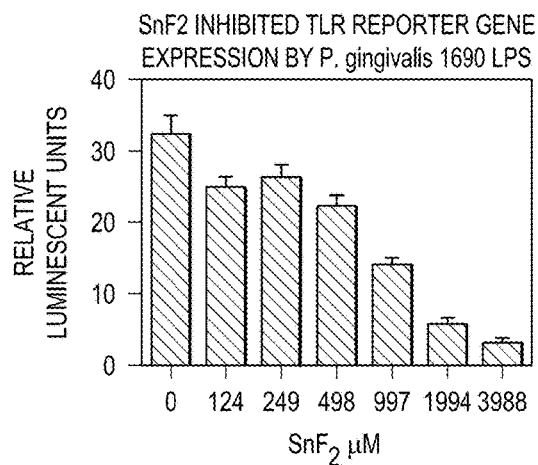


FIG. 5

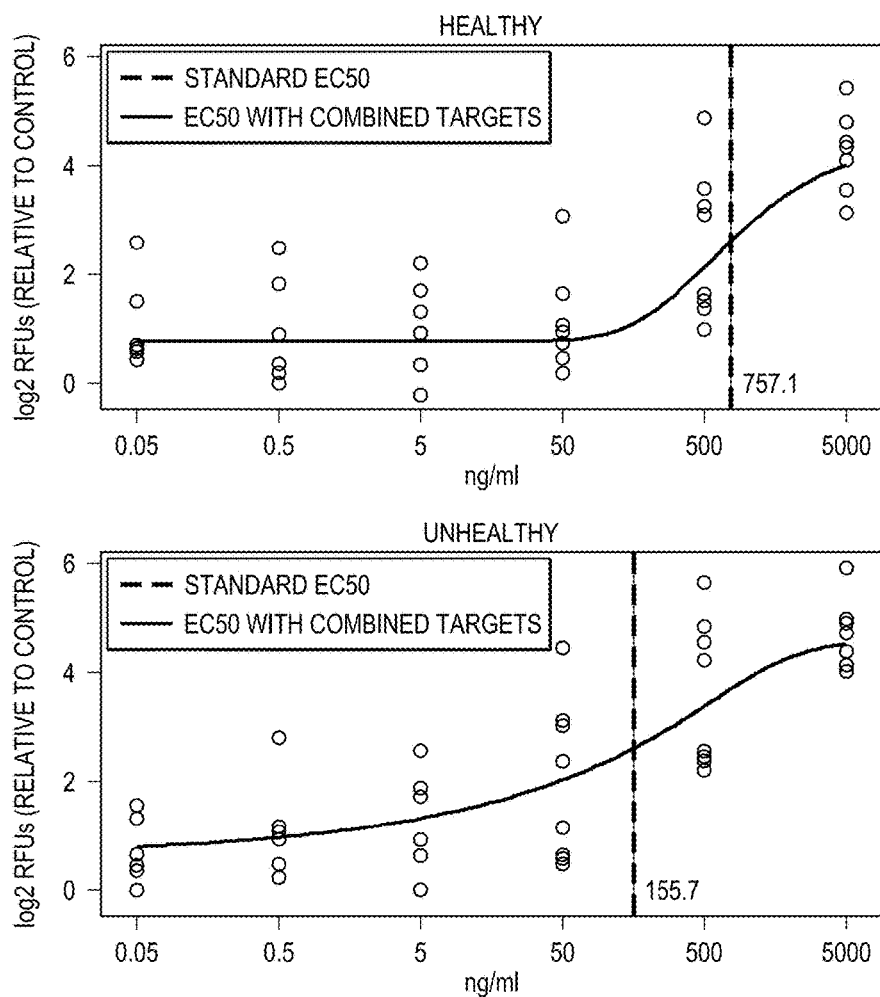


FIG. 6

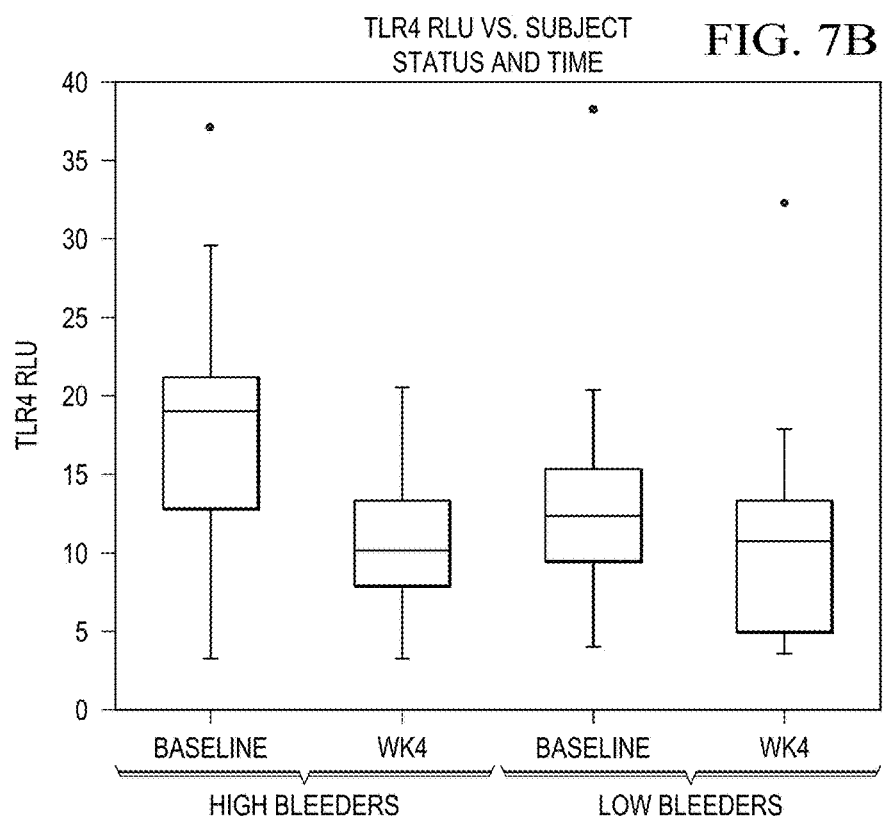
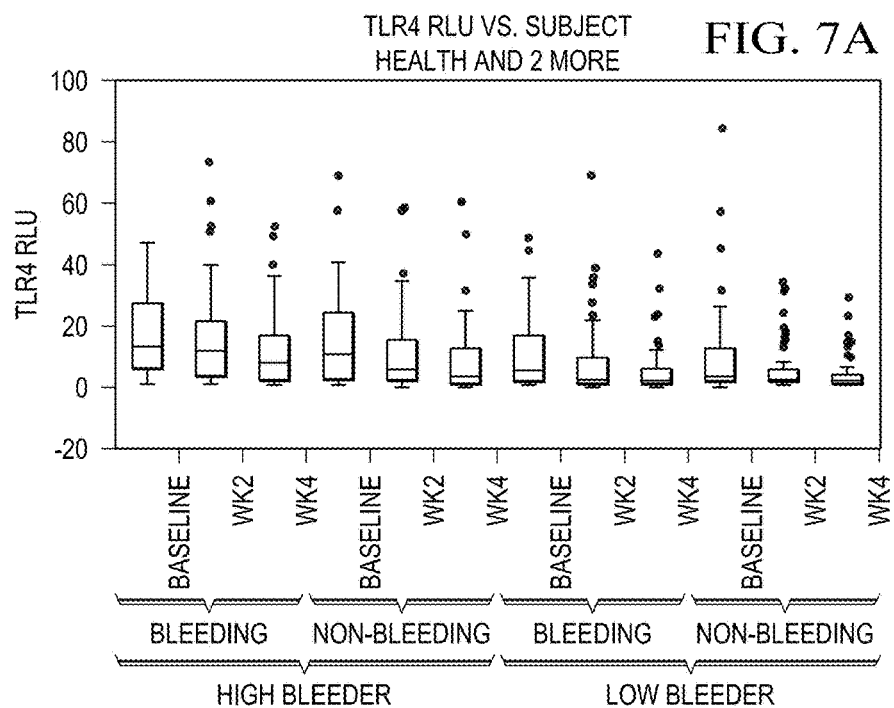


FIG. 8A

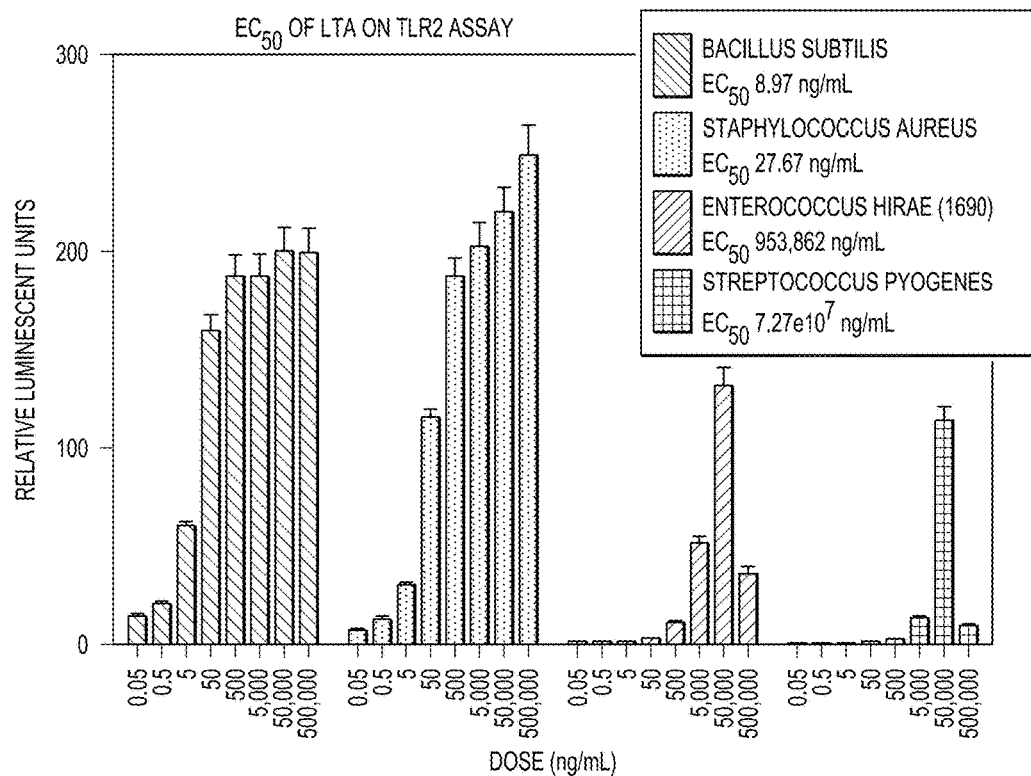
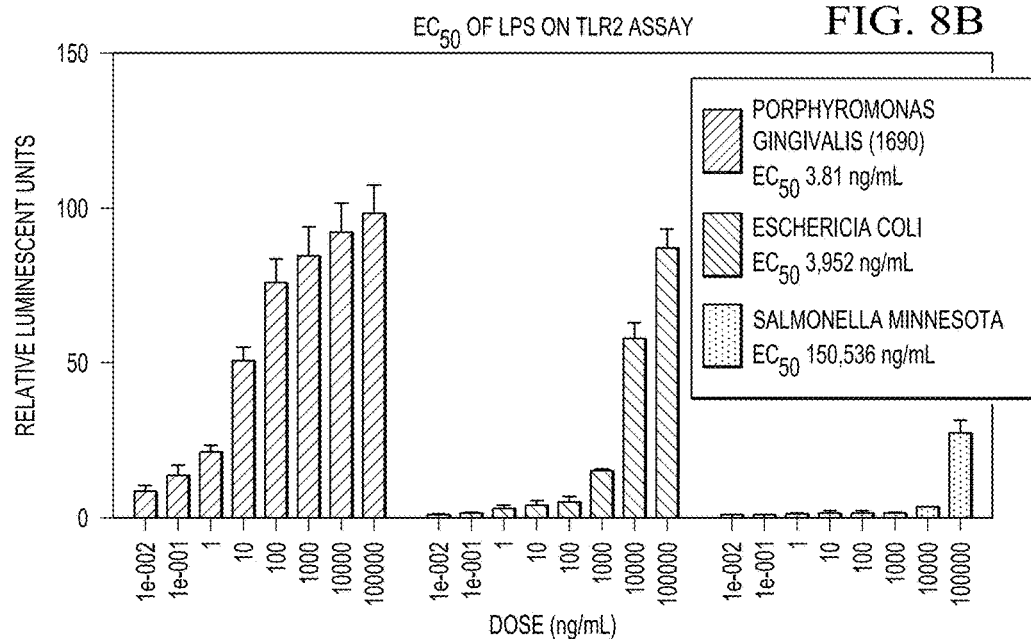
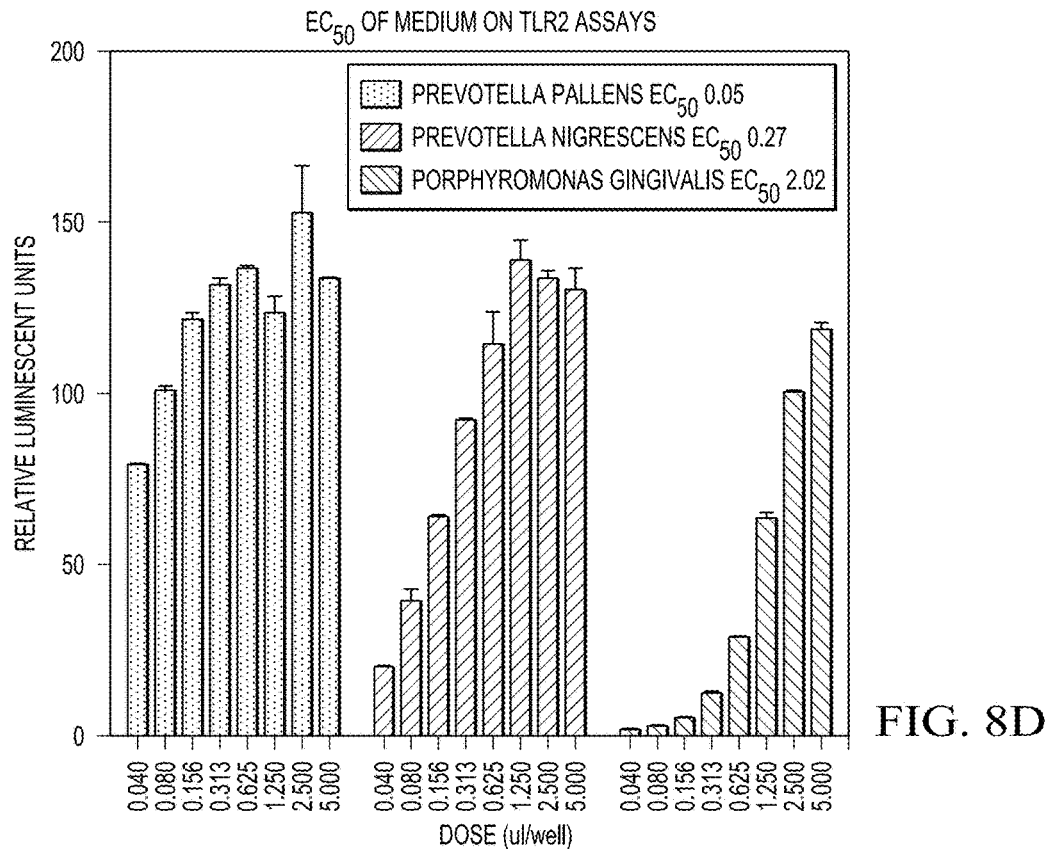
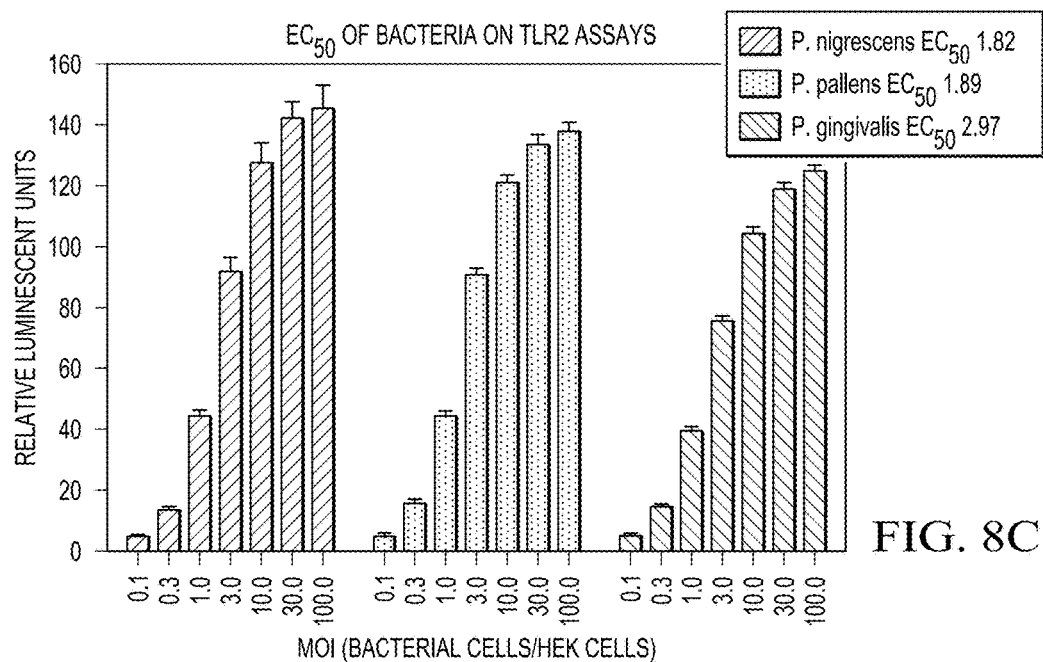
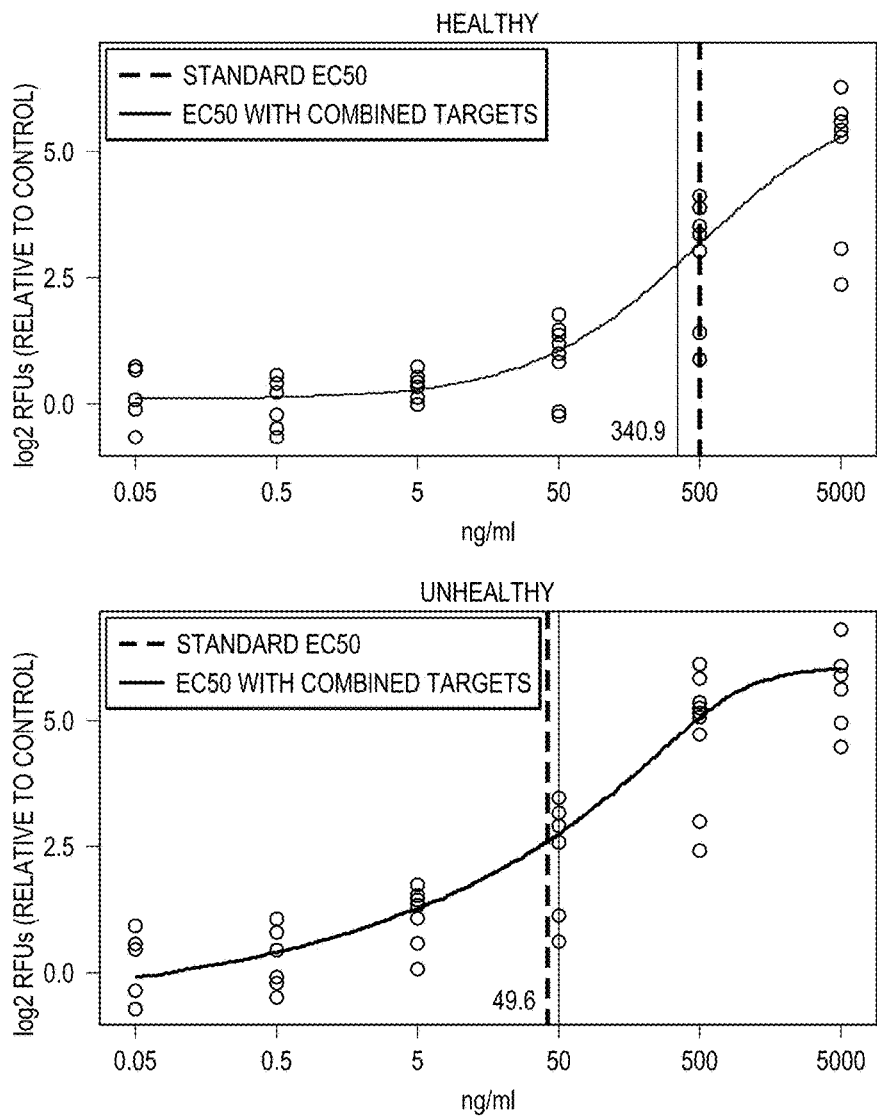


FIG. 8B







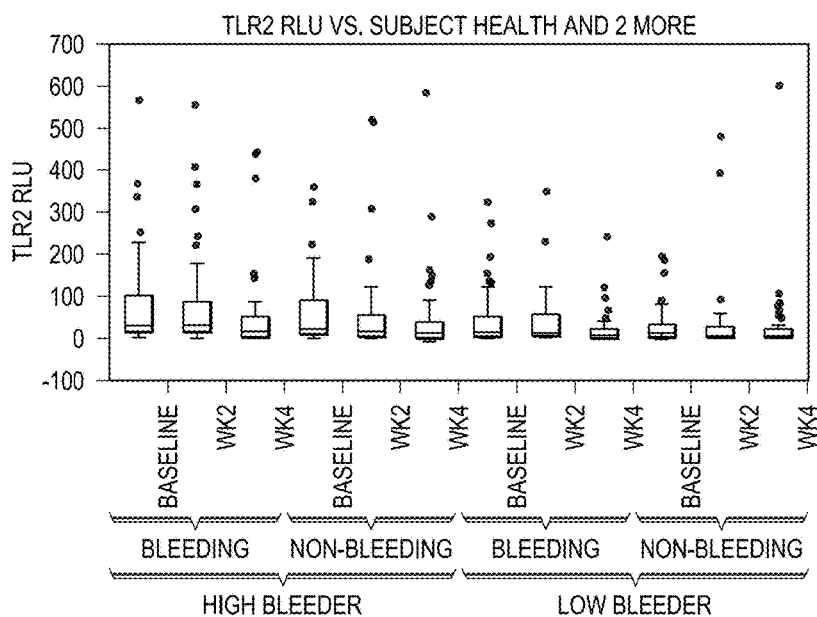


FIG. 10A

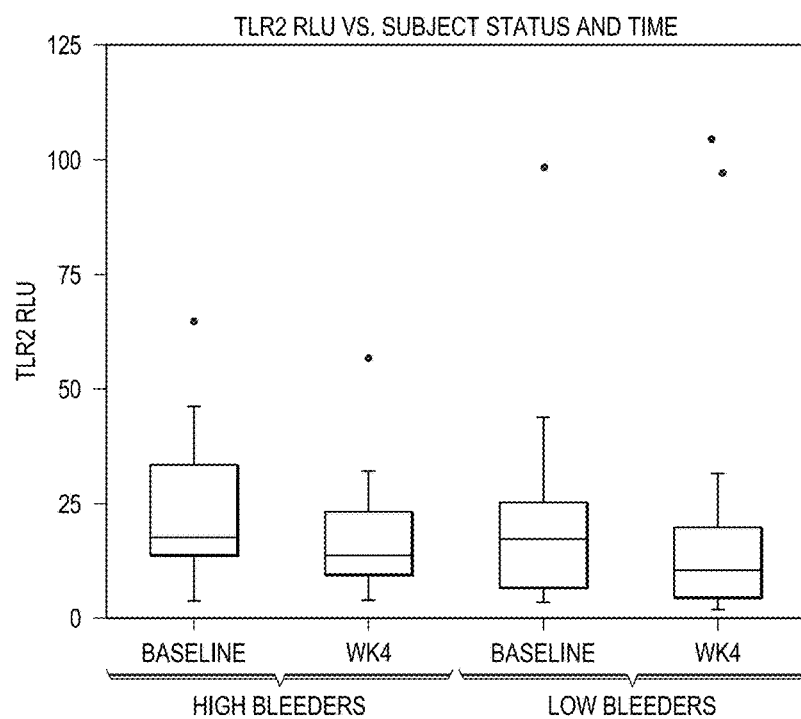


FIG. 10B

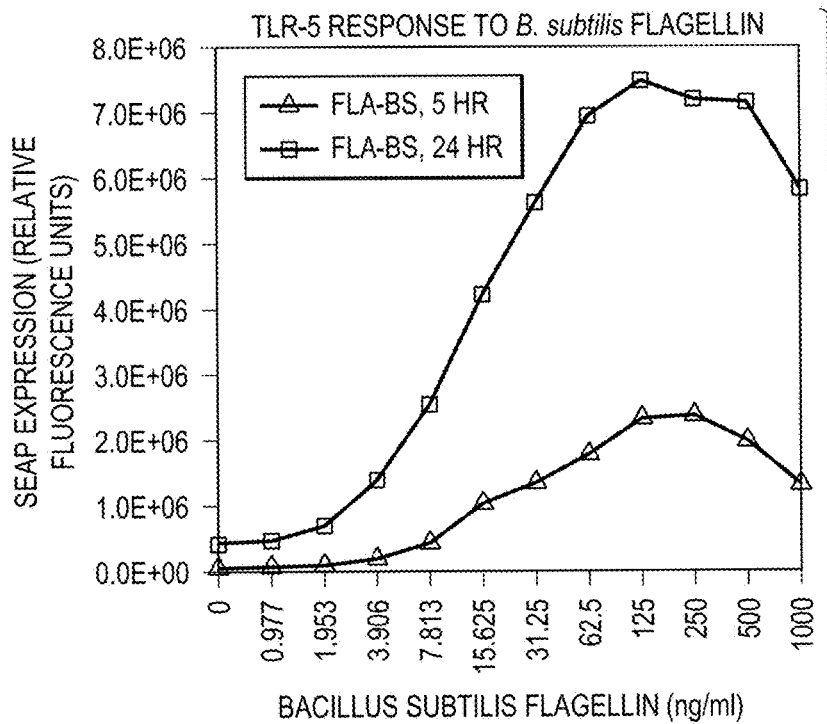
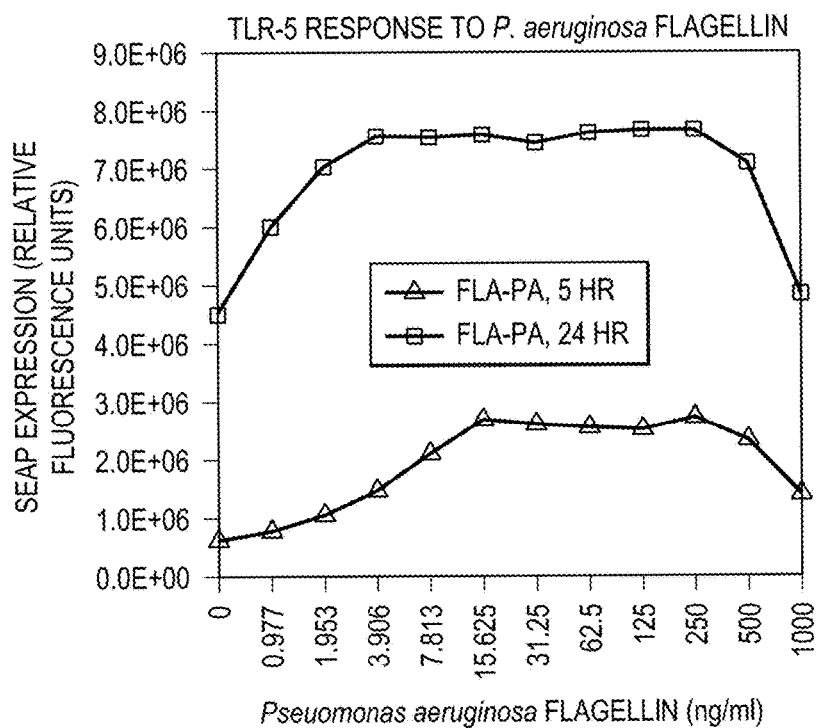


FIG. 11



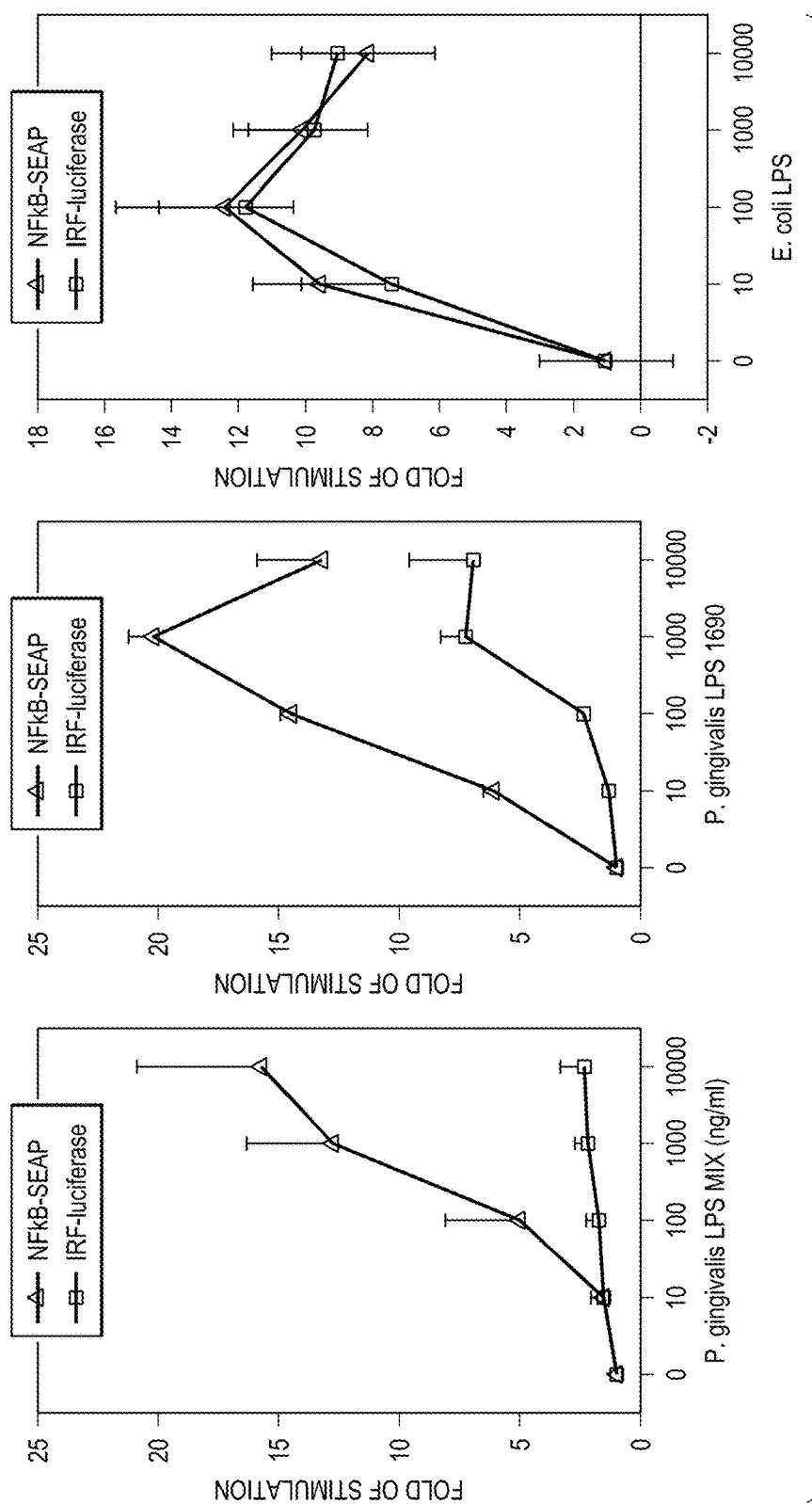


FIG. 12A

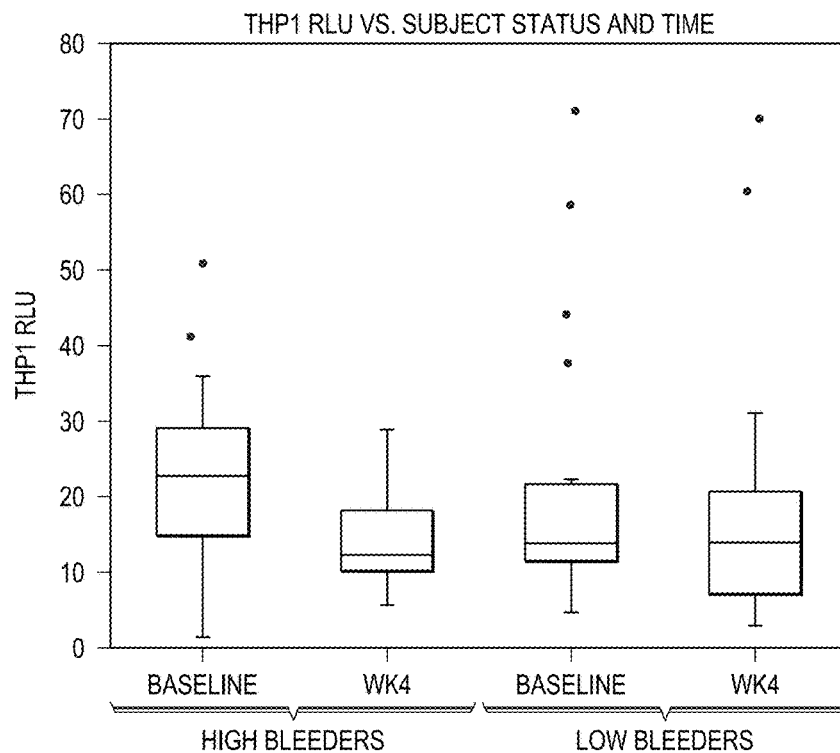
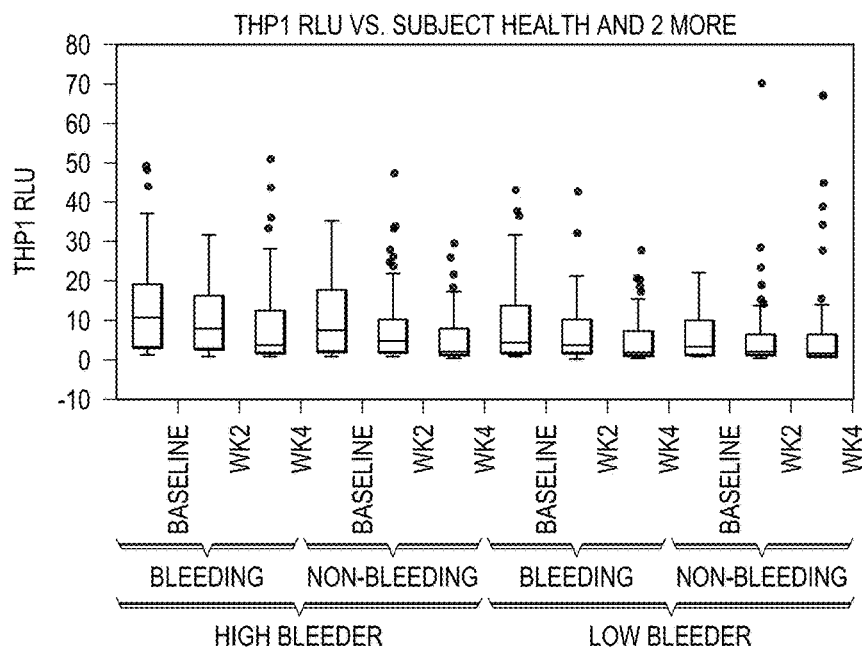


FIG. 12B



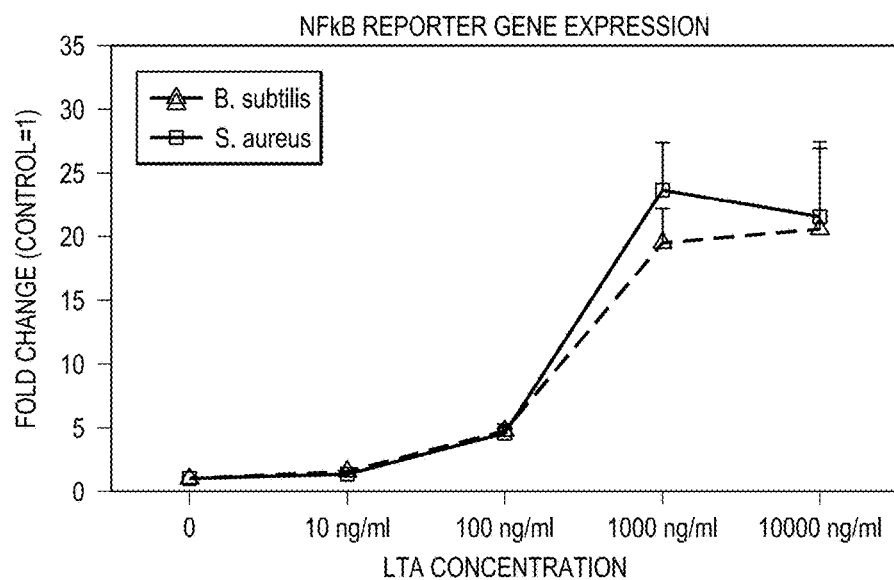


FIG. 13

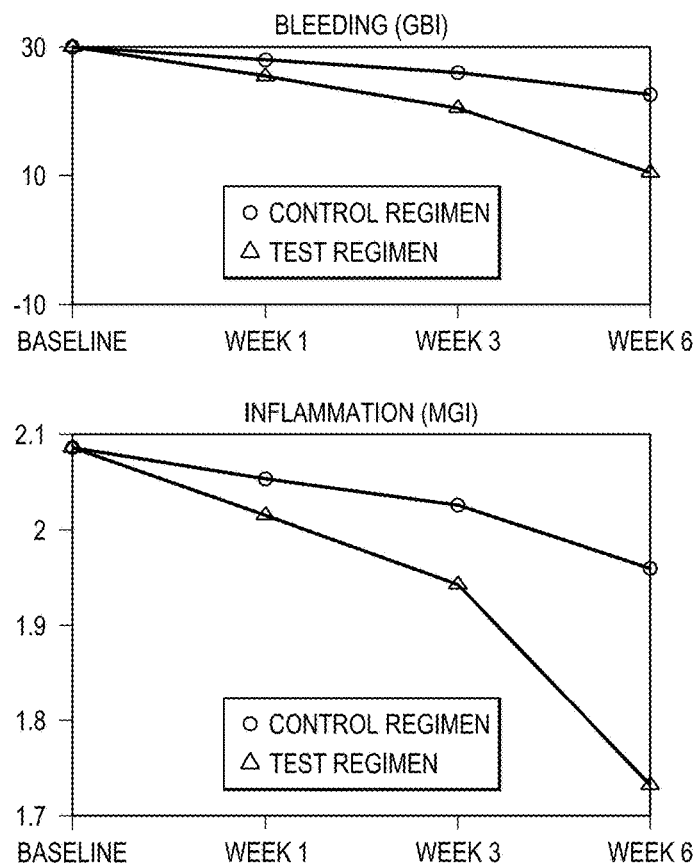


FIG. 14

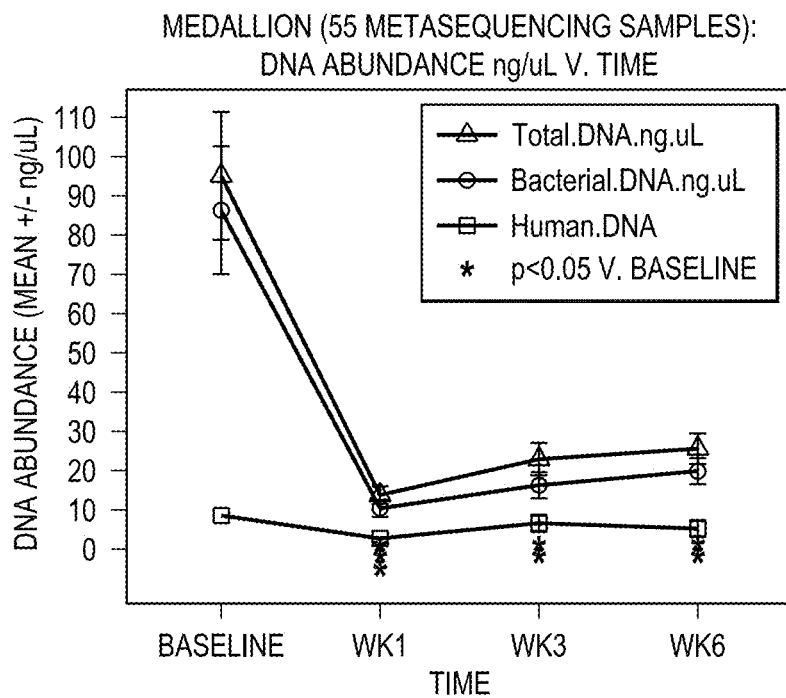


FIG. 15

FIG. 16A-1
FIG. 16A-2
FIG. 16A-3

FIG. 16A

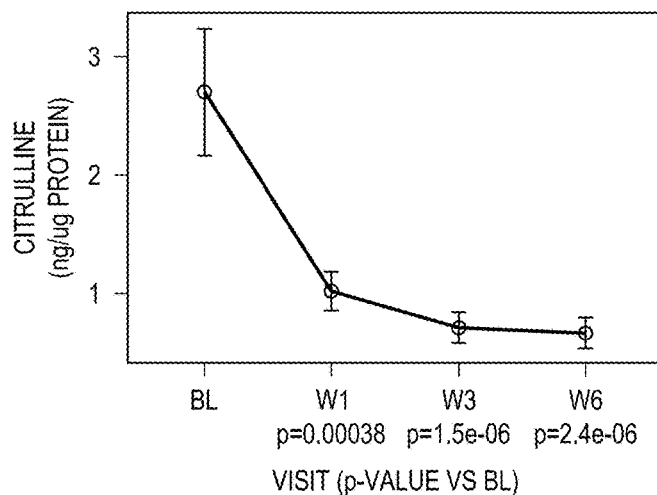


FIG. 16

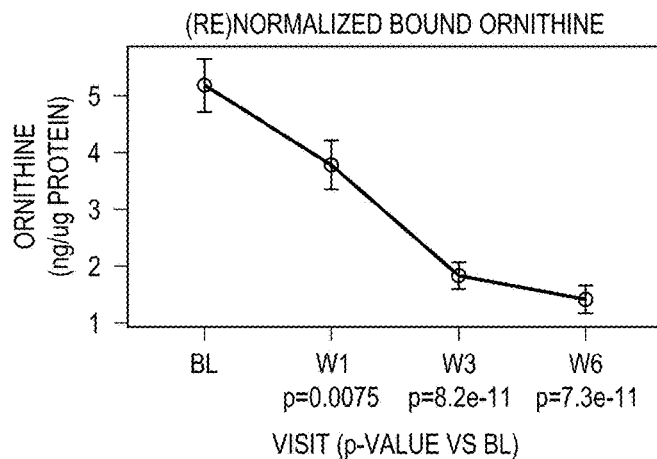


FIG. 17

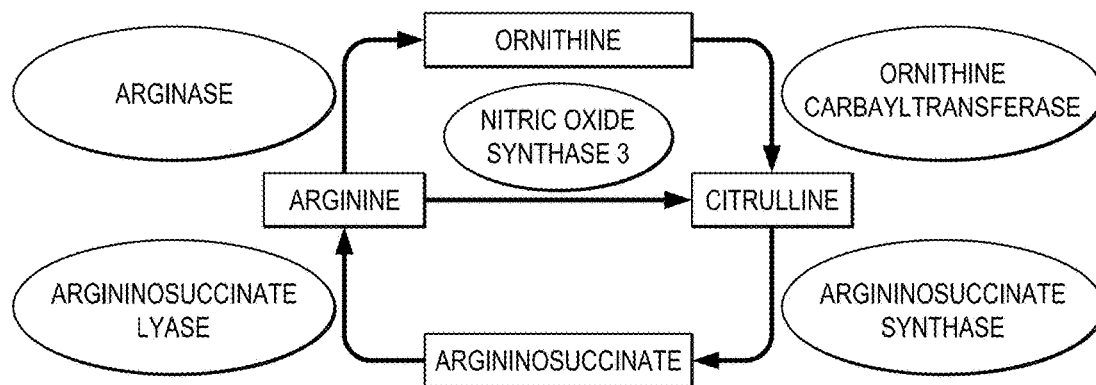
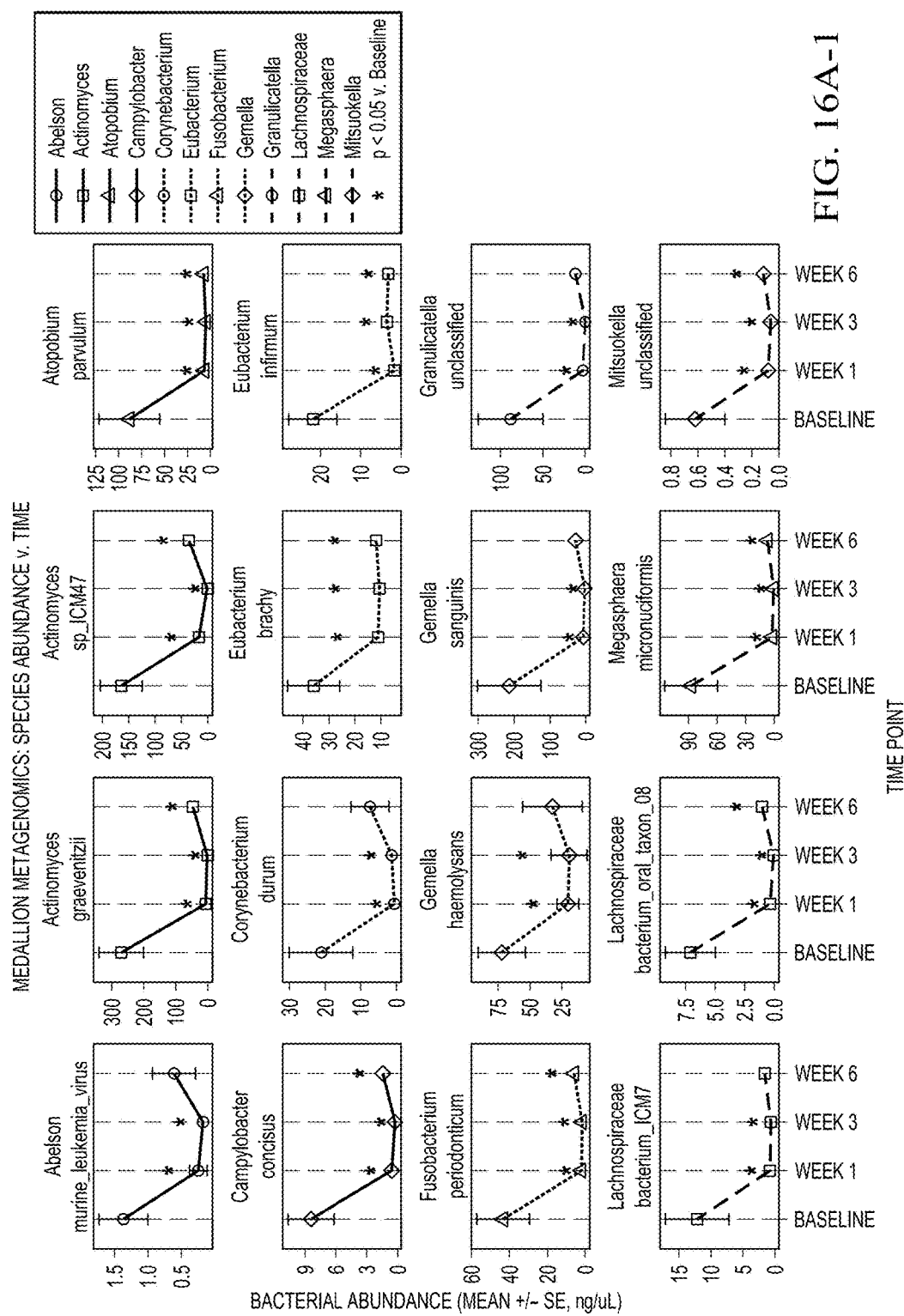
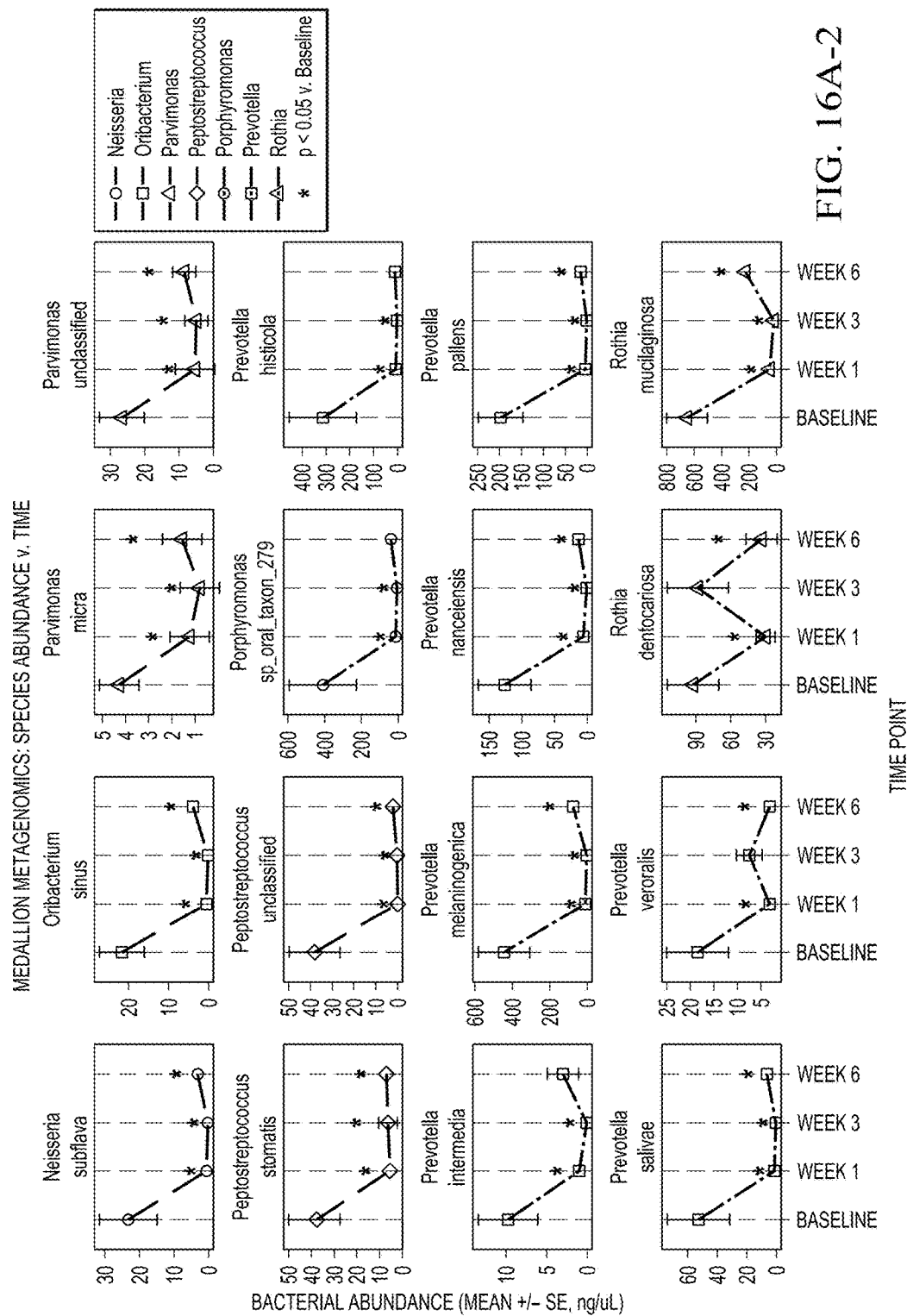


FIG. 18





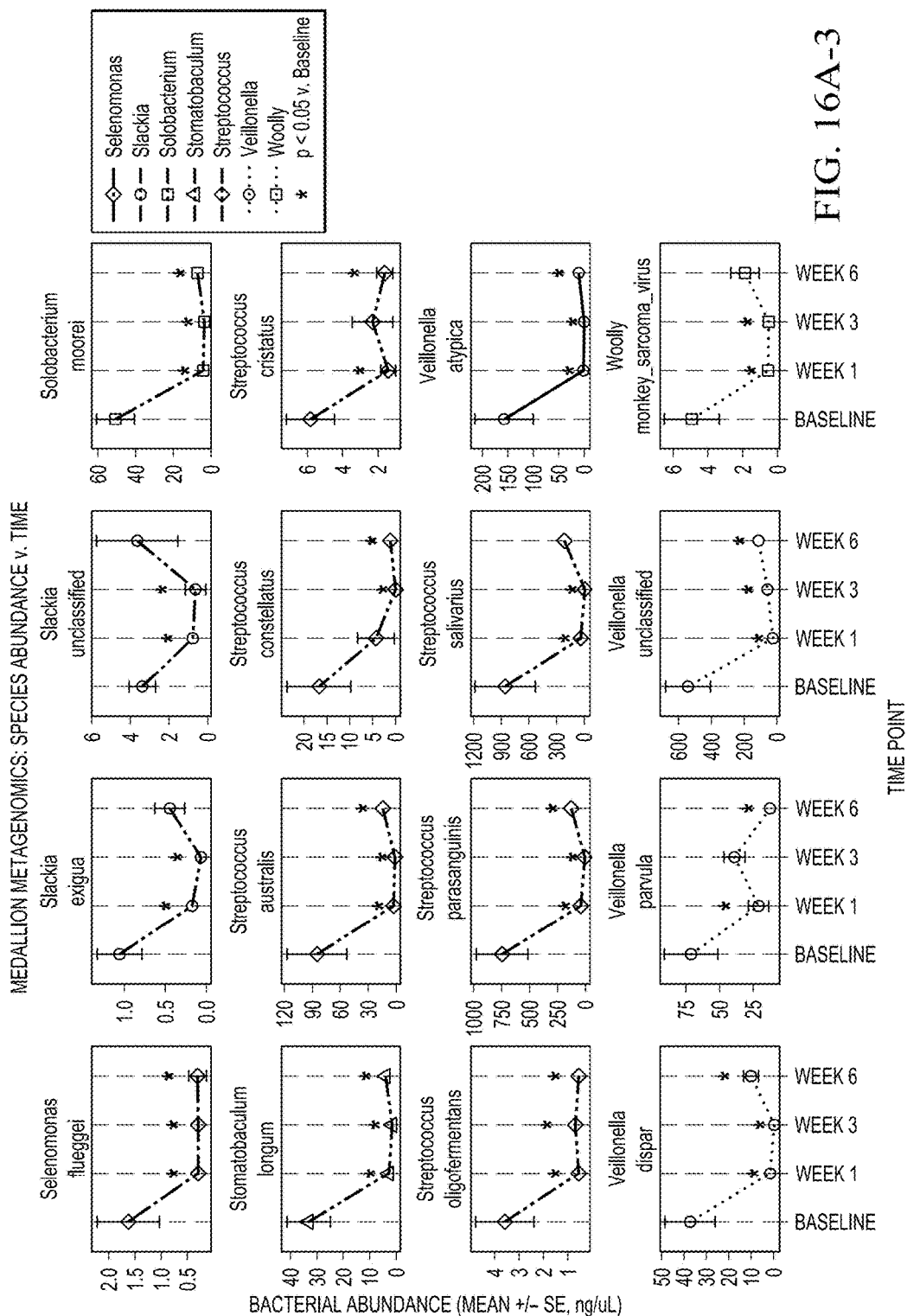


FIG. 19

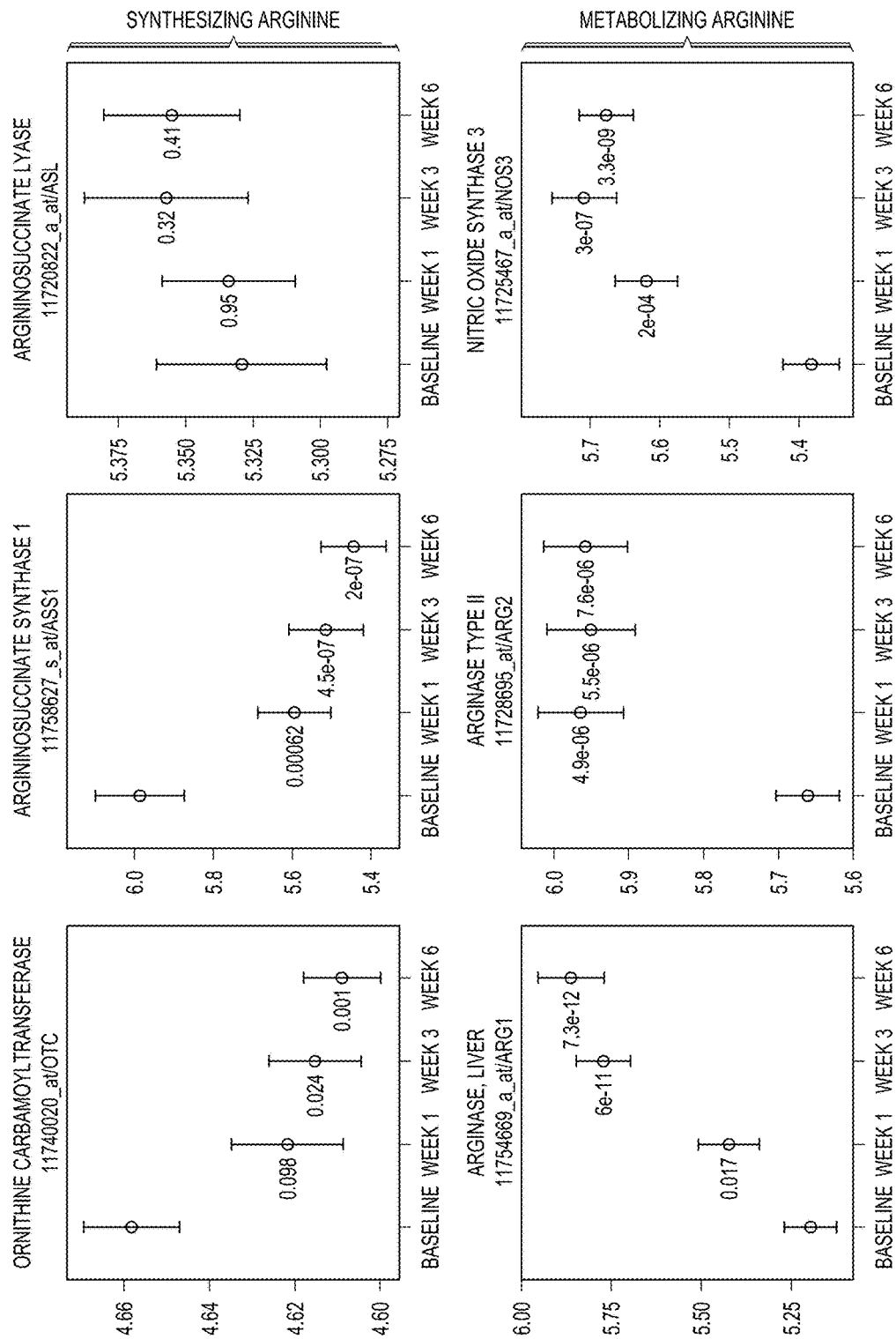


FIG. 20

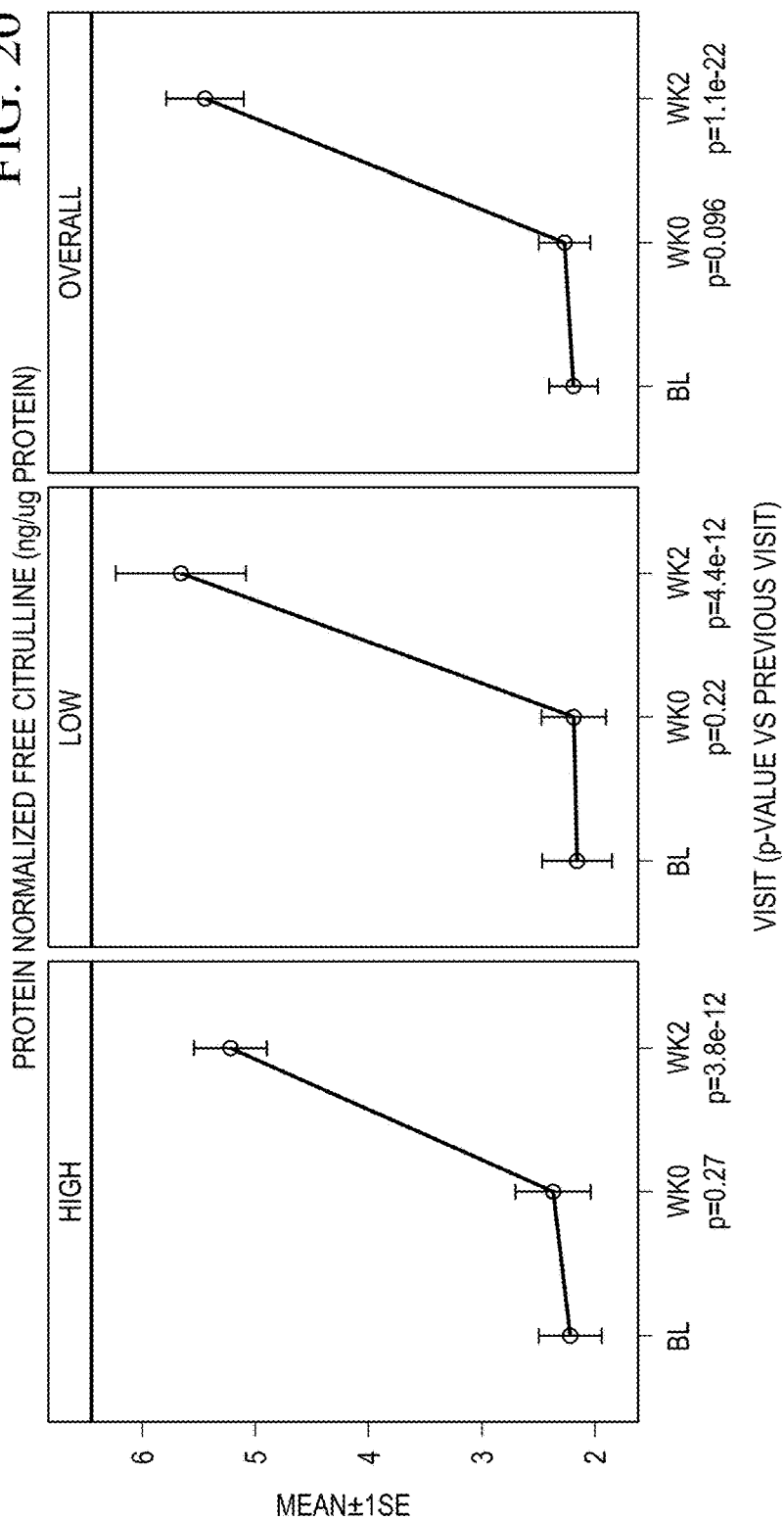
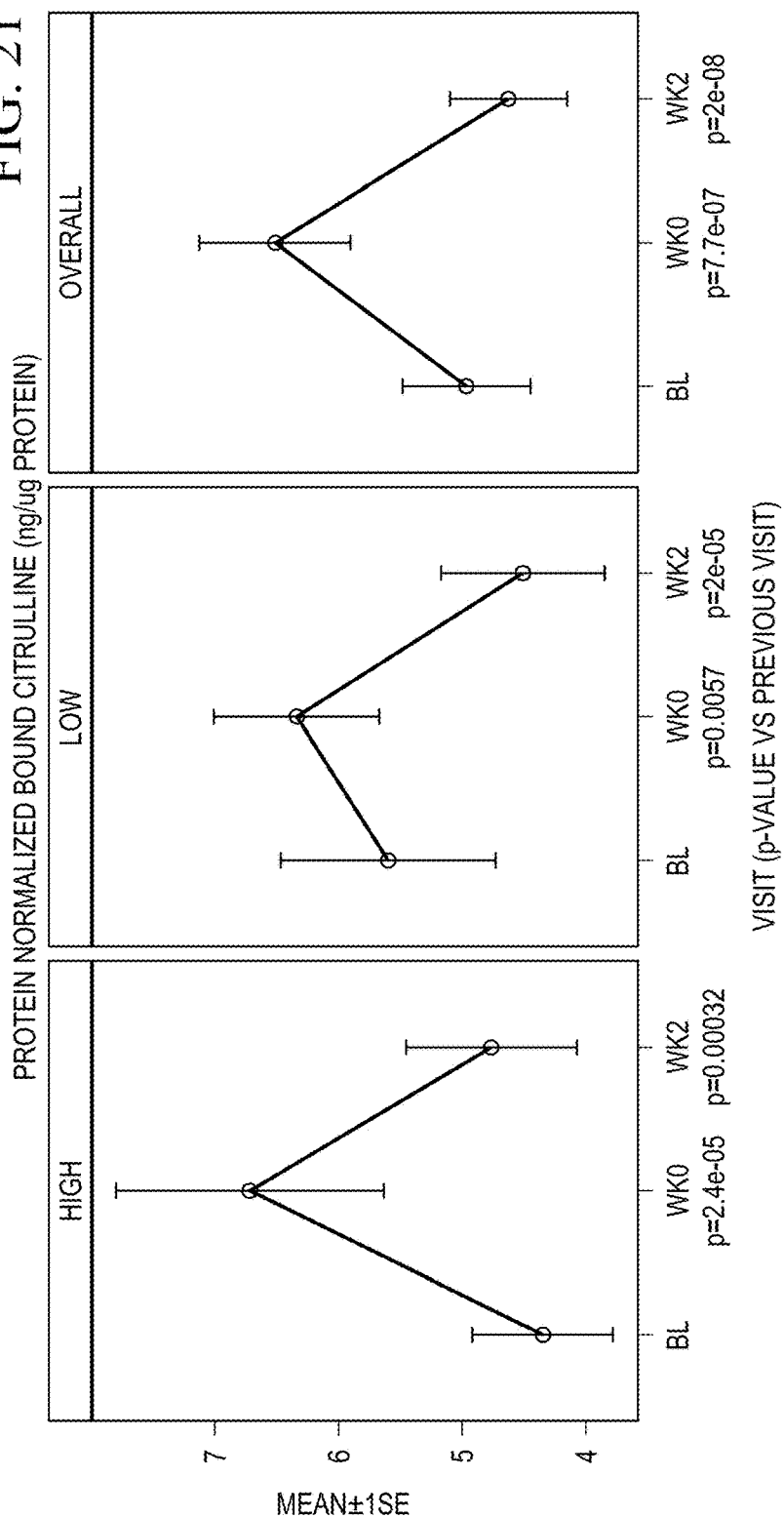
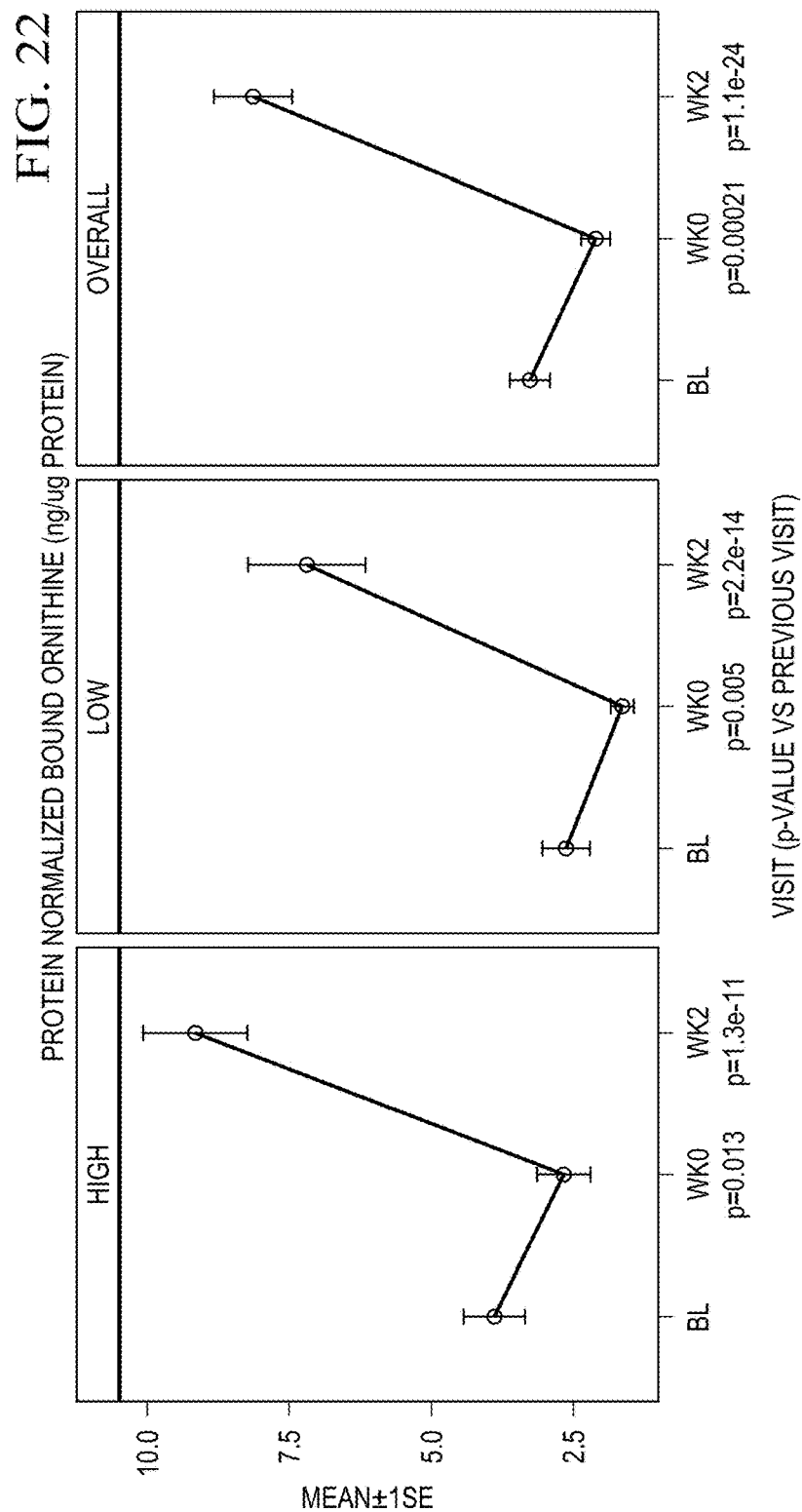


FIG. 21





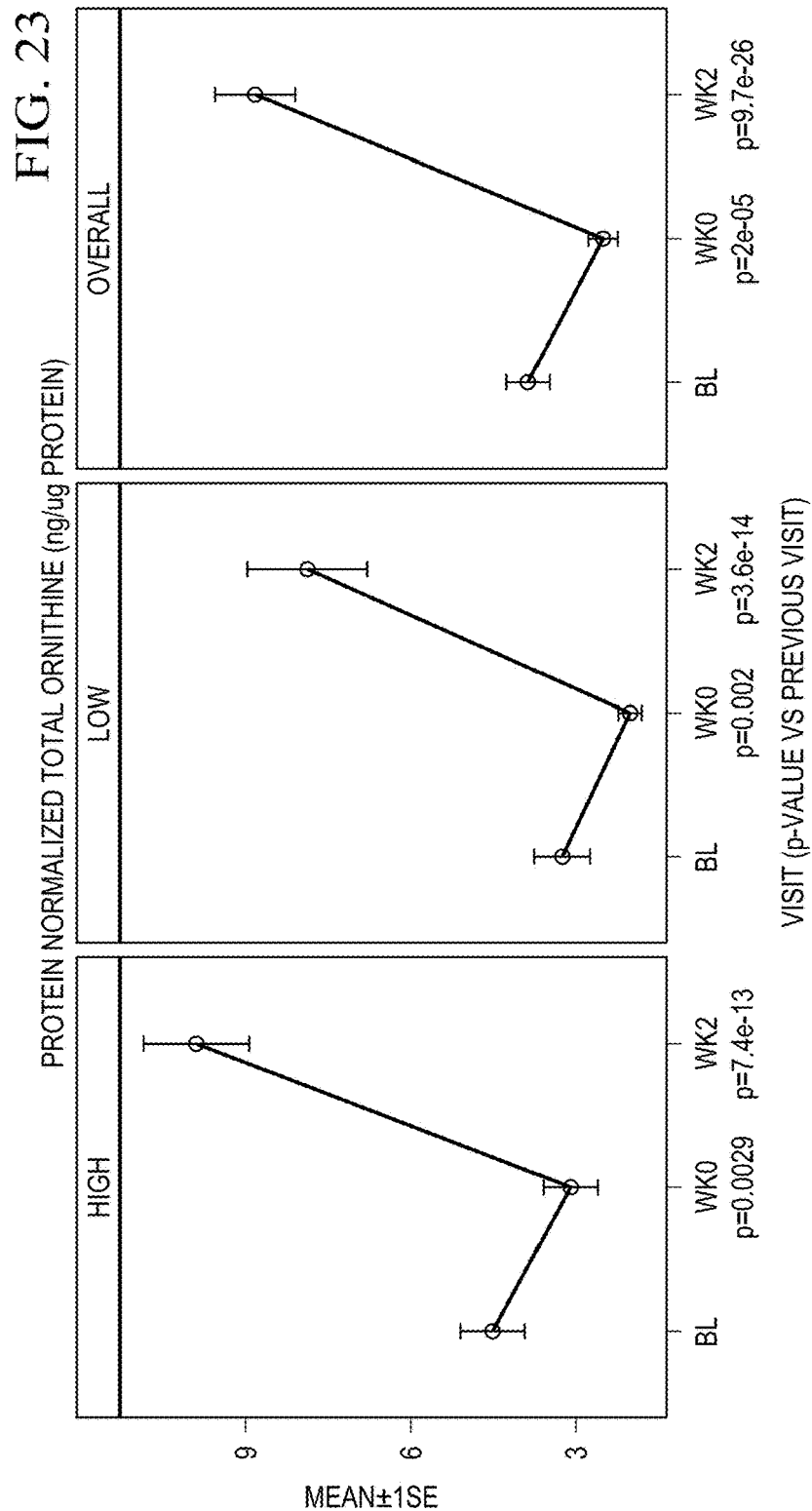


FIG. 24

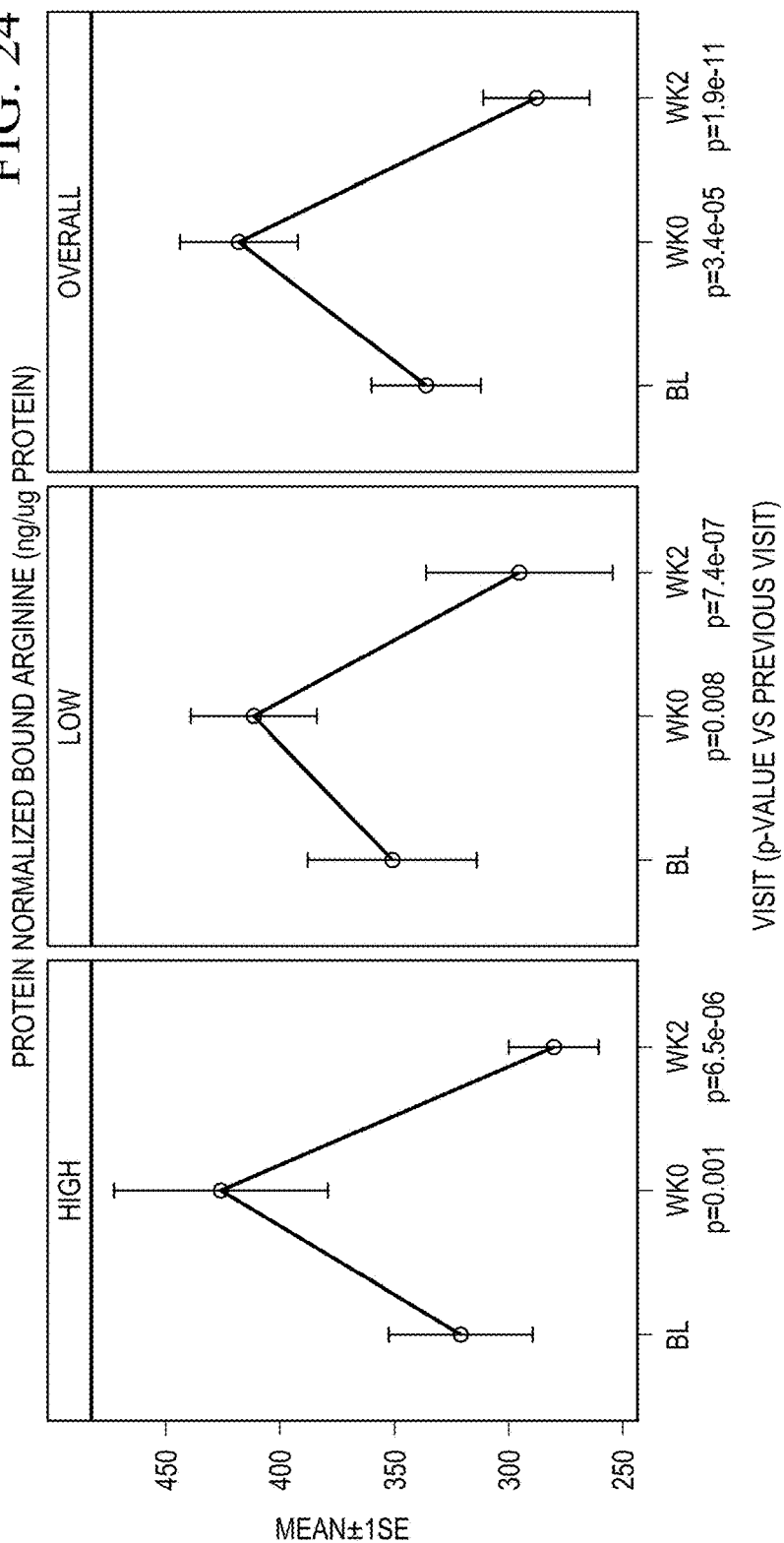
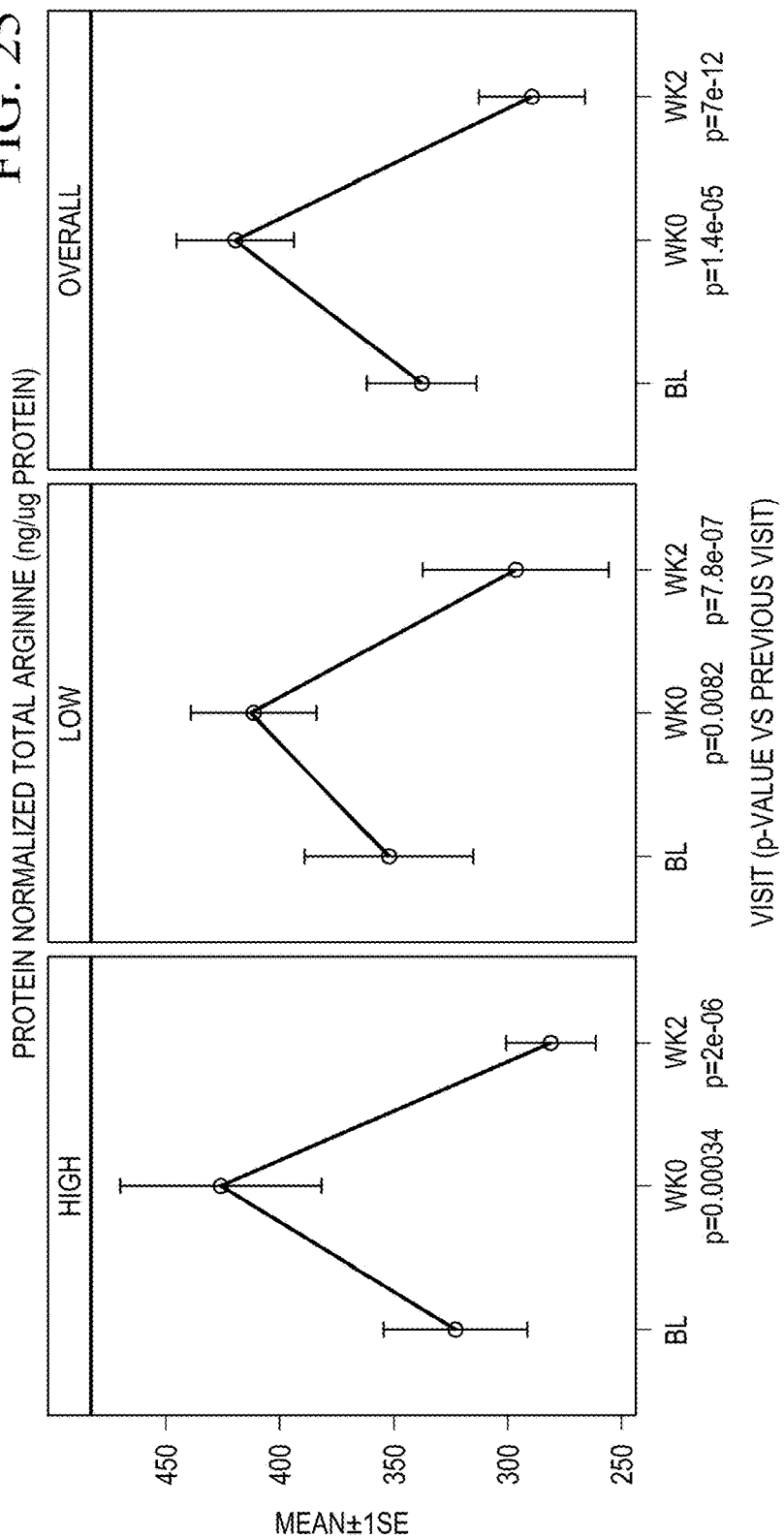


FIG. 25



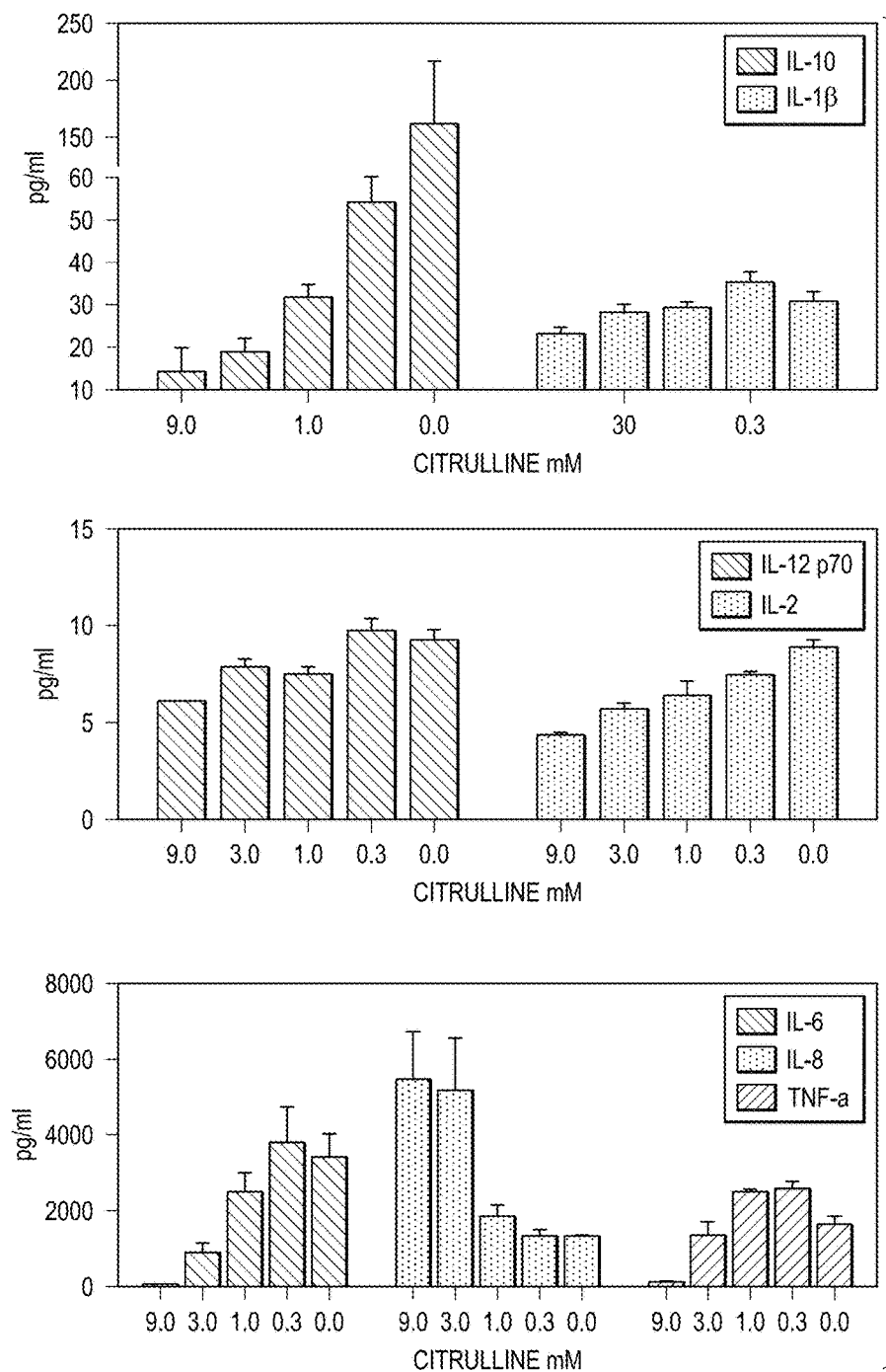
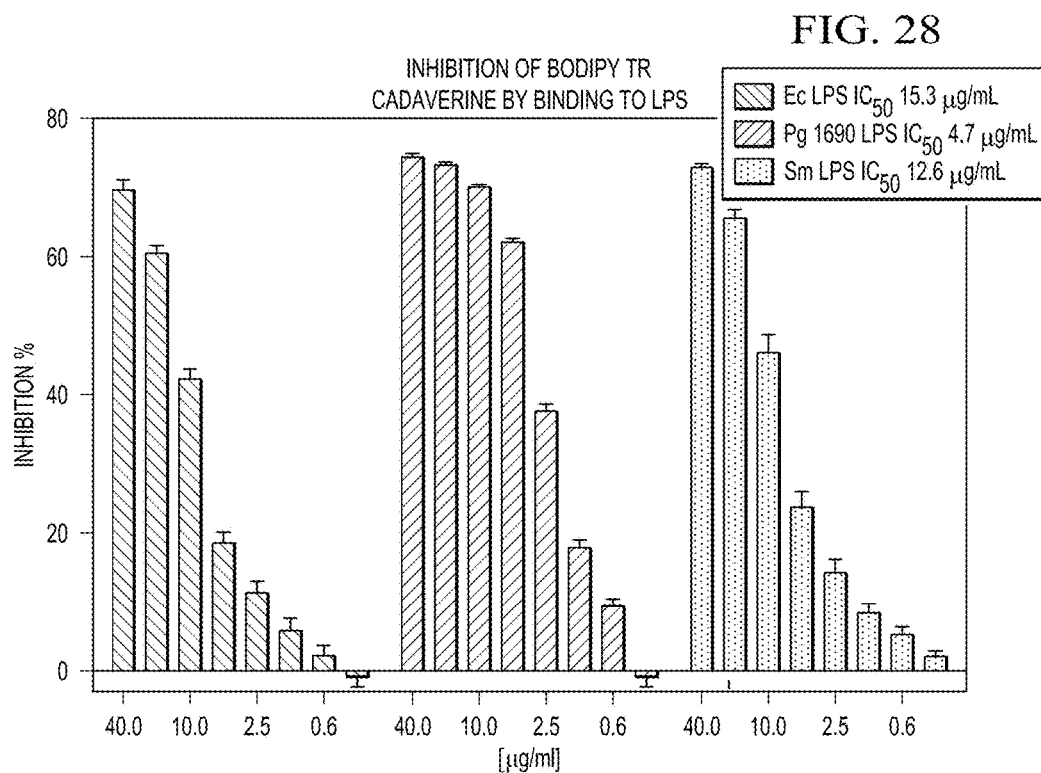
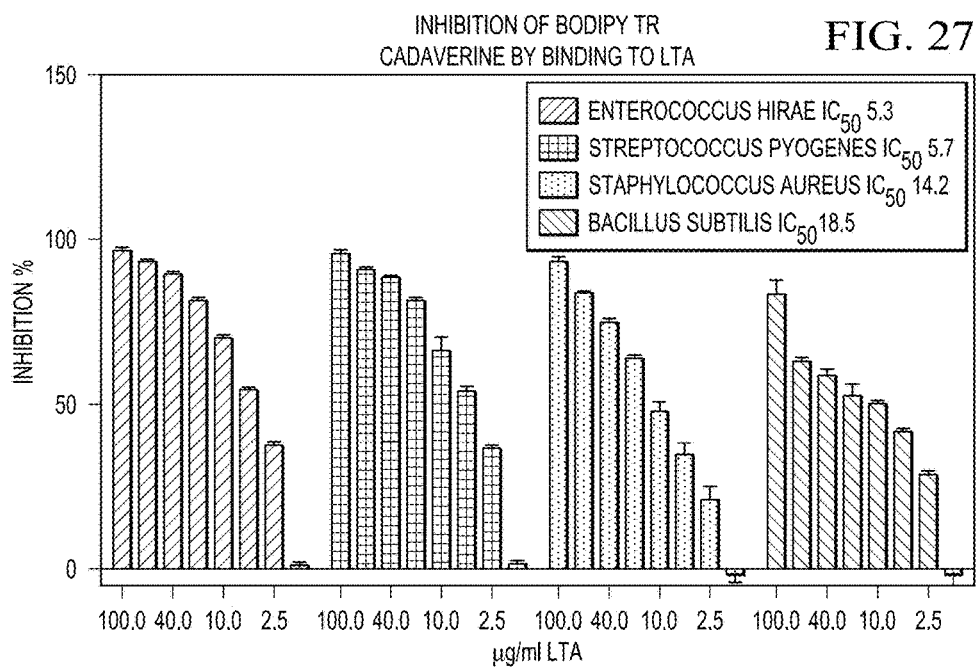


FIG. 26



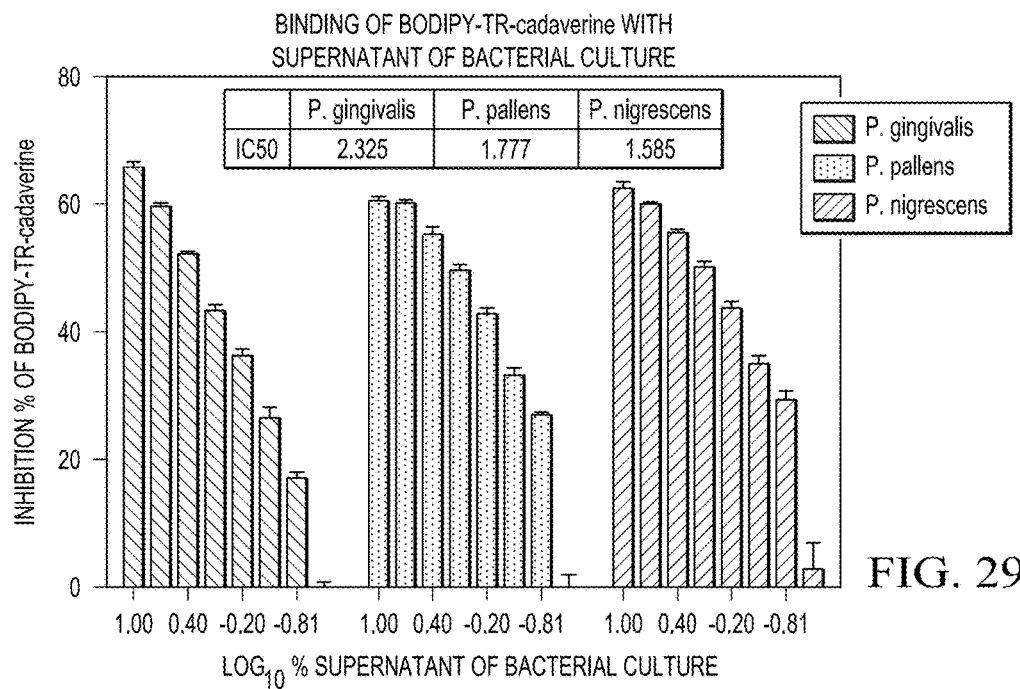


FIG. 29

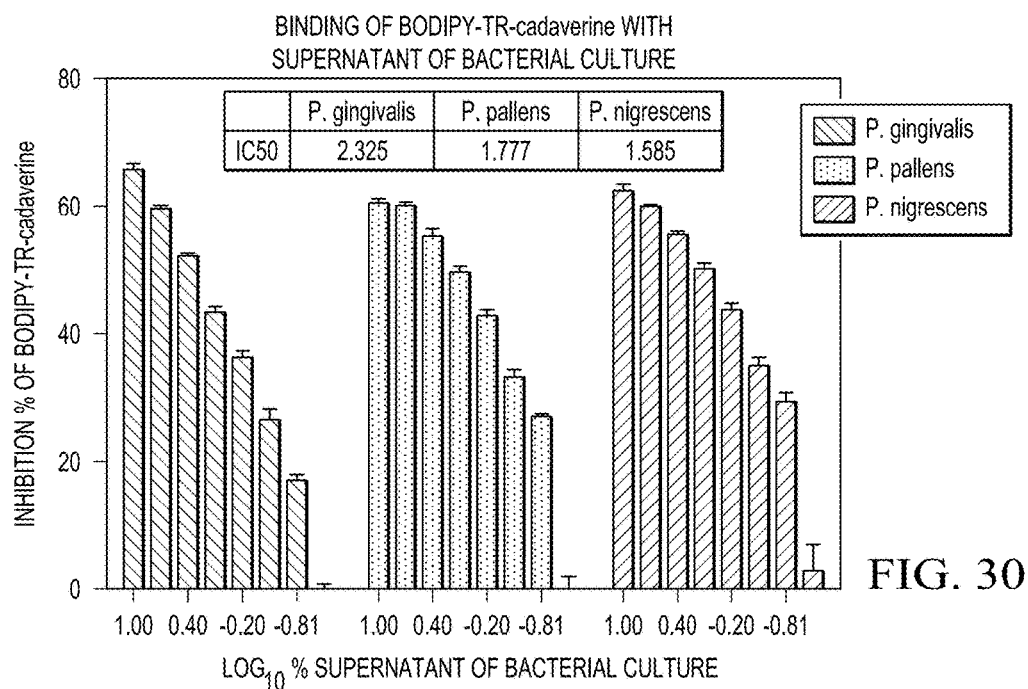


FIG. 30

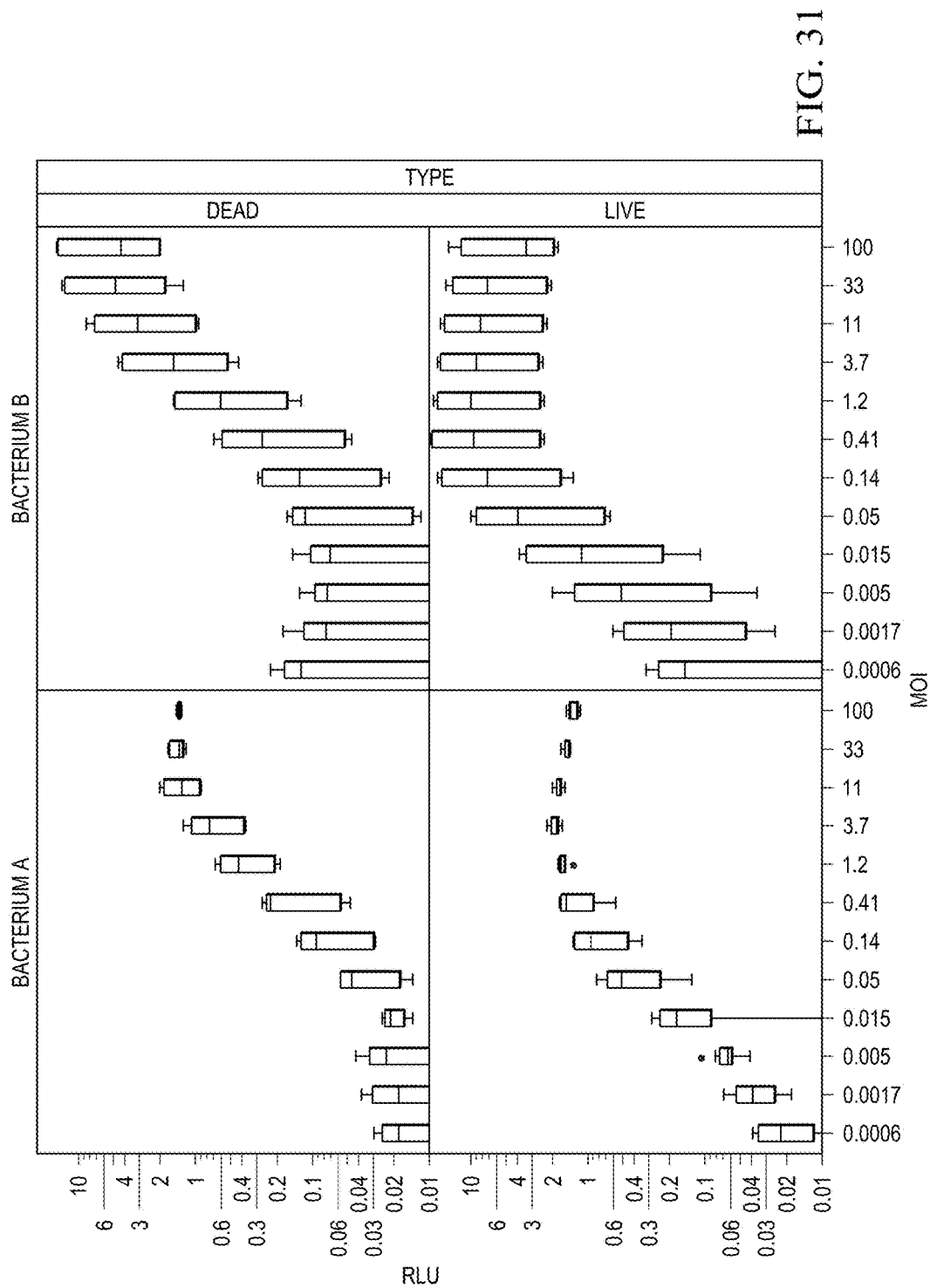


FIG. 31

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DETECTION OF ORAL MICROBIAL VIRULENCE FACTORS

FIELD OF THE INVENTION

The invention relates to a method for detecting and quantifying bacterial virulence factors in oral samples, and for identifying agents for detoxifying oral virulence factors and a method for determining the effectiveness of detoxifying agents.

BACKGROUND OF THE INVENTION

The toxins of bacterial cell walls (Henkel et al, *EXS*. (2010) 100: 1-29) have been associated with health related issues, such as septic shock, fever and malaise (V. Liebers et al., *Am J Ind Med*. (2006) 49(6): 474-91). Examples of Gram-negative cell wall toxins associated with health concerns are endotoxins, such as lipopolysaccharide (LPS), peptidoglycans, and fimbriae; and Gram-positive cell wall toxins associated with health concerns are lipoteichoic acid (LTA) and peptidoglycans. There are many other bacterial toxins, such as enterotoxins and exotoxins, as reported in Henkel et al, *EXS*. 2010; 100: 1-29. For the oral environment, the LPS and LTA seem to be the dominant drivers of a bacterial induced immune response, or at least the best characterized. The immune response mounted by the body in response to these toxins depends on the origin of the toxin and the exposure history of the individual to said toxin. The LPS is a component of Gram-negative bacteria that is different from strain to strain, as has been illustrated with the differences in virulence of *E. coli* (Raetz and Whitfield *Annu. Rev. Biochem* (2002) 71:635-700). LPS is composed of a lipid A fraction, core region, and may have an O-antigen. The Lipid A fraction's fatty acid composition has been shown to determine its virulence in response to its interaction with the Toll-like 4 (TLR4) receptor. The LTA has been linked to various inflammatory responses (Y. Yokoyama, et al., *Acta Otolaryngol Suppl*. (1996) 523:108-111) and associated with Toll-like receptor 2 (TLR2) activation. It is widely believed that only the lysed bacteria liberate LPS that can initiate an inflammatory response (CA2323630). However, Zhang et al. showed that growing bacteria secrete LPS at a level in proportion to their growth phase (H. Zhang et al. (1998) *Infection & Immunity*, 66(11), 5196-5201). Therefore, even a small fraction of the plaque left on the teeth after brushing could seed the inflammatory cascade due to the release of LPS from the Gram negative bacteria present in the plaque.

Methods of detecting specific microbial species have been demonstrated in the art. In US Pub. No. 2012/019735A1, methods were proposed to distinguish disease-causing bacteria via spectrophotometric methods. Though they were able to show the presence of specific microbes, their invention would not allow the user to determine the virulence level of a specific site. Further, their method requires the microbes to be cultured in the lab in order to obtain a sufficient quantity of LTA or LPS for detection. Thus, their invention lacks the ability to detect the non-culturable species present, nor would it allow for measures of toxicity of biological samples.

In U.S. Pat. No. 5,175,089, the use of the Limulus amoebocyte lysate (LAL) endotoxin (LPS) assay was applied to the determination of the amount of endotoxin in the periodontal pocket. Though they were able to show overall amounts of endotoxin present, they lacked the ability to differentiate diseased versus healthy endotoxin and they

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were unable to quantify the level of virulence of the endotoxin. Further, their invention limited them to the Gram-negative endotoxin, as the LTA is not detectable via the LAL kit.

5 In US Pub. No. 2009/0047240, the chaperonin 10 (Cpn10) was used to modulate the clustering of Cpn10 in a cell line (murine RAW264) with labeled antibodies. Though they showed TLR-4, 7, and 9 reporter genes in an HEK cell line, their system would not allow for a more sensitive or
10 low level detection needed for microbial populations with weaker activating LPS, since those genes were under the control of the NFkB binding sites only (a minimal promoter). Their system lacks the sensitivity needed to differentiate biological systems with multiple microbial species and no dominant organism present. Further, their system needs strong NFkB activators to overcome the weak promoter used in their system, thus unable to pick up weaker TLR LPS agonists, such as LPS from *Porphyromonas gingivalis*. Additionally, their system lacked the ability to detect TLR3 agonists, which would be deleterious to the characterization of an inflammatory disease, such as gingivitis.

US Pub. No. 2007/0160544 describes a method for determining orally deleterious bacteria. Their method calls for contacting a gingival cell with bacteria or a bacterial component and measuring an inflammatory marker. According to US Pub. No. 2007/0160544, the presence of a marker indicates inflammation and the labeling of a bacterium as deleterious. Conversely, they say that the absence of a marker indicates the bacterium is not a problem. Though they cited Toll-like receptors, which were known in the art as part of the pathway to generate cytokines, their method would have only allowed for determining the presence of a
25 cytokine.

Since oral cells contain one or more of the receptors to which a bacterial virulence factor would activate, screening on the individual receptors requires the use of engineered cells, such as reporter cells containing the receptor gene of interest. What further complicates the use of native oral cells, such as gingival cells, is that the expression and activation of a receptor, such as a Toll-like receptor, is specific to the function of the cell. Gingival cells are less likely to respond to bacterial virulence factors, due to their constant contact with microbes in the dental plaque. Thus the need exists to have engineered cells where a direct response can be measured via a reporter system.

In addition to quantifying the virulence of microbial components and byproducts, there also exists a need for an in vitro screen of the inflammatory potential of organic and inorganic molecules, which would allow for pharmacokinetic parameters to be determined.

SUMMARY OF THE INVENTION

A method of determining bacterial virulence in an oral cavity is provided that includes providing a reporter cell expressing at least one toll-like receptor; providing a sample of oral matter; combining the sample of oral matter and the reporter cell; and measuring the toll-like receptor activation. The method may also include the additional steps of providing another sample of oral matter from an individual who used an oral care composition prior to providing another sample of oral matter; combining the another sample of oral matter and the reporter cell; measuring the toll-like receptor response; and comparing the toll-like receptor response from the sample of oral matter and another sample of oral matter.

A method for determining the virulence of lipopolysaccharide comprising providing a lipopolysaccharide; providing a reporter cell expressing at least one Toll-like receptor; combining the reporter cell with the lipopolysaccharide; measuring the toll-like receptor activation; and quantifying the lipopolysaccharide.

A method for determining the virulence of lipoteichoic acid comprising providing a lipoteichoic acid; providing a reporter cell expressing at least one Toll-like receptor; combining the reporter cell with the lipoteichoic acid; measuring the toll-like receptor activation; and quantifying the lipoteichoic acid.

The present invention includes methods of improving and/or resolving the state of gingival inflammation utilizing molecules, peptides, or proteins/enzymes that bind to, alter, or chemically modify bacterial virulence factors and/or host response mechanisms. The methods provide a means of quantifying the level of lipopolysaccharide of oral tissues; and utilizes TLR-4 and TLR-2 reporter cells line combined with the detection LPS via a fluorescence assay, such as BODIPY TR cadaverine, or endotoxin detection assay, assay to assign potency and quantification of LPS. The invention also includes assays and protocols which enable communication and demonstrations to consumers and dental professionals utilizing the TLR reporter cells.

The present invention includes methods for determining the potency of lipopolysaccharide comprising: a) providing a lipopolysaccharide sample; b) providing reporter cells expressing one or more Toll-like receptors; c) exposing the cells to the lipopolysaccharide sample; d) measuring the EC50 value of the lipopolysaccharide on activation of a Toll-like receptor; e) quantification of the lipopolysaccharide.

The present invention also includes isolating lipopolysaccharide from a growth culture of Gram negative bacteria. A lipopolysaccharide may be isolated from a biological sample. The biological sample includes, but is not limited to, an oral plaque, saliva, gingival brush samples. Toll-like receptor reporter gene assays, such as TLR4-SEAP and/or TLR2-SEAP, may be used to detect and quantify bacterial toxins, including but not limited to endotoxins, in a biological sample.

A BODIPY TR cadaverine assay may be used to detect and quantify lipopolysaccharide in a biological sample. In addition, LAL (the Limulus amoebocyte lysate assay) assay or endotoxin detection assay may be used to detect and quantify the lipopolysaccharide in a biological sample.

The present invention may be directed toward a method for determining the potency of an oral biofilm comprising: a) providing a biofilm sample; b) providing reporter cells expressing one or more Toll like receptors; c) exposing the cells to the biofilm sample; d) measuring the EC50 value of the biofilm activation of a Toll like receptor; e) quantification of the lipopolysaccharide. The biofilm may be an oral plaque, including but not limited to subgingival plaque, marginal or gumline plaque, supragingival plaque.

The present invention may also be directed toward a method for determining the potency of virulence in an oral sample comprising: a) providing an oral sample; b) providing reporter cells expressing one or more Toll-like receptors; c) exposing the cells to the oral sample; d) measuring the EC50 value of the biofilm activation of a Toll like receptor; e) quantification of the lipopolysaccharide. The oral sample may include saliva, oral lavage or gingival crevicular fluid.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a graph showing secretion of LPS during bacterial growth in culture.

FIG. 1B is a graph showing time course of bacterial growth in culture medium.

FIG. 1C is a graph showing secretion of LPS during bacterial growth in culture.

FIG. 1D is a graph showing secretion of LPS during bacterial growth in culture at 24 hours. *P. pallens* and *P. nigrescens* released more LPS into the medium than *P. gingivalis*.

FIG. 1E is a graph showing the amount of LPS per bacterium during bacterial growth in culture at 24 hours. *P. pallens* and *P. nigrescens* contain more LPS than *P. gingivalis*.

FIG. 1F is a picture showing the taking of gingival plaque samples using Periopaper.

FIG. 2A is a graph showing cell culture and virulence determination of lipopolysaccharide from different bacteria.

FIG. 2B is a graph showing activation of TLR4 reporter gene in culture.

FIG. 2C is a graph showing activation of TLR4 reporter gene in culture.

FIG. 3 is a graph showing stannous fluoride inhibition of TLR4 reporter gene expression.

FIG. 4 is a graph showing stannous fluoride prevention of *P. gingivalis* LPS from activating TLR2 reporter gene expression.

FIG. 5 is a graph showing percentage reduction of *P. gingivalis* LPS on activation of TLR-4 by stannous fluoride.

FIG. 6 is a graph showing EC50 determination of subgingival plaques that activate TLR4-SEAP signal transduction. Dental plaques from unhealthy sites had a smaller EC50 than those from healthy sites, suggesting the dental plaques from unhealthy sites contain more virulence factors.

FIG. 7A is a graph showing TLR4 Evaluation of Human Subgingival Plaque (Healthy vs. Gingivitis).

FIG. 7B is a graph showing TLR4 Evaluation of Human oral lavage (Healthy vs. Gingivitis). and

FIG. 8A is a graph showing cell culture and virulence determination of LTA from different bacteria.

FIG. 8B is a graph showing cell culture and virulence determination of LPS from different bacteria.

FIG. 8C is a graph showing cell culture and virulence determination of LPS from different bacteria.

FIG. 8D is a graph showing cell culture and virulence determination of LPS from different bacteria.

FIG. 9 is an EC50 graph showing determination of subgingival plaques that activate TLR2-SEAP signal transduction.

FIG. 10A is a graph showing TLR2 Evaluation of Human Subgingival Plaque (Healthy vs. Gingivitis).

FIG. 10B is a graph showing TLR2 Evaluation of Human oral lavage (Healthy vs. Gingivitis).

FIG. 11 is a graph showing cell culture and virulence determination of flagellins from different bacteria.

FIG. 12A is a graph showing LPS Stimulated TLR Activity in Human Monocytes THP-1.

FIG. 12B is a graph showing Evaluation of Human oral lavage (Healthy vs. Gingivitis) using the IRF-Luc reporter gene in THP-1 cells.

FIG. 12C is a graph showing Evaluation of Human Subgingival Plaque (Healthy vs. Gingivitis) in THP-1 cells using the NFkB reporter gene.

FIG. 13 is a graph showing detection of LTA of different bacteria in THP1 dual reporter cells.

FIG. 14 is a graph showing clinical measurements.

FIG. 15 is graphs showing decreases in bacterial abundance in the supragingival plaques during six weeks of regimen treatment.

FIG. 16 is a graph showing citrulline concentrations in Buccal-brush samples over a six-week regimen treatment.

FIG. 16A is a figure showing the relationship between FIG. 16A-1, FIG. 16A-2, and FIG. 16A-3.

FIG. 16A-1 is graphs showing decreases in bacterial abundance in the supragingival plaques during six weeks of regimen treatment.

FIG. 16A-2 is graphs showing decreases in bacterial abundance in the supragingival plaques during six weeks of regimen treatment.

FIG. 16A-3 is graphs showing decreases in bacterial abundance in the supragingival plaques during six weeks of regimen treatment.

FIG. 17 is a graph showing protein bound ornithine in Buccal brush samples during treatment.

FIG. 18 is a diagram showing enzymes in the ornithine, citrulline and arginine cycle.

FIG. 19 is a graph showing expression of ornithine, citrulline and arginine genes during a 6-week treatment.

FIG. 20 is a graph showing citrulline concentrations increased in Buccal-brush samples in experimental gingivitis.

FIG. 21 is graphs showing protein bound citrulline in Buccal brush samples in experimentally induced gingivitis.

FIG. 22 is graphs showing concentrations of protein bound ornithine in Buccal-brush samples in experimentally induced gingivitis.

FIG. 23 is graphs showing concentrations of total ornithine in Buccal-brush samples in experimentally induced gingivitis.

FIG. 24 is graphs showing concentrations of protein bound arginine in Buccal-brush samples in experimentally induced gingivitis.

FIG. 25 is a graph showing concentrations of total arginine in Buccal-brush samples in experimentally induced gingivitis.

FIG. 26 is a graph showing citrulline inhibited cytokine production stimulated by *P. gingivalis* LPS 1690 in THP-1 cells.

FIG. 27 is graphs showing bacterial LTA reduces fluorescent intensity of BC in a dose dependent manner.

FIG. 28 is graphs showing LPS reduces the fluorescent intensity of BC in a dose-dependent manner.

FIG. 29 is graphs showing the supernatant of bacterial culture reduces the fluorescent intensity of BC in a dose-dependent manner.

FIG. 30 is graphs showing bacteria reduce the fluorescent intensity of BC in a dose-dependent manner.

FIG. 31 is graphs showing EC50 values.

DETAILED DESCRIPTION OF THE INVENTION

As disclosed herein, it was surprisingly discovered that one or more methods could be used to detect and quantify the virulence in subgingival and supragingival plaques, thus distinguishing healthy gingivae from an inflamed site suffering from the symptoms of gingivitis. The state of health of the gingivae can be directly related to the level of bacterial toxins present, for example, endotoxins, and thus a reduction of these toxins, as discussed herein, in the oral cavity, as determined by screening on non-gingival engineered cells, would improve overall oral health. Additionally, the way an individual responds to the virulence factors can be quantified utilizing the individual's metabolic pathways, such as by quantifying products of the urea cycle.

Gingivitis is defined per the FDA monograph (12 CFR Part 356, Vol. 68, No. 103 (2003)) as "An inflammatory lesion of the gingiva that is most frequently caused by dental plaque. Gingivitis is characterized by tissue swelling and redness, loss of stippling (a normal state in which the surface of healthy gingiva is comprised of small lobes), glossy surface, and increased tissue temperature. The gingiva also may bleed upon gentle provocation, such as tooth brushing or may bleed spontaneously. Gingivitis is usually not painful." Within the monograph, plaque is defined as being composed of multiple bacterial species. Those species exert a constant inflammatory pressure on the host tissues.

When the inflammation progresses to the state of gingivitis, there exists a need to quantify how severe the gingivitis is and how effective treatments from oral hygiene products are in reducing the inflammatory response. The reduction in inflammatory response due to activation of membrane bound receptors across the gingival cells is termed detoxification; and measuring the level of detoxification, which is lacking in the art, is needed to educate consumers on the efficacy of their oral hygiene.

Pathogenesis of gingivitis involves both bacteria and host responses. The present invention discloses methodologies measuring the virulence factors in the dental plaques in vitro, and also measuring effects of virulence factors on gingival tissues in vivo. These methodologies allow understanding of what virulence factor types are present in the dental plaques, and how the host responds. Importantly, the measuring of virulence factors provides a detailed assessment on the severity of gingivitis in terms of virulence factors of the microbes in dental plaques and the health status of the host. In addition, these methods help evaluate the effectiveness of a technology in preventing and treating gingivitis.

The present invention includes a methodology comprising one or more of the following steps: (1) using the endotoxin detection assay kit or BODIPY-TR cadaverine method to quantify total LPS and LTA present in a biological sample and to detect technologies that inhibit the endotoxin detection assay; (2) using the Toll like receptor assays to determine potency of purified virulence and oral dental plaques, and to measure the efficacy of technologies that neutralize toxicity of virulence factors; (3) using meta-sequencing to identify and quantitate bacteria in supragingival plaques, (4) measuring ornithine and citrulline in gingival swab samples to determine the healthy status of gingival tissue, (5) measuring protein and mRNA levels to determine the levels of host responses, and determining citrulline activities in inhibiting LPS-induced production of proinflammatory cytokines.

The above-mentioned methods and chemistry may be applied in a strip form to the outer surface of the tooth and gumline. The strip may contain color or fluorescence reagents to interact with the virulence factors present and thus allow for semi-quantitative determination of the virulence present.

This would allow for rapid assessment of the level of severity of gingivitis and/or periodontal disease or to ascertain the effectiveness of oral products. This execution of the described methods allows for consumers to ascertain the state of their dental health at home or allows for professionals, such as dentists, to rapidly measure the state of a patient's oral health.

The methods described above can be used to determine the cellular impact of organic and inorganic molecules, as long as there is an interaction between the molecule and the targeted receptor. Further, the methods could be used to

ascertain if a molecule would cause an irritation or inflammatory response. The receptor in question would be used in a reporter system, as described herein, and the molecular impact of the molecule in question determined. The EC50 value of the molecule in question would be determined using the receptor associated with the biological response, thus reducing or eliminating the need to do animal testing.

All percentages and ratios used hereinafter are by weight of total composition, unless otherwise indicated. All percentages, ratios, and levels of ingredients referred to herein are based on the actual amount of the ingredient, and do not include solvents, fillers, or other materials with which the ingredient may be combined as a commercially available product, unless otherwise indicated.

All measurements referred to herein are made at 25° C. (i.e. room temperature) unless otherwise specified

As used herein, the word "include," and its variants, are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that may also be useful in the materials, compositions, devices, and methods of this invention.

As used herein, the word "or" when used as a connector of two or more elements is meant to include the elements individually and in combination; for example X or Y, means X or Y or both.

By "personal care composition" is meant a product, which in the ordinary course of usage is applied to or contacted with a body surface to provide a beneficial effect. Body surface includes skin, for example dermal or mucosal; body surface also includes structures associated with the body surface for example hair, teeth, or nails. Examples of personal care compositions include a product applied to a human body for improving appearance, cleansing, and odor control or general aesthetics. Non-limiting examples of personal care compositions include hair coloring compositions, oral care compositions, after shave gels and creams, pre-shave preparations, shaving gels, creams, or foams, moisturizers and lotions, cough and cold compositions, leave-on skin lotions and creams, shampoos, conditioners, shower gels, bar soaps, toilet bars, antiperspirants, deodorants, depilatories, lipsticks, foundations, mascara, sunless tanners and sunscreen lotions.

By "oral care composition", as used herein, is meant a product, which in the ordinary course of usage, is not intentionally swallowed for purposes of systemic administration of particular therapeutic agents, but is rather retained in the oral cavity for a time sufficient to contact dental surfaces or oral tissues. Examples of oral care compositions include dentifrice, mouth rinse, mousse, foam, mouth spray, lozenge, chewable tablet, chewing gum, tooth whitening strips, floss and floss coatings, breath freshening dissolvable strips, or denture care or adhesive product. The oral care composition may also be incorporated onto strips or films for direct application or attachment to oral surfaces.

The term "dentifrice", as used herein, includes tooth or subgingival-paste, gel, or liquid formulations unless otherwise specified. The dentifrice composition may be a single phase composition or may be a combination of two or more separate dentifrice compositions. The dentifrice composition may be in any desired form, such as deep striped, surface striped, multilayered, having a gel surrounding a paste, or any combination thereof. Each dentifrice composition in a dentifrice comprising two or more separate dentifrice compositions may be contained in a physically separated compartment of a dispenser and dispensed side-by-side.

The term "teeth", as used herein, refers to natural teeth as well as artificial teeth or dental prosthesis.

Virulence factors are molecules produced by pathogenic microbes that contribute to the pathogenicity of the organism and enable them to invade and proliferate in the host, and evade host immune surveillance. Virulence factors include, but are not limited to the following: Gram positive and Gram negative cell wall components, such as lipopolysaccharide and lipoteichoic acids, bacterial DNA, flagellin, peptidoglycan, adhesins, invasins, and antiphagocytic factors, hemolysins, bacterial metabolites, fimbriae, outer membrane vesicles, bacterial proteins or bacterial enzymes. A reduction in virulence or "detoxification", based on reduced activation of Toll-like receptors, can be used to measure the effectiveness of various treatments.

The term "detoxification" or "detoxify" or "detox" as used herein, refers to the neutralization, reduction, or removal of microbial virulence factors as measured by a reduction in the activation of a receptor known to be responsive with a virulence factor from an engineered cell, such as a reporter cell line. In certain embodiments a determination of virulence is assigned based on the activation of one or more of the Toll-like receptors.

The present invention includes obtaining a sample of oral matter. The oral matter can include gum-line plaque, subgingival plaque, supragingival plaque, interstitial plaque, gingival crevicular fluid (GCF), gingival biopsy, saliva, or tongue swab. The oral matter may be obtained by any method known in the art, for example, subgingival plaque sample may be collected physically by scraping or by using paper points. The plaque may be collected off of the tooth beneath gums from the sulcus, the developed periodontal pocket, or at the gumline. For example, each paper point can be placed in the pocket between the tooth and the gingiva for 10 seconds. After 10 seconds, a paper point can be removed and placed into a pre-labeled 1.5 ml tube with 700 μ l phosphate-buffered saline. The sampling procedure can be repeated with three more paper points. After all four paper points are collected, the 1.5 ml tube will be closed, vortexed for 30 seconds and placed on dry ice until the samples are stored in a -80° C. freezer. Other methods of collection could include a mechanical device to help release the plaque from the tooth surface, such as a sonic scaler. The oral matter may be obtained both before and after treatment of an oral site from which the oral matter is obtained. An oral site from which the oral matter is obtained includes host tissues and bacterial matters. Further treatment of an oral site may be more than once and may include multiple different treatments, for example a regimen, such as brushing teeth followed by mouthrinse. In addition to obtaining oral matter before and after completed treatments, oral matter may be obtained between separate treatments, for example between the brushing of teeth followed by the use of mouth rinse.

The sample of oral matter is combined with a Toll-like receptor in a reporter cell. Examples of Toll-like Receptors that can be used in the present invention include TLR 2, TLR 4, TLR 5, and TLR 9. Human TLR cDNA (TLR 1, 2, 3, 4, and 5) was first cloned in 1998 and their sequences were published (Rock F L, Hardiman G, Timans J C, Kastelein R A, Bazan J F. A family of human receptors structurally related to *Drosophila* Toll. Proc Natl Acad Sci USA. 1998 Jan. 20; 95:588-93). Rock et al. discovered that cytoplasmic domains of *Drosophila* Toll and human interleukin 1 receptors shared high sequence homology, and hypothesized that both molecules trigger related signaling pathways tied to the nuclear translocation of Rel-type transcription factors. They cloned a class of putative human receptors with a protein architecture that is similar to *Drosophila* Toll in both intra- and extracellular segments. They hypothesized that the five

human Toll-like receptors—named TLRs 1-5—are probably the direct homologs of the fly molecule and, as such, could constitute an important and unrecognized component of innate immunity in humans. Soon after the DNA sequences for TLR1 to 5 were published, other TLR DNA sequences were also revealed. For example, TLR6 cDNA sequence was reported in 1999 (Takeuchi O I, Kawai T, Sanjo H, Copeland N G, Gilbert D J, Jenkins N A, Takeda K, Akira S. A novel member of an expanding toll-like receptor family. *Gene*. 1999 Apr. 29; 231(1-2):59-65). cDNA sequences of human TLR7, TLR8 and TLR9 were reported in 2000 (Chuang TH1, Ulevitch R J. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur Cytokine Netw*. 2000 September; 11(3):372-8).

SEQ ID NO	Sequence
1	Human Toll-Like Receptor 1 Amino Acid sequence
2	Human Toll-Like Receptor 2 Amino Acid sequence
3	Human Toll-Like Receptor 3 Amino Acid sequence
4	Human Toll-Like Receptor 4 Amino Acid sequence
5	Human Toll-Like Receptor 5 Amino Acid sequence
6	Human Toll-Like Receptor 6 Amino Acid sequence
7	Human Toll-Like Receptor 7 Amino Acid sequence
8	Human Toll-Like Receptor 8 Amino Acid sequence
9	Human Toll-Like Receptor 9 Amino Acid Sequence

A sequence listing that sets forth the amino acid sequences for SEQ ID NO: 1 to 9 herein is being filed concurrently with the present application as an ASCII text file titled “13837M_AA_Sequence_Listing_ST25.” The ASCII text file was created on 29 Mar. 2016 and is 68 Kbytes in size. In accordance with MPEP § 605.08 and 37 CFR § 1.52(e), the subject matter in the ASCII text file is incorporated herein by reference.

TLR proteins can form heterodimers or homodimers. There are 10 TLR genes identified in humans. Their gene products form homodimers or heterodimers in cell membranes. For example, TLR1 can form heterodimers with TLR2. Similarly, TLR6 also can assemble a heterodimer with TLR2. On other hand, TLR4 forms a homodimer. Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate immune system as well as the digestive system. They are membrane-spanning, non-catalytic receptors usually expressed in cells such as macrophages, dendrite cells, and gingival epithelial cells that recognize structurally conserved molecules derived from microbes. Once these microbes have breached physical barriers such as the skin or intestinal tract mucosa or oral epithelial cells they are recognized by TLRs, which activate immune cell responses. Toll-like receptors were targeted because they are the primary sensors of virulence factors produced by the microbes in the dental plaques. TLR 1, 2, 4, 5 and 6 are expressed in the cell plasma membranes, providing easy access for the virulence factors to be sensed by the host receptors. TLR 3, 7, 8, and 9 are located on the membranes of endosomes; and as virulence factors are phagocytosed into the cells, they also gain access to TLR 3, 7, 8 and 9

A reporter cell refers to a eukaryotic cell, such as, but not limited to, HEK 293T, human monocyte (THP1), Chinese hamster ovary (CHO) cell, murine cells, or monkey kidney epithelial (Vero) cells engineered to express a predetermined number of TLR receptors, for example a single TLR receptor; which is in contrast to gingival cells that express multiple functional TLR receptors. Thus, one type of engineered reporter cells respond to only one type of virulence

factors in the dental plaques. In contrast, gingival cells express several types of functional TLRs, and can't be used to identify a single type of virulence factor in dental plaques. The output from gingival cells is the sum of various virulence factors in the dental plaques. HEK 293T cells can be used as reporter cells, as they are easy to maintain and have similar gene expression profiles to oral epithelial cells, making them a closer match to the gene expression of gingival cells, such that the results will mirror in vivo results. In contrast to naturally occurring gingival cells the reporter cells of the present invention are easy to maintain in the lab, and stable in phenotypes. Further, the reporter cells make detection of virulence factors simpler, are more reproducible, increase accuracy, provide higher throughput are more specific and more quantifiable.

Individual TLR receptor genes are stably transfected to HEK 293 cells as described by Invivogen (/PDF/HEK_Blue_htrl4_TDS.pdf). HEK-Blue™ hTLR4 Cells are designed for studying the stimulation of human TLR4 (hTLR4) by monitoring the activation of NF-kB. HEK-Blue™ hTLR4 Cells were obtained by co-transfection of the hTLR4 gene, the MD-2/CD14 co-receptor genes and a secreted embryonic alkaline phosphatase (SEAP) reporter gene into HEK293 cells. The SEAP reporter gene is placed under the control of an IL-12 p40 minimal promoter fused to five NF-kB and AP-1-binding sites (Supplement 1. HEK-Blue™ hTLR4 Cells SEAP Reporter 293 cells expressing the human TLR4 gene, Catalog # hkb-htrl4, Version #15C04-MM (/PDF/HEK_Blue_htrl4_TDS.pdf). As compared to measuring an immune response the reporter genes of the present invention allow rapid, specific and reproducible measurements of virulence factors.

The level of receptor activation can be determined by any method known in the art for the type of reporter gene used. For example if an NFkB-SEAP reporter gene is used, one could measure the production of SEAP in the culture medium. The reporter cells can be treated with virulence factors, or dental plaque matters collected before or after treatments. Expression of the reporter gene will be stimulated and SEAP secreted into the medium when stimulated by virulence factors. The level of reporter gene product SEAP can be readily measured with commercial kits, and will be proportional to the amount of particular types of virulence factors. Similarly, if an NFkB-luciferase, NFkB-beta-lactamase, or other reporter genes are used, available kits can be used to measure the reporter gene products.

The potency can then be determined based on parameters such as, EC50 and fold of stimulation. EC50 provides a measurement on the amount of virulence factors needed to mount an inflammatory response, and the fold of stimulation is indicative of the severity of inflammatory responses the virulence factors cause. The EC50 is used to determine potency; wherein “potency” as defined by the Merck Manual, refers to the concentration (EC50) of a chemistry required to produce 50% of the chemistry's maximal effect as depicted by a graded dose-response curve. EC50 equals Kd (Dissociation constant, which is a measure of 50% of the substance in question bound to the receptor) when there is a linear relationship between occupancy and response. Often, signal amplification occurs between receptor occupancy and response, which results in the EC50 for response being much less (ie, positioned to the left on the abscissa of the log dose-response curve) than Kd for receptor occupancy. Potency depends on both the affinity of a compound for its receptor, and the efficiency with which a compound-receptor interaction is coupled to response. The dose of a compound required to produce an effect is inversely related to potency.

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In general, low potency is important only if it results in a need to administer a compound in large doses that are impractical. Quantal dose-response curves provide information on the potency of a compound that is different from the information derived from graded dose-response curves. In a quantal dose-response relationship, the EC50 is the dose at which 50% of individuals exhibit the specified quantal effect.

In the present invention, the activities of endotoxins or lipopolysaccharides can be measured using an endotoxin detection assay, or LAL assay. The Limulus Amebocyte Lysate (LAL) test has been used to detect LPS. LAL is derived from the blood cells, or amebocytes, of the horseshoe crab, *Limulus polyphemus*. At present, some main endotoxin detection agents are derived from recombinant proteins. Thereinafter, endotoxin detection assay and LAL assay are used interchangeably.

A. A method of determining bacterial virulence in an oral cavity comprising:

- a. providing a reporter cell expressing at least one toll-like receptor;
- b. providing a sample of oral matter;
- c. combining the sample of oral matter and the reporter cell;
- d. measuring the toll-like receptor activation.

B. The method of paragraph A, wherein the toll-like receptor is at least one of TLR1 to TLR9.

C. The method according to paragraph A or B, wherein the reporter cell expresses at least one of TLR2 or TLR4.

D. The method according to any one of paragraphs A to C, wherein the toll-like receptor activates a reporter gene, preferably wherein the reported gene is a secreted embryonic alkaline phosphatase (SEAP) reporter gene.

E. The method according to any one of paragraphs A to D, wherein the sample of oral matter is at least one of gum-line plaque, subgingival plaque, supragingival plaque, interstitial plaque, gingival crevicular fluid (GCF), gingival biopsy, saliva, or tongue swab, preferably wherein the sample of oral matter comprises virulence factor that is at least one of Gram positive cell wall components, Gram negative cell wall components, bacterial DNA, flagellin, peptidoglycan, bacterial metabolites, fimbriae; outer membrane vesicles bacterial proteins, or bacterial enzymes.

F. The method of paragraph E, wherein the sample of oral matter comprises virulence factor that is at least one of lipopolysaccharide or lipoteichoic acid.

G. The method according to any of paragraphs A to F comprising the additional steps of:

- e. providing another sample of oral matter from an individual who used an oral care composition prior to providing another sample of oral matter;
- f. combining the another sample of oral matter and the reporter cell;
- g. measuring the toll-like receptor response;
- h. comparing the toll-like receptor response from the sample of oral matter and another sample of oral matter.

H. The method of paragraph G, where the sample of oral matter and the another sample of oral matter are from the same individual.

I. A method for determining the virulence of lipopolysaccharide comprising:

- a. providing a lipopolysaccharide;
- b. providing a reporter cell expressing at least one Toll-like receptor;
- c. combining the reporter cell with the lipopolysaccharide;

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d. measuring the toll-like receptor activation;

e. quantifying the lipopolysaccharide.

J. The method of paragraph I, wherein the Toll-like receptor activation is determined by measuring at least one of EC50 or fold of stimulation.

K. The method according to paragraph I or J, wherein the Toll-like receptor is at least one of TLR1 to TLR9.

L. The method according to any of paragraphs I to K, wherein the reporter cell expresses at least one of TLR2 or TLR4.

M. The method according to any of paragraphs I to L, wherein the Toll-like receptor activates a reporter gene.

N. The method according to paragraph M, wherein the reported gene is a secreted embryonic alkaline phosphatase (SEAP) reporter gene.

O. The method according to any of paragraphs I to N, wherein the lipopolysaccharide is quantified using at least one of limulus amebocyte lysate assay or BODIPY TR cadaverine assay.

EXAMPLES

Example 1—Determination of Secreted Lipopolysaccharide by Bacteria

Growth of bacteria: A 1 ml aliquot of a 24 hour culture of *E. coli* ATCC 8739 was used to inoculate 100 ml of Luria-Bertani (LB) broth in a 250 ml baffled flask. This culture was then incubated at 37° C. with agitation (220 rpm) and sampled at 30 minute intervals. Samples were assessed for turbidity (OD₆₀₀) in a SpectraMax platereader M3 (Molecular Devices, Sunnydale, Calif.), which is one method of monitoring the growth and physiological state of microorganisms. The sample turbidity was then recorded and the samples were centrifuged at 5000 RPM for 10 min at room temperature. The supernatant, thereinafter referred to as “supernatant of bacterial culture”, was subsequently analyzed for LPS content using the procedure as described below.

Twenty ml aliquots of MTGE broth (Anaerobe Systems, Morgan Hill, Calif.) were inoculated with *P. gingivalis* ATCC 33277, *P. pallens* ATCC 700821, or *P. nigrescens* ATCC 25261. These cultures were incubated overnight in a Whitely A45 Anaerobic Workstation (Don Whitley Scientific, Frederick, Md.) at 37° C. with an 85:10:5 N₂:CO₂:H₂ gas ratio. One ml aliquots of these starter cultures were then used to inoculate 99 ml of membrane-Tryptone Glucose Extract (m-TGE) broth in a 250 ml baffled flask. These cultures were then incubated under agitation (200 rpm) as previously described and sampled at regular intervals. Samples were assessed for turbidity (OD₆₀₀) in a Tecan Infinite m200 Pro (Tecan Trading AG, Switzerland) and then centrifuged at 16,100×g for 10 min at room temperature. Supernatants were decanted and passed through a 0.22 μm filter prior to analysis for LPS content.

In the experiment, only OD600 was measured. For the sake of consistency in following experiments, we converted OD600 readings into bacterial numbers, even though the relationship between OD600 readings and bacterial numbers is varied for each bacterium. The number of bacteria was estimated based on spectrophotometer readings at OD₆₀₀ (OD₆₀₀ of 1.0=8×10⁸ cells/ml).

The Limulus Amebocyte Lysate Assay (LAL) is an assay to determine the total amount of lipopolysaccharide (LPS) in the sample tested (Pierce LAL Chromogenic Endotoxin Quantitation Kit, ThermoFischer Scientific, Waltham, Mass.). The assay was performed following manufacturer's

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instruction. Ninety-six-well microplates were first equilibrated in a heating block for 10 min at 37° C. Fifty µl each of standard or sample was dispensed into the microplate wells and incubated with plate covered for 5 min at 37° C. Then 50 µl LAL was added to each well. Plates were shaken gently and incubated for 10 min at 37° C. 100 µl of chromogenic substrate was added and incubated for 6 min at 37° C. Finally, 50 µl Stop Reagent was added and the absorbance was measured at 405-410 nm on Spectramax M3 plate reader (Molecular Device, Sunnyvale, Calif.).

FIGS. 1A, 1C, and 1D show the ability of microbes to shed LPS as part of their normal growth cycle. This data shows the need to deliver chemistry to the subgingival plaque to effectively mitigate the LPS, since tooth brushing generally does not remove the subgingival plaque.

The LPS, as measured by the LAL kit reported in endotoxin unit per ml (EU/ml), was shed by the bacteria (*E. coli* K12) as depicted in FIG. 1A. The growth media began to be depleted of complex sugars around 120 minutes, as reflected in the bacterial growth curve in FIG. 1B, where the LPS shedding started to decline. This data gave a reason to believe that a mature biofilm/plaque could supply a constant level of LPS to the host cells, if food sources were present. The LPS would then have the ability to induce an inflammatory response from the host cells.

Importantly, LPS are secreted into the supernatant of bacterial culture (FIG. 1D). LPS also exists in bacterial walls (FIG. 1E). Again, this data further enforce the need to deliver chemistry to the subgingival plaque to effectively mitigate the LPS, since tooth brushing generally does not remove the subgingival plaque.

Example 2—LAL Assay to Quantify Virulence Factors in Dental Plaques

Seven panelists, with at least three bleeding sites, took part in the testing. A licensed dental hygienist collected subgingival plaque samples. Samples were taken at the tooth/gum interface (buccal surfaces only) using care to avoid contact with the oral soft tissues. Six subgingival plaque sites were sampled from each panelist (3 healthy and 3 unhealthy sites). Unhealthy teeth had bleeding sites with pockets greater than 3 mm and healthy sites had no bleeding with pocket depth less than 2 mm. Prior to sampling, panelists were instructed to abstain for 12 hours from oral hygiene and refrain from eating, chewing gum, drinking (except small sips of water). Next, panelists had their marginal plaque collected with a curette at the sampling sites. Then, from the same site, subgingival plaque samples were collected with 3 consecutive paper points as shown in FIG. 1F. The sampling sites were isolated with cotton rolls and gently air-dried. Paper points (PROFLOW incorporated, Amityville, N.Y.) were gently placed for 10 seconds into the pocket until a minimum of resistance was felt. After 10 seconds, paper points were removed and placed into pre-labeled 1.5 ml tubes. The same sampling procedure was repeated with 2 more paper points (paper points go into separate tubes). The first, second and third sample paper points from a healthy site of all panelists were pooled separately into three tubes, labeled as paper point 1, 2 and 3, respectively. Similarly the unhealthy site samples were also pooled.

TABLE 1 showed that unhealthy dental plaques contained more endotoxins than the healthy dental plaques. One ml PBS was added to each pooled sample in the 1.5 ml tube. Bacteria were lysed in a MolBio Fast Prep bead beater (MP Biomedicals, Santa Ana, Calif.). Samples were centrifuged

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for 10 min at 10,000 RPM at 4° C., supernatants were collected and analyzed with LAL assay kits following manufacturer's instruction as described in EXAMPLE 1.

TABLE 1

Protein concentrations and endotoxin levels in the pooled dental plaque samples.	
Dental plaque	Endotoxin (endotoxin unit)
Healthy paperpoint 1 sub plaque	1284
Healthy paperpoint 2 sub plaque	476
Healthy paperpoint 3 sub plaque	361
Healthy Marginal Plaque	23180
Unhealthy paperpoint 1 sub plaque	3371
Unhealthy paperpoint 2 sub plaque	1732
Unhealthy paperpoint 3 sub plaque	16111
Unhealthy Marginal Plaque	80277

It was expected that the marginal plaques in unhealthy sites had more endotoxins than those in the healthy sites (TABLE 1) within the same subjects. Three samples were taken from subgingival pockets with three paper points sequentially, named paper point 1, 2 and 3. Again, the subgingival plaques taken by the paper point 1 had more endotoxins in the unhealthy sites than in the healthy sites (TABLE 1). The same is true for the samples taken by paper point 2 and 3. Importantly, dental plaques in the unhealthy subgingival pockets possessed more endotoxins than plaques from healthy pockets. This may explain why unhealthy gingiva are prone to bleeding upon probing.

Example 3—Technology Development Using the LAL Assay Kit

The LAL assay, as described in EXAMPLE 1, was modified for development of technology which inhibits LPS from activating a proenzyme in the LAL assay. The Thermo Scientific Pierce LAL Chromogenic Endotoxin Quantitation Kit is a quantitative endpoint assay for the detection of LPS, which catalyzes the activation of a proenzyme in the modified Limulus Amebocyte Lysate (LAL). The activated proenzyme then splits p-Nitroaniline (pNA) from the colorless substrate, Ac-Ile-Glu-Ala-Arg-pNA. The product pNA is photometrically measured at 405-410 nm. If SnF2 binds to LPS, the latter can't react with the proenzyme in the LAL kit. Consequently, the proenzyme is not activated, and the colorless substrate Ac-Ile-Glu-Ala-Arg-pNA will not split and no color product is produced. *P. gingivalis* LPS 1690 (1 ng/ml), or *E. coli* LPS (1 ng/ml), and stannous fluoride and other materials (50 and 500 µM), as listed in TABLE 2, were dissolved in endotoxin-free water. Then 50 µl LAL was added to each well. Plates were shaken gently and incubated for 10 min at 37° C. 100 µl of chromogenic substrate was added and incubated for 6 min at 37° C. Finally, 50 µl Stop Reagent was added and the absorbance was measured at 405-410 nm on Spectramax M3 plate reader (Molecular Device, Sunnyvale, Calif.).

As shown in TABLE 2, SnF2 and some other compounds inhibited LPS activities in LAL assays

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TABLE 2

Inhibition of LPS activities on LAL Assays				
Samples	Inhibition of LAL activity %			
	P. gingivalis LPS 1690 1 ng/ml		E. coli LPS 1 ng/ml	
	500 uM	50 uM	500 uM	50 uM
Tin (II) fluoride	60	49	92	87
stannous chloride	48	21	89	65
Cetylpyridinium chloride	103	77	103	46
monohydrate				
Chlorhexidine	102	38	97	57
zinc citrate, dihydrate	104	57	104	82
zinc lactate	58	0	66	0
potassium oxalate	8	0	1	6
Triclosan (irgasan)	0	0	10	0
1-Hydroxypyridine-2-thione	0	0	2	6
zinc salt				
sodium fluoride	0	0	4	5
Carboxymethyl cellulose	0	0	2	0
sodium				

Example 4—Binding of BODIPY-TR-Cadaverine to LPS and Displacement of LPS Binding by Stannous Fluoride

In addition to LAL quantification of LPS, the BODIPY method can be utilized to assess the level of LPS. Detoxifying technologies are able to target and neutralize bacterial virulence factors, such as LPS and LTA. To develop such LPS and LTA sequestration technologies, a high throughput screening was employed to identify molecules that disrupt the activation of a Toll-like receptor by LPS and LTA, and other virulence factors. The high throughput screen utilizes the fluorescent dye BODIPY-TR-cadaverine 5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl)amino)pentylamine hydrochloride (BC fluorescent dye), purchased from Life Technologies (Carlsbad, Calif.), as described previously by Wood, Miller and David (2004) (Comb Chem High Throughput Screen 2004 May; 7(3):239-49. Anti-endotoxin agents. 1. Development of a fluorescent probe displacement method optimized for the rapid identification of lipopolysaccharide-binding agents.). The experiment was carried out at 22° C. *E. coli* LPS (15 µg/ml) was mixed in a well of a 96-well solid black flat bottom plate (Corning Incorporated, Corning, N.Y.) with SNF2 and cetylpyridinium chloride in 30 µl of 50 mM Tris buffer at pH 7.4 for 10 mM, and then 20 µl of 60 µM BC fluorescent dye was added right before fluorescence measurement at 22° C., or room temperature. Fluorescence measurements were performed in a SpectraMax M3 automated 96 well plate reader (Molecular Device, Sunnyvale, Calif.). The excitation wavelength was 580 nm and the fluorescence emission was measured at 620 nm.

As shown in TABLE 3, leading anti-gingivitis technologies, such as stannous fluoride, displaced LPS from BODIPY TR cadaverine.

TABLE 3

Displacement of LPS from binding to BODIPY TR cadaverine		
Concentrations	Displacement %	
mM	Stannous fluoride	Cetylpyridinium chloride
0.16	2.45	9.98
0.31	2.79	31.11

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TABLE 3-continued

Displacement of LPS from binding to BODIPY TR cadaverine		
Concentrations	Displacement %	
mM	Stannous fluoride	Cetylpyridinium chloride
0.63	12.69	68.05
1.25	49.44	91.12

Example 5—Cell Culture and Virulence Determination of Lipopolysaccharide from Different Bacteria

Reporter gene cell lines, human HEK 293T cells, were purchased from Invivogen of San Diego, Calif. The HEK 293T cells were stably transfected with at least two exogenous genes, a TLR4 structural gene, and a SEAP reporter gene, which is under the control of NFκB transcriptional factors. The cell line is named here as TLR4-SEAP. The reporter gene encodes a secreted enzyme, called embryonic alkaline phosphatase or SEAP. The SEAP reporter is placed under the control of the interferon-β minimal promoter fused to five NFκB and AP-1-binding sites. Furthermore, the TLR4-SEAP cell line also contains a CD14 co-receptor gene, which is needed to transfer LPS to TLR4 receptors. The recombinant TLR binds its ligand, or distinct pathogen-associated molecule, initiates a chain of responses, leading to recruitment of NFκB and AP1 transcription factors to the reporter gene promoter, which induce expression of SEAP.

Cell culture and treatment: 500,000 gene reporter cells were grown and maintained in 15 ml growth medium, comprised of DMEM medium supplemented with 10% fetal calf serum in T75 flasks for three days at 37° C., 5% CO₂, and 95% humidity. For treatment, wells of a 96-well plate were seeded with 10,000 cells/well in 100 µL of growth medium. The cells were incubated for 72 hours at 37° C., 5% CO₂, and 95% humidity until day 4. On day 4, medium was changed to assay medium (90 µl), which is the DMEM medium without fetal calf serum. LPS, bacteria and the culture medium of bacterial growth, as described in EXAMPLE 1, were first resolved or mixed with the assay medium. 10 µl of the bacteria, LPS and culture medium of bacterial growth were added to the TLR4-SEAP cells. Samples were taken 24 hours later, following addition of LPS, bacteria, and culture medium. Expression of the reporter gene (SEAP) was quantified with a commercially available kit (SEAP Reporter Gene Assay of Cayman Chemical Co., Ann Arbor, Mich.).

EC50 was calculated using GraphPad Prism software (GraphPad Software, La Jolla, Calif.). Samples with lower EC50 are more potent in activating the TLR4 reporter gene than those with higher EC50. As shown in FIG. 2A, LPS from *E. coli* has lower EC50 than *P. gingivalis*, thus, was far more potent than *P. gingivalis* (Pg). *Salmonella Minnesota* LPS is not as potent as that of *E. coli*, but is far more potent than those of *P. gingivalis* LPS 1690 and 1435. Each species of bacteria produces multiple forms of LPS. Each form of LPS from the same species of bacteria has different potency in stimulating TLR4-downstream signaling pathways. For example, Pg 1690 LPS is more potent than Pg1435/50. LPS is a component in bacterial cell walls. Likely, *E. coli* cell wall is more virulent in inducing production of proinflammatory cytokines in host cells than *P. gingivalis* when they are in direct contact with host blood cells. *P. gingivalis* had

far higher EC50 than *P. pallens* and *P. nigrescens* as shown in FIG. 2B in stimulating TLR4 reporter gene expression, suggesting that *P. pallens* and *P. nigrescens* are more likely to promote production of proinflammatory cytokines in host cells than *P. gingivalis*.

Bacteria release LPS into the supernatant of bacterial culture. As shown in FIG. 2C, the supernatant of *P. pallens* has an EC50 that is similar to that of *P. nigrescens*, but far lower than that of *P. gingivalis*, in stimulating expression of TLR4 reporter gene. Again, those results imply that the products of *P. pallens* and *P. nigrescens* are more likely to promote production of proinflammatory cytokines in host cells than those of *P. gingivalis*.

Example 6—Development of Technology for Inhibition of LPS Effects on Stimulating Expression of NFkB-Regulated Reporter Genes

Stannous fluoride is a leading anti-gingivitis technology in P&G toothpaste products. Tests were conducted to understand whether stannous fluoride could reduce LPS's ability to trigger proinflammatory responses in host cells. TLR4-SEAP reporter cells were prepared using the same conditions as described in EXAMPLE 5 in the presence or absence of LPS. Production of SEAP was quantified also as described in EXAMPLE 5.

FIG. 3 shows the effect of stannous at various concentrations from 62.5 uM to 1,000 uM on 100 ng/ml *E. coli* LPS, as reported by activation of TLR-4. At stannous concentrations of 500 uM or higher, the level of *E. coli* induction of TLR-4 was decreased.

FIG. 4 shows the effects of stannous at various concentrations from 62.5 uM to 1,000 uM on *P. gingivalis* LPS, as reported by activation of TLR-2. At stannous concentrations of 1000 uM, the level of *P. gingivalis* induction of TLR-2 was decreased.

The data in FIG. 5 shows reduction of LPS activity by the stannous ion, from a stannous fluoride salt. The data showed that stannous fluoride, at 1.6 mM and 3.2 mM, reduce about 50% of *P. gingivalis* LPS (500 ng/ml) activation on the TLR4 reporter system (One asterisk means $P < 0.05$, two asterisks mean $P < 0.01$).

Example 7—Cell Culture and Virulence Determination of EC50 in Activation of TLR4-SEAP Signal Transduction by Clinical Samples

The method described in EXAMPLE 5 is effective at determining the potency of LPS from different bacteria. The same method was used to determine the EC50 of clinical samples, as described in EXAMPLE 2. As shown in FIG. 6, dental plaques from unhealthy sites had a smaller EC50 than those from healthy sites, suggesting the dental plaques from unhealthy sites contain more virulence factors.

The same method described in EXAMPLE 5 was used to examine the clinical samples in another study. A clinical study was conducted to evaluate sample collection methods and measurement procedures. It was a controlled, examiner-blind study. Forty panelists met the inclusion criteria, wherein in order to be included in the study, each panelist must:

- Provide written informed consent to participate in the study;
- Be 18 years of age or older;
- Agree not to participate in any other oral/dental product studies during the course of this study;

Agree to delay any elective dentistry (including dental prophylaxis) until the study has been completed;

Agree to refrain from any form of non-specified oral hygiene during the treatment periods, including but not limited to the use of products such as floss or whitening products;

Agree to return for all scheduled visits and follow study procedures;

Must have at least 16 natural teeth;

Be in good general health, as determined by the Investigator/designee based on a review of the health history/update for participation in the study.

For Unhealthy Group (high bleeder group):

Have at least 20 bleeding sites (sites with a score of 1 or 2 on the GBI index); Have minimum 3 sampling sites with bleeding and pocket depth > 3 mm but not deeper than 4 mm;

Have minimum 3 sampling sites without bleeding and with pocket depth < 2 mm

For Healthy Group (low bleeder group):

Have maximum 3 bleeding sites (sites with a score of 1 or 2 on the GBI index);

No pockets deeper than 2 mm. Twenty (20) panelists were qualified as healthy—with up to 3 bleeding sites and with all pockets less than or equal to 2 mm deep and twenty (20) panelists were qualified as unhealthy—with greater than 20 bleeding sites with at least 3 pockets greater than or equal to 3 mm but not deeper than 4 mm with bleeding, and at least 3 pockets less than or equal to 2 mm deep with no bleeding for sampling. All panelists had up to 6 sites identified as "sampling sites." The "sampling sites" had supragingival and subgingival plaque collected at Baseline, Week 2 and Week 4. Subgingival plaque samples were taken from a gingival sulcus from the pre-identified sites. Prior to sample collection, the site had supragingival plaque removed with a curette. The site was dried and subgingival plaque samples were collected with another dental curette (e.g., Gracey $^{13}/_{14}$, $^{15}/_{16}$, $^{11}/_{12}$, 7/8, $^{1}/_{2}$.) Each Gracey curette is designed to adapt to a specific area or tooth surface. For example, Gracey $^{13}/_{14}$ is designed to adapt to the distal surfaces of posterior teeth. Samples from each site were placed in a pre-labeled 2.0 ml sterile tube containing 300 μ l of DPBS buffer with about 50 of sterile 1 mm glass beads. Samples were stored at 4° C. The subgingival samples were stored at -80° C. until analyzed. The samples were thawed at room temperature and dispersed in a TissueLyser II (Qiagen, Valencia, Calif., USA) at 30 shakes per second for 3 min. Protein concentrations of the dispersed subgingival samples were measured using a Pierce microBCA Protein kit (ThermoFisher Scientific, Grand Island, N.Y., USA) following the manufacturer's instruction.

Oral lavage samples were collected at wake up (one per panelist) by rinsing with 4 ml of water for 30 seconds and then expectorating the contents of the mouth into a centrifuge tube. These samples were frozen at home until they were brought into the site in a cold pack. Each panelist collected up to 15 samples throughout the study. Saliva samples were frozen at -70° C. from submission.

All panelists were given investigational products: Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush. Panelists continued their regular oral hygiene routine, and did not use any new products starting from the baseline to the end of four week treatment study. During the

four week treatment period, panelists brushed their teeth twice daily, morning and evening, in their customary manner using the assigned dentifrice and soft manual toothbrush.

The subgingival plaques from the above clinical study were applied to the TLR4 reporter cells in a procedure as described in EXAMPLE 5. FIG. 7A shows the results of a four-week study of 40 panelists going from baseline out over four weeks of treatment with Crest ProHealth Clinical toothpaste. The subgingival plaque samples in bleeding sites on the high bleeders group stimulated high expression of TLR4 reporter gene. More virulence in a sample elicits higher RLU (relative luminescent units) readings in the TLR4 reporter gene assay. As shown in FIG. 7A, the baseline samples of the high bleeders group had higher RLU than those of the low bleeders on both the bleeding and non-bleeding sites. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in both high and lower bleeders groups at both bleeding and non-bleeding sites.

The oral lavage samples were applied to the TLR4 reporter cells in a procedure as described in EXAMPLE 5. As shown in FIG. 7B, oral lavage (Healthy vs. Gingivitis) samples were evaluated in the TLR4-SEAP reporter assay. The baseline samples of the high bleeders group had higher RLU than those of the low bleeders. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in the high bleeder group.

Example 8—Cell Culture and Virulence Determination of Lipoteichoic Acids from Different Bacteria

The reporter gene cell lines, human HEK 293T cells, were purchased from Invivogen of San Diego, Calif. The HEK 293T cells were stably transfected with at least two exogenous genes, a TLR2 structural gene, and SEAP reporter gene which is under the control of NFkB transcriptional factors. The cell line is named here as TLR2-SEAP. The reporter gene encodes a secreted enzyme, called embryonic alkaline phosphatase or SEAP. The SEAP reporter is placed under the control of the interferon- β minimal promoter fused to five NFkB and AP-1-binding sites. Furthermore, a CD14 co-receptor gene was transfected into the reporter gene cells expressing TLR2, as CD14 has been identified as a co-receptor for TLR2 ligands to enhance the TLR response. The CD14 co-receptor is needed to transfer LTA to TLR2 receptors. The recombinant TLR2 binds its ligand, or distinct pathogen-associated molecule, initiates a chain of responses, leading to recruitment of NFkB and AP1 transcription factors to the reporter gene promoter, which induce expression of SEAP.

Cell culture and treatment: 500,000 gene reporter cells were grown and maintained in 15 ml growth medium, comprising DMEM medium supplemented with 10% fetal calf serum in T75 flasks for three days at 37° C., 5% CO₂, and 95% humidity. For treatment with LTA, wells of a 96-well plate were seeded with 10,000 cells/well in 100 μ L of growth medium. The cells were incubated for 72 hours at 37° C., 5% CO₂, and 95% humidity until day 4. On day 4, medium (100 μ L) was changed to DMEM medium without fetal calf serum. LTA, LPS and bacterial cells, as described in EXAMPLE 7, were added. Samples were taken 24 hours later, following addition of samples. Expression of the

reporter gene (SEAP) was quantified with a commercially available kit (SEAP Reporter Gene Assay of Cayman Chemical Co., Ann Arbor, Mich.).

As shown in FIGS. 8A, 8B, 8C and 8D, LTA, LPS, bacteria and the supernatant of bacterial culture could bind to TLR2 and activate TLR2 downstream signaling pathways in a dose-dependent manner. As shown in FIG. 8A, *B. subtilis* (BS) LTA is more potent than that of *Enterococcus hirae*. As shown in FIG. 8B, *P. gingivalis* LPS also activated expression of the TLR2 reporter gene. For example, Pg1690, as shown in FIG. 8B, activated TLR2-SEAP signal transduction, and stimulated SEAP production. But as shown in FIG. 8B, *E. coli* LPS did not activate the TLR2-SEAP reporter cells. It should also be noted that *P. pallens*, *P. nigrescens* and *P. gingivalis* have similar EC50 in stimulating expression of TLR2 reporter gene (FIG. 8C). However, the released TLR2 ligands from the three different bacteria have very different EC50 on activation of TLR2 reporter gene (FIG. 8D).

Example 9—Cell Culture and Virulence Determination of EC50 in Activation of TLR2-SEAP Signal Transduction by Clinical Samples

The method described in EXAMPLE 8 is effective in determining the EC50 of LTA and other TLR2 ligands from different bacteria. The same method was used to determine the EC50 of clinical samples, as described in EXAMPLE 2. As shown in FIG. 9, dental plaques from unhealthy (bleeding) sites had smaller EC50 than those from healthy (non-bleeding) sites, suggesting the dental plaques from unhealthy sites contain more virulence factors.

Clinical samples as described for FIG. 7A of EXAMPLE 7 were examined using the TLR2-SEAP reporter gene assay. The results are shown in FIG. 10A. The subgingival samples in unhealthy (bleeding) sites from the unhealthy group (high bleeders) had more virulence factors than other sites. The baseline samples of the high bleeders group had higher RLU than those of the low bleeders on both the bleeding and non-bleeding sites. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in both high and low bleeders groups at both bleeding sites.

The clinical samples as described for FIG. 7B of EXAMPLE 7 were examined using the TLR2-SEAP reporter gene assay. As shown in FIG. 10B, oral lavage (Healthy vs. Gingivitis) was evaluated. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in the high bleeder group.

Example 10—Cell Culture and Virulence Determination of Flagellins from Different Bacteria

The reporter gene cell lines, human HEK 293T cells, were purchased from Invivogen of San Diego Calif. The HEK 293T cells were stably transfected with two exogenous genes, a TLR5 structural gene, and SEAP reporter gene which is under the control of NFkB transcriptional factors. The cell line was named as TLR5-NFkB-SEAP. The reporter gene encodes a secreted enzyme, called embryonic alkaline phosphatase or SEAP. The SEAP reporter is placed under the control of the interferon- β minimal promoter fused to five NFkB and AP-1-binding sites. The recombinant TLR5 binds

to its ligand, or distinct pathogen-associated molecule, and initiates a chain of responses leading to recruitment of NFkB and AP1 transcription factors to the reporter gene promoter, which induce expression of SEAP.

Cell culture and treatment: 500,000 gene reporter cells were grown and maintained in DMEM medium supplemented with 10% fetal calf serum in T75 flasks for three days at 37° C., 5% CO₂, and 95% humidity. For treatment with flagellin, wells of a 96-well plate were seeded with 10,000 cells/well in 100 µL of growth medium. The cells were incubated for 72 hours at 37° C., 5% CO₂, 95% humidity until day 4 after cells were seeded onto wells of a 96-well plate. On day 4, medium (100 µL) was changed to DMEM medium without fetal calf serum. *S. subtilis* and *S. aureus* flagellin were added at a range of concentration from 0.97 ng to 1 µg/ml. Samples were taken at 6 and 24 hours later after adding flagellin. Expression of reporter gene (SEAP) was quantified with a commercially available kit (SEAP Reporter Gene Assay of Cayman Chemical Co., Ann Arbor, Mich.).

FIG. 11 shows both flagellins (*B. subtilis* and *P. aeruginosa*) could activate TLR5 downstream signaling pathways. Flagellin from *B. subtilis* stimulated TLR5-SEAP signaling transduction in a dose-dependent manner. The EC₅₀ of flagellin on TLR5 was 0.02 ng/ml at 5 hours and 0.014 ng/ml at 23 hours. The EC₅₀ of flagellin from *Pseudomonas aeruginosa* on TLR5 was 0.0049 at 5 hours and 0.0019 at 23 hours. Flagellin from *P. aeruginosa* was more potent on stimulating TLR5 signaling pathways.

Example 11—Cell Culture and Virulence Determination of LPS from Different Bacteria in THP1 Dual Reporter Cells

Bacterial cell wall and membrane components are recognized by TLR2. TLR2 recognizes the microbial motifs PGN (peptidoglycan)/lipoproteins/dectin and LPS. TLR1 and TLR6 form heterodimers with TLR2 and bind to triacylated lipoproteins and diacylated lipoproteins, respectively. THP1 NFkB-SEAP and IRF-Lucia™ Reporter Monocytes were purchased from Invivogen, San Diego, Calif. THP1-Dual cells were derived from the human THP-1 monocyte cell line by stable integration of two inducible reporter constructs. THP1-Dual cells feature the Lucia gene under the control of an ISG54 (interferon-stimulated gene) minimal promoter in conjunction with five interferon-stimulated response elements. THP1-Dual cells also express a SEAP reporter gene driven by an IFN- γ minimal promoter fused to five copies of the NF- κ B consensus transcriptional response element and three copies of the c-Rel binding site. As a result, THP1-Dual cells allow the simultaneous study of the NFkB pathway, by monitoring the activity of SEAP, and the interferon regulatory factor (IRF) pathway, by assessing the activity of Lucia (IRF-Luc). Both reporter proteins are readily measurable in the cell culture supernatant. This THP-1 cell line possesses functional TLR1, TLR2, TLR4, TLR5, TLR6 and TLR8, purchased from Invivogen. TLR4 senses LPS from Gram-negative bacteria while TLR5 recognizes bacterial flagellin from both Gram-positive and Gram-negative bacteria, TLR8 detects long single-stranded RNA.

Culture and treatment: The THP1-dual cells were cultured in 15 ml growth medium (RPMI 1640 with 10% heat-inactivated fetal bovine serum) in a T75 flask at 37° C. and 5% CO₂. Cells were passed every 3 to 4 days by inoculating 300,000-500,000 cells/ml into a fresh T75 flask with 15 ml of fresh growth medium. To determine the effect of bacterial

components on reporter gene expression, wells in 96-well plates were seeded at 100,000 cells in 90 µl of growth medium. 10 µl of bacterial wall and membrane components, or heat-killed whole bacteria, were added to each well. After incubation for 18 hours at 37° C. and 5% CO₂, secreted luciferase and SEAP were quantified with commercially available assay kits (QUANTI-Luc of Invivogen, San Diego, Calif. for luciferase; SEAP Reporter Gene Assay of Cayman Chemical Co., Ann Arbor, Mich. for SEAP).

DHP1-dual reporter cells were treated with three different preparations of LPS as shown in FIG. 12A. All three LPS (ng/ml) activated production of NFkB-SEAP reporter genes in a dose-dependent manner. In addition, Pg 1690 LPS and *E. coli* LPS also stimulated expression of the IRF-luciferase reporter gene. TLR4 ligands, upon binding to TLR4 receptors, activate at least two signaling pathways. One is a common pathway NFkB-SEAP, which can be activated by all TLR ligands upon binding to their specific receptors. For example, TLR2 ligand, LTA, can bind to TLR2 receptors and activate the NFkB-SEAP signaling pathway. Similarly, TLR4 ligand, LPS, upon binding to TLR4 receptors, also is able to activate the NFkB-SEAP signaling transduction. As shown in FIG. 12A, *E. coli* LPS is a more potent ligand than *P. gingivalis* 1690 LPS on activation of both NFkB-SEAP and IRF-luciferase signaling transduction. THP-1 cells produce several functional TLR receptors. And all TLR receptors can activate the NFkB pathway, thus promoting expression of the NFkB-SEAP reporter gene. The reading of NFkB-SEAP is the collective actions of all TLR receptors, such as TLR2, TLR1, TLR6 and TLR4. All LPS from different bacteria stimulated NFkB-SEAP reporter gene. IRF-luciferase reporter gene, on the other hand, is driven by a limited number of TLR receptors, primarily TLR3, TLR4, TLR7, TLR8 and TLR9. Both *P. gingivalis* LPS 1690 and *E. coli* LPS stimulated expression of IRF-luciferase in a dose-dependent fashion.

The THP-1 reporter cells were used to examine the clinical samples as described for FIG. 7B of EXAMPLE 7. As shown in FIG. 12B, oral lavage (Healthy vs. Gingivitis) was evaluated using the IRF-Luc reporter gene in THP-1 cells. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in both high and lower bleeders groups.

The THP-1 reporter cells were used to examine the clinical samples as described for FIG. 7A of EXAMPLE 7. As shown in FIG. 12C, the subgingival Plaque (Healthy vs. Gingivitis) was examined using the NFkB reporter gene in THP-1 cells. The baseline samples of the high bleeders group had higher RLU than those of the low bleeders. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in the bleeding sites in both high and lower bleeders groups.

Example 12—Cell Culture and Virulence Determination of LTA from Different Bacteria and Biofilms in THP1 Dual Reporter Cells

THP1 dual reporter cells also express TLR2, TLR1 and TLR6 receptors. Bacterial cell wall and some membrane components are recognized by TLR2, TLR1 and TLR6. TLR2 recognizes the microbial motifs PGN (peptidoglycan)/lipoproteins/dectin and LPS. To determine whether LTA from different bacteria have different effects on stimu-

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lating NFkB-SEAP reporter gene expression in the THP1 dual reporter cells, the cells were prepared and treated in the same procedures as described in EXAMPLE 11. As shown in FIG. 13, LTA from both *B. subtilis* and *S. aureus* had similar potency in promoting reporter gene expression in the THP1 dual reporter cells.

Example 13—Distinguishing Bacterial Components from Different Bacteria in Human Primary Gingival Epithelial Cells

Primary human gingival epithelial cells were purchased from Zen-bio (Research Triangle Park, N.C.), and maintained in 15 ml of growth medium (CellnTec medium supplemented with CellnTec Growth Supplement, purchased from CellnTec Advanced Cell Systems AG, Bern, Switzerland) in T75 flasks at 37° C. under a 5% CO₂ atmosphere. As the experiment was done at 24 h, 48 h and 72 h time point and with assay media, CellnTec medium alone or with supplements, six 96 well plates were seeded with 7,500 cells/well in 100 µl of CELLnTEC growth medium at 37° C. under a 5% CO₂ atmosphere. The growth medium was changed to assay medium right before adding LPS or bacterial DNA. For example, if the assay medium was CellnTec medium without supplements, 100 µl of CellnTec medium was added without supplements in each well. If the assay medium was the growth medium, 100 µl of growth medium was added to each well. The *P. gingivalis* LPS and bacterial DNA were added to the cells. At 24 h, 48 h and 72 h, medium was collected for analysis. Cytokines were measured using Elisa kits from Meso Scale Discovery (Rockville, Md.), as per the manufacturer's instructions.

As shown in TABLE 4, human primary gingival epithelial cells were treated with *P. gingivalis* DNA at 0, 0.3, 1 and 2 µg/ml. The cultures were harvested at 24 and 48 hours after treatment and six proinflammatory cytokines (interferon-γ, IL-1β, IL-2, IL-10, IL-12p70 and TNF-α) were analyzed using ELISA kits from Meso Scale Discovery. As shown in TABLE 4, expression of interferon-γ, IL-1β, IL-2, IL-10, IL-12p70 and TNF-α was low, or almost undetectable (each value is the mean of three replicate in one experiment). They were not viable biomarkers in distinguishing bacterial DNA. The results in TABLE 4 illustrate the lack of inflammatory response from human gingival epithelial cells, thus demonstrating the need to use an engineered cell.

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TABLE 4

Screening of Human Gingival Epithelial Cells for Inflammatory Markers treated with <i>P. gingivalis</i> DNA								
Production of cytokines (pg/ml) in primary human gingival epithelial cells treated with <i>P. gingivalis</i> genomic DNA								
		<i>P. gingivalis</i>				<i>P. gingivalis</i>		
		genomic DNA	24 hours	48 hours		genomic DNA	24 hours	48 hours
Interferon gamma	0 ug	0	0.25	IL-1 beta	0 ug	0	0.5	
	0.3 ug	0	0.33		0.3 ug	0	0.33	
	1 ug	0	0		1 ug	0	0	
	2 ug	0.33	0.67		2 ug	0	0	
IL-10	PG DNA 0.5	0.5	1	IL-12p70	PG DNA 0	0	0.25	
	0 ug				0 ug			
	PG DNA 0.3 ug	0.67	0.33		PG DNA 0.3 ug	0.67	0	
	PG DNA 1 ug	0	0		PG DNA 1 ug	0	0.67	
IL-2	PG DNA 0.33	0.33	0.33	TNF-alpha	PG DNA 2 ug	0	0.33	
	2 ug				PG DNA 1	1	1.5	
	PG DNA 0.5	0.5	1		0 ug			
	0 ug				PG DNA 1.67	1.67	1.33	
	PG DNA 0.3 ug	0.33	1		0.3 ug			
	PG DNA 1 ug	0.33	0.67		PG DNA 0.67	0.67	1	
	2 ug				1 ug			
	PG DNA 0.33	0.33	0.67		PG DNA 1.33	1.33	1.33	
	2 ug				2 ug			

Human primary gingival epithelial cells were treated with *P. gingivalis* LPS at 0, 0.3, 1 and 2 µg/ml in the procedures described above. As shown in TABLE 5, again, expression of interferon-γ, IL-1β, IL-2, IL-10, IL-12p70 and TNF-α was low, or almost undetectable. They were not viable biomarkers in distinguishing bacterial LPS in primary human gingival epithelial cells. The results in TABLE 5 further illustrate how human gingival cells were not sufficiently sensitive to bacterial virulence factors for an assay, and thus the need to utilize a cell line capable of demonstrating a dose dependent response to virulence factors.

TABLE 5

Screening of Human Gingival Epithelial Cells for Inflammatory Markers treated with <i>P. gingivalis</i> LPS								
Production of cytokines (pg/ml) in primary human gingival epithelial cells treated with <i>P. gingivalis</i> LPS								
		24 h	48 h			24 h	48 h	
Interferon gamma	PG IPS 0 ug	0.00	0.25	IL-1 beta	PG IPS 0 ug	0.00	0.50	
	PG LPS 0.3 ug	0.00	0.00		PG LPS 0.3 ug	0.00	0.67	
	PG LPS 1 ug	0.33	0.33		PG LPS 1 ug	0.00	0.00	
	PG LPS 2 ug	0.00	0.00		PG LPS 2 ug	0.00	1.00	
IL-10	PG IPS 0 ug	0.50	1.00	IL-12p70	PG IPS 0 ug	0.00	0.25	
	PG LPS 0.3 ug	0.67	0.67		PG LPS 0.3 ug	0.00	0.00	
	PG LPS 1 ug	0.00	0.67		PG LPS 1 ug	0.00	0.33	
	PG LPS 2 ug	0.33	0.67		PG LPS 2 ug	0.67	0.00	
IL-2	PG IPS 0 ug	0.50	1.00	TNF-alpha	PG IPS 0 ug	1.00	1.50	
	PG LPS 0.3 ug	0.33	0.67		PG LPS 0.3 ug	1.33	0.67	
	PG LPS 1 ug	0.33	1.00		PG LPS 1 ug	2.00	1.33	
	PG LPS 2 ug	1.33	1.00		PG LPS 2 ug	0.67	1.33	

Example 14—Gingivitis-Enriched Bacteria Reduced in Abundance in Six Weeks of Gingivitis Treatment

A randomized, two-group clinical study was conducted with 69 panelists (35 in the negative control group and 34 in the test regimen group). Panelists were 39 years old on average, ranging from 20 to 69, and 46% of the panelists were female. Treatment groups were well balanced, since there were no statistically significant ($p \geq 0.395$) differences for demographic characteristics (age, ethnicity, gender) or starting measurements for Gingival Bleeding Index (GBI); mean=29.957 with at least 20 bleeding sites, and Modified Gingival Index (MGI); mean=2.086. All sixty-nine panelists attended each visit and completed the treatment process. The following treatment groups were compared over a 6-week period:

Test regimen: Crest® Pro-Health Clinical Plaque Control (0.454% stannous fluoride) dentifrice, Oral-B® Professional Care 1000 with Precision Clean brush head and Crest® Pro-Health Refreshing Clean Mint (0.07% CPC) mouth rinse. Control regimen: Crest® Cavity Protection (0.243% sodium fluoride) dentifrice and Oral-B® Indicator Soft Manual toothbrush.

Dental plaques were collected from the same panelists in the test regimen in the clinical study as described in EXAMPLE 2. A supragingival sample was taken from each panelist with a sterile curette at the tooth/gum interface, using care to avoid contact with the oral soft tissue. Plaques were sampled from all available natural teeth (upper arch only) until no plaque was visible. Following sampling, the plaque samples were placed into a pre-labeled (panelist ID, sample initials, visit, and date) Eppendorf tube with 1 ml of PBS/Glycerol buffer and about 50 of sterile 1 mm glass beads, stored on ice until all samples were collected. The samples were then transferred to a -70° C. freezer for storage until further processing. Genomic DNA was isolated from supragingival plaque samples using QIAamp® genomic DNA kits (Qiagen, Germany) following manufacturer's instruction. Metasequencing was carried out at BGI Americas Corporation (Cambridge, Mass.). All data were analyzed at Global Biotech of Procter & Gamble Company in Mason, Ohio.

Clinical measurements: Bleeding sites (GBI) were decreased in the test regimen significantly on week 1, 3 and 6 in comparison to the control regimen (FIG. 14). Similarly, Inflammation (MGI) grades also decreased in the test regimen (FIG. 14).

Genomic DNA of the supragingival plaques in the test regimen was sequenced. As shown in FIG. 15, abundance of

certain bacteria in the supragingival plaques changed in the six week treatments. Certain bacteria, such as *Porphyromonas* sp oral taxon 279 and *Prevotella pallens*, were decreased in weeks 1 and 3 (FIG. 15). The amount of each bacterial species was plotted over the four time periods of the treatment. The amount of certain bacteria, such as *Peptostreptococcus stomatis* and *Prevotella intermedia*, was reduced during the six week of treatment as shown in FIG. 15. This is also shown in FIG. 16A-1, FIG. 16A-2, and FIG. 16A-3.

Example 15—Production of Cytokines, Chemokines and Other Bioactive Proteins Decreased as Gingivitis Symptoms were Alleviated During 6 Week of Treatment

In the same clinical study as described in EXAMPLE 14, gingival-brush samples were collected from the same panelists as in EXAMPLE 14. Before sampling, panelists rinsed their mouths for 30 seconds with water. A dental hygienist then sampled the area just above the gumline using a buccal swab brush (Epicentre Biotechnologies cat.# MB100SP). The swab was immediately placed into 1 ml extraction buffer [PBS, 0.25M NaCl, 1× Halt™ Protease Inhibitor Single-Use Cocktail (Lifetechnologies, Grand Island, N.Y.)] in a 1.5 ml Eppendorf tube vortexed for 30 seconds, and immediately frozen on dry ice and stored in a -80° C freezer until analysis. The samples were taken out of freezer, thawed and extracted by placing the samples on a tube shaker for 30 minutes at 4° C. The tubes were centrifuged at 15000 RPM for 10 min in Eppendorf Centrifuge 5417R (Eppendorf, Ontario, Canada) to pellet any debris. The extract (800 μ l) was analyzed for protein concentrations using the Bio-Rad protein assay (BioRad, Hercules, Calif.).

Forty proteins were measured in the gingival samples using V-PLEX Human Biomarker 40-Plex Kit (Meso Scale Diagnostics Rockville, Md.). The assay was performed following the manufacturer's instruction.

Among the proteins measured in the gingival samples, most proteins in the Proinflammatory Panel 1 (human), Cytokine Panel 1 (human), Chemokine Panel 1 (human), Angiogenesis Panel 1 (human), and Vascular Injury Panel 2 (human) had significant changes in their abundance during the 6-week treatment (TABLE 6). Those include FN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF- α , GM-CSF, IL-5, IL-16, IL-7, IL-12/IL-23p40, IL-1 α , VEGF-A, IL-17A, IL-15, TNF- β , IL-8 (HA), MCP-1, MCP-4, Eotaxin, IP-10, MDC, Eotaxin-3, TARC, MIP-1 α , MIP-1 β , VEGF-C, VEGF-D, Tie-2, Flt-1/VEGFR1, PlGF, FGF (basic), SAA, CRP, VCAM-1, and ICAM-1.

TABLE 6

Changes in abundance of proteins in the gingival-brush samples								
	Mean				$\alpha = 0.05$			
	Baseline	Week 1	Week 3	Week 6	Baseline	Week 1	Week 3	Week 6
ICAM-1	16.035	12.209	10.090	9.767	A	B	B, C	C
IL-1 α	3.554	2.331	2.181	1.891	A	A, B	B, C	C
IL-1 β	53.666	35.575	24.295	24.440	A	B	C	C
TNF- β	0.0013	0.0010	0.0008	0.0007	A	B	C	C
IL-12p70	0.172	0.148	0.118	0.127	A	A, B	C	B, C
IL-13	0.805	0.762	0.624	0.648	A	A, B	C	B, C
IL-4	0.127	0.115	0.090	0.096	A	A, B	C	B, C
IL-5	0.004	0.003	0.002	0.003	A	B	C	B, C
CRP	15.637	12.743	12.385	5.809	A	A	A	B
Eotaxin	0.077	0.064	0.059	0.059	A	A, B	B	B
GM-CSF	0.010	0.008	0.008	0.008	A	B	B	B

TABLE 6-continued

Changes in abundance of proteins in the gingival-brush samples								
IFN γ	0.530	0.446	0.378	0.386	A	A, B	B	B
IL-10	0.875	0.490	0.423	0.244	A	A, B	B	B
IL-15	0.005	0.003	0.003	0.003	A	B	B	B
IL-16	0.466	0.345	0.342	0.295	A	B	B	B
IL-6	0.196	0.192	0.168	0.150	A	A	A, B	B
IL-7	0.004	0.003	0.003	0.003	A	B	B	B
IL-8	856.276	652.066	567.361	572.602	A	B	B	B
MCP-1	0.053	0.047	0.039	0.039	A	A, B	B	B
MDC	0.399	0.407	0.345	0.339	A	A	B	B

	Mean					$\alpha = 0.05$			
	Baseline	Week 1	Week 3	Week 6		Baseline	Week 1	Week 3	Week 6
SAA	7.039	6.905	6.092	5.162	A	A	A, B	B	
Tie-2	0.273	0.239	0.267	0.221	A	A, B	A	B	
VCAM-1	4.971	3.706	3.156	2.892	A	B	B	B	
VEGF	0.625	0.511	0.478	0.480	A	B	B	B	
VEGF 2	0.772	0.661	0.620	0.582	A	B	B	B	
VEGF-D	0.057	0.052	0.051	0.045	A	A, B	A, B	B	
VEGF-C	0.145	0.149	0.125	0.137	A, B	A	B	A, B	
TARC	0.020	0.029	0.019	0.019	A	B	A	A	
bFGF	0.020	0.015	0.012	0.013	A	A	A	A	
Eotaxin-3	0.095	0.108	0.091	0.094	A	A	A	A	
Flt-1	0.390	0.518	0.433	0.415	A	B	A, B	A	
IL-12p40	0.039	0.031	0.028	0.031	A	A	A	A	
IL-2	0.166	0.199	0.210	0.162	A	A	A	A	
IL-8 (HA)	47.508	44.362	41.260	39.119	A	A	A	A	
IP-10	0.540	1.688	0.740	0.606	A	A	A	A	
MCP-4	0.023	0.023	0.020	0.022	A	A	A	A	
MIP-1 α	0.091	0.091	0.084	0.080	A	A	A	A	
MIP-1 β	0.091	0.100	0.110	0.094	A	A	A	A	
TNF α	2.009	2.067	2.021	1.670	A	A	A	A	

Example 16—One Hundred Seventy Metabolites were Identified in Gingival Samples

The same gingival-brush samples as described in EXAMPLE 15 were used for metabolomic analyses. Fourteen panelists were selected randomly from each treatment group to determine if any metabolite concentrations were changed in gingival samples during the first 3 weeks of treatment. Both baseline and week 3 samples were sent to Metabolon, Inc. (Durham, N.C.) for metabolomic measure-

ment. 170 metabolites were identified and quantified. As shown in TABLE 7, some metabolite concentrations were changed during the first 3 weeks of treatment. Citrulline concentrations in the gingival samples were reduced after three weeks of treatment in the treatment regimen group. Similarly, ornithine was also reduced in the treatment regimen group after three weeks of treatment. Reduction of citrulline and ornithine was likely associated with alleviation of gingivitis.

TABLE 7

Comparison of metabolites in gingival brush samples between baseline and week 3 during gingivitis treatment						
Biochemical Name	Baseline mean	3 week mean	3 week/baseline	p-value	q-value	Mass
13-HODE + 9-HODE	1.0877	0.7088	0.65	0.0601	0.1338	295.2
1-arachidonoylglycerophosphoethanolamine	1.2294	0.8274	0.67	0.038	0.1035	500.3
1-oleoylglycerophosphoethanolamine	0.7378	1.0747	1.46	0.0767	0.1548	478.3
2-methylbutyrylcarnitine (C5)	1.7769	0.6997	0.39	0.0034	0.0546	246.1
adenosine 5'-monophosphate (AMP)	1.4092	0.8451	0.6	0.0295	0.0956	348.1
alanine	0.8721	1.102	1.26	0.0318	0.0973	115.9
arginyllucine	1.4447	0.6819	0.47	0.0084	0.0777	288.3
arginylphenylalanine	0.9616	0.3335	0.35	0.0119	0.0777	322.2
asparagyllucine	0.9295	0.6122	0.66	0.0698	0.1465	246.2
citrulline	1.0147	0.71	0.7	0.0104	0.0777	176.1
deoxycarnitine	3.2381	0.6088	0.19	0.0003	0.0168	146.1
EDTA	1.5985	0.8384	0.52	0.0138	0.0777	291.1
erythritol	1.625	0.8085	0.5	0.0582	0.1325	217
fructose	1.9933	1.1106	0.56	0.0847	0.1605	217
glutamine	1.2459	0.8366	0.67	0.0374	0.1035	147.2
glutathione, oxidized (GSSG)	1.0161	1.4669	1.44	0.087	0.1605	613.1

TABLE 7-continued

Comparison of metabolites in gingival brush samples between baseline and week 3 during gingivitis treatment						
Biochemical Name	Baseline mean	3 week mean	3 week/ baseline	p-value	q-value	Mass
glycerol	1.3783	0.8308	0.6	0.0391	0.1035	205
lauryl sulfate	1.685	0.8623	0.51	0.0397	0.1035	265.2
leucine	1.2158	0.9359	0.77	0.0613	0.1338	132.2
leucylleucine	0.9505	0.4393	0.46	0.0251	0.0877	245.1
lysylleucine	1.2009	0.5275	0.44	0.0036	0.0546	260.2
lysylphenylalanine	1.1682	0.4563	0.39	0.0095	0.0777	294.3
maltose	0.8727	1.4481	1.66	0.022	0.0877	204.1
maltotriose	1.0456	1.8347	1.75	0.0858	0.1605	204
mannitol	1.3004	0.7982	0.61	0.042	0.107	319.1
ornithine	1.2916	0.7069	0.55	0.0367	0.1035	141.9
palatinitol	1.4395	0.8272	0.57	0.0782	0.1549	204
phosphate	1.4008	0.8376	0.6	0.0208	0.0877	298.9
proline	1.405	0.99	0.7	0.0033	0.0546	116.1
propionylcarnitine	1.2565	0.7688	0.61	0.0201	0.0877	218.2
pyroglutamine	1.3424	0.7873	0.59	0.0136	0.0777	129.2
serylisoleucine	1.1753	0.7169	0.61	0.0814	0.1583	219.2
spermidine	1.1613	0.8678	0.75	0.0687	0.1465	146.2
succinate	1.2929	0.8113	0.63	0.0754	0.1548	247
threonylleucine	1.1513	0.4931	0.43	0.0044	0.0594	231.2
threonylphenylalanine	1.7693	0.918	0.52	0.0233	0.0877	267.2
trehalose	2.3563	0.9084	0.39	0.0054	0.0647	361.2
tryptophan	1.1518	0.9089	0.79	0.0487	0.1185	205.1
tyrosine	1.383	1.0299	0.74	0.0161	0.0787	182.1
valine	1.1598	0.9271	0.8	0.0304	0.0956	118.1
valylvaline	0.9347	0.8231	0.88	0.0508	0.1207	215.2
X-13671	0.5035	0.918	1.82	0.0545	0.1267	315.3
X-14588	1.3647	0.8378	0.61	0.024	0.0877	151
X-16103	1.3643	0.8461	0.62	0.0297	0.0956	99.3
X-17266	1.3158	0.576	0.44	0.0003	0.0168	530.4
X-17375	1.4785	0.8387	0.57	0.0189	0.0877	357.1
X-18472	0.6138	1.1441	1.86	0.0011	0.0405	827.1
X-18779	1.3756	0.8035	0.58	0.0162	0.0787	209.1
X-19607	1.5237	0.7167	0.47	0.002	0.0537	366.1
X-19609	1.3284	0.7721	0.58	0.016	0.0787	204
X-19612	1.3896	0.7843	0.56	0.01	0.0777	427.2
X-19613	1.3412	0.7535	0.56	0.0099	0.0777	429.3
X-19614	1.3378	0.7343	0.55	0.0454	0.113	570.1
X-19807	1.3478	0.8411	0.62	0.0244	0.0877	93
X-19808	1.3348	0.8368	0.63	0.0254	0.0877	95
X-19850	1.3576	0.7519	0.55	0.011	0.0777	334.2
X-19857	1.3357	0.8032	0.6	0.038	0.1035	230
X-20000	1.2784	0.7536	0.59	0.0133	0.0777	81.2

Example 17—Citrulline was Reduced in Regimen Treatment Over 6 Weeks

Quantitation of citrulline and ornithine from the extracts of the Gingival-brush samples was conducted using gradient hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC/MS/MS). Gingival-brush samples were obtained from the same human panelists in the clinical study as described in EXAMPLE 14, and were placed into extraction buffer as described in EXAMPLE 15. The supernatants were subject to both HILIC/MS/MS and BCA analysis. For free citrulline and ornithine analysis, the extracts of the Gingival-brush samples were analyzed either directly (50 μ l undiluted sample solution) in 50/50 acetonitrile/ultra-pure water with 0.754% formic acid or diluted fivefold. For total citrulline and ornithine analysis, the extracts of the Gingival-brush samples were first hydrolyzed using 6 N HCl (50 μ L of extract with 450 μ L of 6N HCl), no shaking, and placed on a hot plate at 110° C. for 16 hours. The hydrolyzed samples were then dried down under vacuum at room temperature (Savant speedvac of Lifetech-
nology, Grand Island, N.Y.) and then reconstituted in 1 ml of

dilution solution (50/50 acetonitrile/ultra-pure water with 0.754% formic acid) for analysis. The standards and the samples were analyzed using gradient hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC/MS/MS). Analytes and the corresponding ISTDs (stable isotope labeled internal standard) were monitored by electrospray ionization (ESI) in positive mode using the selected-reaction-monitoring schemes shown in TABLE 8. A standard curve was constructed by plotting the signal, defined here as the peak area ratio (peak area analyte/peak area ISTD), for each standard versus the mass of each analyte for the corresponding standard. The mass of each analyte in the calibration standards and Gingival-brush extract samples were then back-calculated using the generated regression equation. The concentration of protein bound citrulline or ornithine was calculated as the result of subtracting the concentration of free citrulline or ornithine from the concentration of total citrulline or ornithine, respectively. The result was reported as the concentration of citrulline or ornithine or the result was standardized by dividing by the amount of citrulline or ornithine by the amount of the total proteins that were found in the extract.

TABLE 8

Multiple Reaction Monitoring (MRM) transitions for analytes and their corresponding stable isotope labeled internal standards			
Analytes	MRM	Internal Standards	MRM
Citrulline	176 → 159	d ₇ -Citrulline	181 → 164
Ornithine	133 → 70	d ₆ -Ornithine	139 → 76

All samples from all panelists of the Test regimen [Crest® Pro-Health Clinical Plaque Control (0.454% stannous fluoride) dentifrice, Oral-B® Professional Care 1000 with Precision Clean brush head and Crest® Pro-Health Refreshing Clean Mint (0.07% CPC) mouth rinse] were analyzed. As shown in FIG. 16, citrulline levels reduced rapidly in the first week of treatment, and then continued to decline gradually in weeks 3 and 6 of treatment. These results are consistent with clinical observations, where gingival bleeding sites (GBI) and the gingival inflammation (MGI) were reduced over the 6-week treatment period.

Example 18—Levels of Proteins Containing Ornithine Decreased Over 6 Week Treatment

The same samples as described in EXAMPLE 17 were analyzed using procedures as described in EXAMPLE 17. Gingivitis was treated for 6 weeks. Baseline (BL) represents diseased status. Symptoms of gingivitis were alleviated from week 1 to week 6 treatments. Protein bound ornithine (the difference between total and the free ornithine) was higher in gingivitis as shown in FIG. 17. Protein bound ornithine was reduced gradually as gingivitis was decreased in severity.

Example 19—Expression of Enzymes in the Ornithine-Citrulline-Arginine Cycle was Changed in Gingival Samples During the 6 Week Treatment

Gingival samples were collected as described in EXAMPLES 15, from the same panelists as in EXAMPLE 15, and were used to examine the expression of genes during 6 weeks of treatments with Test regimen [Crest® Pro-Health Clinical Plaque Control (0.454% stannous fluoride) dentifrice, Oral-B® Professional Care 1000 with Precision Clean brush head and Crest® Pro-Health Refreshing Clean Mint (0.07% CPC) mouth rinse] and Control regimen [Crest® Cavity Protection (0.243% sodium fluoride) dentifrice and Oral-B® Indicator Soft Manual toothbrush].

After harvesting the samples, the brush was completely immersed in the RNAlater solution (1 ml in a 1.5 ml Eppendorf tube) for keeping RNA from degrading during transport and storage (Qiagen, Valencia, Calif.). The micro-centrifuge tubes were vortexed/mixed for 30 seconds, immediately frozen on dry ice, stored and transferred on dry ice to the lab for biomarker analysis. RNA isolation and microarray analysis was performed as described previously in a publication (Offenbacher S, Barros S P, Paquette D W, Winston J L, Biesbrock A R, Thomason R G, Gibb R D, Fulmer A W, Tiesman J P, Juhlin K D, Wang S L, Reichling T D, Chen K S, Ho B. J Periodontol. 2009 December; 80(12):1963-82. doi: 10.1902/jop.2009.080645. Gingival transcriptome patterns during induction and resolution of experimental gingivitis in humans).

The ornithine-citrulline-arginine cycle consists of four enzymes (FIG. 18). The main feature of the cycle is that three amino acids (arginine, ornithine, and citrulline) can be

converted to each other. The first enzyme is ornithine transcarbamoylase, which transfers a carbamoyl group from carbamoyl phosphate to ornithine to generate citrulline. This reaction occurs in the matrix of the mitochondria. Expression of ornithine transcarbamoylase was reduced in the treatment (FIG. 19). The second enzyme is argininosuccinate synthetase. This enzyme uses ATP to activate citrulline by forming a citrullyl-AMP intermediate, which is attacked by the amino group of an aspartate residue to generate argininosuccinate. This and subsequent two reactions occur in the cytosol. Again, expression of argininosuccinate synthetase decreased during the treatment. The third enzyme is argininosuccinate lyase, which catalyzes cleavage of argininosuccinate into fumarate and arginine. The last enzyme is arginase. Arginases cleave arginine to produce urea and ornithine. In a contrast to the decreased expression of ornithine transcarbamoylase and argininosuccinate synthetase genes, arginase I and II increased (FIG. 19).

Arginine is also a substrate for nitric oxide synthase, which oxidizes arginine to produce citrulline and nitric oxide. Expression of nitric oxide synthase gene increased too (FIG. 19).

Example 20—Citrulline Increased in Gingival Samples in Experiment Gingivitis

Experimental gingivitis: Another clinical study was carried out to determine whether citrulline is increased in experimentally induced gingivitis in healthy human panelists. This was a case-control study enrolling 60 panelists. The study population included two groups as follows: Group 1 or high bleeders group, thirty (30) panelists with at least 20 bleeding sites, where bleeding is a GBI site score of 1 or 2 at baseline. Group 2 or low bleeders group, thirty (30) panelists with 2 or less bleeding sites, where bleeding is a GBI site score of 1 or 2.

The study consisted of two Phases: Health/Rigorous Hygiene Phase with dental prophylaxis, polishing and rigorous oral hygiene; and Induced Gingivitis Phase without oral hygiene. At the Screening visit, panelists underwent an oral soft tissue assessment and had a gingivitis evaluation (Modified Gingival Index (MGI) and Gingival Bleeding Index (GBI). At Visit 2 qualifying panelists received an oral soft tissue exam followed by a gingivitis evaluation and gingival plaques and gum swabs were collected for the qPCR, protein and RNA host biomarker analysis. Following that, all panelists received dental prophylaxis and entered the Health/Rigorous Hygiene Phase, lasting two weeks. After two weeks of rigorous hygiene, panelists entered the Induced Gingivitis Phase, lasting for three weeks. Oral soft tissue exams and gingivitis were re-evaluated and all samples (gum swabs) were collected at Baseline, WK0 and WK2.

Gingival sample collection—A gum swab was collected from each side of the upper arch using the procedures as described in EXAMPLE 15. Gum swabs were collected close to the gum line from the buccal sites only (preferably from four adjacent teeth—preferably from premolar and molar areas). Panelists rinsed for 30 seconds with 15 ml of Listerine rinse to clean the surface of sampling area. After the Listerine rinse, panelists rinsed for 30 seconds with 20 ml of water. Following that, selected sites were isolated with a cotton roll and gently dried with an air syringe and two gum swabs were taken with collection brushes/swabs from the gingiva region close to the gumline of the selected teeth. The samples were placed in a pre-labeled (panelist ID, sample ID, visit, and date) 1.5 ml micro-centrifuge tube

containing 800 μ l DPBS (Dulbecco's phosphate-buffered saline) (Lifetechnologies, Grand Island, N.Y.) with protease inhibitors, including AEBSEF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) 2 mM, aprotinin 0.3 μ M, Bestatin 130 μ M, EDTA (Ethylenediaminetetraacetic acid) 1 mM, E-64 1 μ M, and leupeptin 1 μ M. The vials were vortexed/mixed for 30 seconds, immediately frozen on dry ice, stored and transferred on dry ice to the lab for biomarker analysis. Samples from three visits were analyzed using the procedures described in EXAMPLE 17, and shown in FIG. 20. Those three visits were baseline, Week 0, (right after the Health/Rigorous Hygiene Phase and before the induced gingivitis phase) and week 2 (at the end of Induced Gingivitis Phase). Free citrulline levels were low in both the high and low bleeders groups at the baseline and week 0, but rose quickly in the induced gingivitis in both groups at week 2.

Example 21—Levels of Proteins Containing Citrulline Decreased in Experimentally Induced Gingivitis

The same procedures were used as described in EXAMPLE 17. The samples were the same as described in EXAMPLE 20. Protein bound citrulline was lower at the baseline than that at week 0 in both high and low bleeders groups as shown in FIG. 21 in gingival tissue. It was low in experimental gingivitis in both groups at week 2.

Example 22—Levels of Proteins Containing Ornithine Increased in Gingival Samples in Experiment Gingivitis

The same clinical samples from experimental gingivitis (EXAMPLE 20) were analyzed using the procedures described in EXAMPLE 17. The bound ornithine was the lowest at week 0 (FIG. 22) in both groups. Its levels at the baseline were higher than those at week 0. The bound ornithine reached peaks when gingivitis was induced in both groups at week 2. Also it is worth noting the total ornithine (Free and protein bound ornithine) was increased in the induced gingivitis (FIG. 23) in both groups.

Example 23—Levels of Proteins Containing Arginine Decreased in Gingival Samples in Experimentally Induced Gingivitis

The same procedures were used as described in EXAMPLE 17. The samples were the same as described in EXAMPLE 20. The protein bound arginine was the lowest in induced gingivitis (FIG. 24) in both groups. Its levels were higher in WK0 than at Baseline in both groups. The total arginine in the gingival brush samples displayed the same patterns as the protein bound one (FIG. 25).

Example 24—Citrulline Inhibited LPS Stimulated Production of Proinflammatory Cytokine in Human THP-1 Cells

Citrulline was purchased from Sigma-Aldrich (St. Louis, Mo.). THP1-Dual™ cells were purchased from Invivogen (San Diego, Calif.). Cells were cultured following the manufacturer's instruction, as described in EXAMPLE 11. For treatment, 0.3 mM to 9 mM of citrulline were first added to the culture medium. Then, 300 ng/ml of *P. gingivalis* LPS 1690 were added 60 minutes later. After 24 hours of treat-

ment, media was collected and analyzed for cytokine production using 9-plex kit (Meso Scale Diagnostics Rockville, Md.).

P. gingivalis LPS 1690 stimulated cytokine production, as shown in FIG. 26. Citrulline inhibited *P. gingivalis* LPS 1690 effects on proinflammatory cytokine production in a dose-dependent manner. Those cytokines include IL-6, TNF- α , IL-12p70, IL-10, IL-2, IFN-r and IL-1 β .

Example 25—Binding of BODIPY-TR-Cadaverine to Bacteria and their Products

Bacteria and their products can activate TLR2 and TLR4 reporter genes as described in EXAMPLES 5 and 8. Here, experiments were carried out to determine whether different bacteria and their products have different IC₅₀ in reducing fluorescent intensity of BODIPY-TR-cadaverine, hereinafter referred to as BC, in a procedure as described in EXAMPLE 4. Briefly, high throughput screen utilizes the fluorescent dye BODIPY-TR-cadaverine 5-(((4-(4,4-difluoro-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl)amino) pentylamine hydrochloride (BC fluorescent dye), purchased from Life Technologies (Carlsbad, Calif.), as described previously by Wood, Miller and David (2004) (Comb Chem High Throughput Screen 2004 May; 7(3):239-49. Anti-endotoxin agents. 1. Development of a fluorescent probe displacement method optimized for the rapid identification of lipopolysaccharide-binding agents.). The experiment was carried out at room temperature. *E. coli* LPS (15 μ g/ml) was mixed in a well of a 96-well solid black flat bottom plate (Corning Incorporated, Corning, N.Y.) with SnF2 and cetylpyridinium chloride in 30 μ l of 50 mM Tris buffer at pH 7.4 for 10 min, and then 20 μ l of 60 μ M BC fluorescent dye was added right before fluorescence measurement at 21° C., or room temperature. Fluorescent measurements were performed in a SpectraMax M3 automated 96 well plate reader (Molecular Device, Sunnyvale, Calif.). The excitation wavelength was 580 nm and the fluorescence emission was measured at 620 nm. The half maximal inhibitory concentration (IC₅₀) is a measure of the effectiveness of bacterial products inhibiting BC emitting fluorescence. It indicates how much of a bacterial material is needed to inhibit BC fluorescence by half using GraphPad Prism software (GraphPad Software, La Jolla, Calif.). As shown in FIGS. 27 and 28, each bacterial product decreases the fluorescent intensity of BC in a dose-dependent manner. Similarly, the whole bacterial cells and the supernatant of bacterial growth also reduced the fluorescence of BC in a dose-dependent fashion (FIGS. 29 and 30).

Example 26—Determination of Bacterial Virulence

Growth of bacteria: Two bacteria, Bacterium A and Bacterium B, were cultured in Tryptic Soy Broth medium (Sigma-Aldrich, St. Louis, Mo.) at 37° C. with shaking at 200 rpm. The bacteria were harvested at 24 hours, and suspended in 0.5 ml of phosphate-buffered saline, labeled "live". Half ml of "Live" bacteria was transferred to a 1.5 ml microtube, and heated to 80° C. for 30 min. The heat-treated bacteria were labeled "Heat-Inactivated", or "Dead".

Measurement of TLR responses in THP-1 gene reporter cells (NFkB-SEAP): The Live and Heat-Inactivated bacteria were applied to THP-1 cells as described in EXAMPLE 11. As shown in FIG. 31, EC₅₀ of Bacterium A and B on activation of NFkB-SEAP reporter gene in THP-1 cells was determined. Both Live and Heat-inactivated (Dead) bacteria stimulated expression of the NFkB-SEAP reporter gene.

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Bacterium B had a lower EC50 than Bacterium A in activating expression of the NFkB-SEAP reporter gene.

Cytokine production and measurement: Human peripheral blood mononuclear cells (hPBMC) were obtained from All Cells company (All Cells, Alameda, Calif.) as Leukapheresed blood. Leukapheresed blood was mixed with an equal part of DMEM+glutaGRO supplemented with 9.1% fetal bovine serum and 1% penicillin/streptomycin (Thermo Fisher, Waltham, Mass.). hPBMC were isolated from the 1:1 mixture of blood and culture medium by collecting the buffy coat of a centrifuged Histopaque®-1077 (Sigma-Aldrich, St. Louis, Mo.) buffer density gradient. The cells (200,000 cells) were cultured in 200 µl of DMEM+glutaGRO supplemented with 9.1% fetal bovine serum and 1% penicillin/streptomycin, and treated with Live and Heat-Inactive bacteria (6,250,000 colony-forming units). The medium was harvested at 24 hours after adding the bacteria, and analyzed for proinflammatory cytokines in a kit following manufacturer's instruction (Meso Scale Diagnostics, Rockville, Md.).

As shown in TABLE 9, both live bacterium A and B stimulated production of cytokines in hPBMC. Bacterium B was far more potent than Bacterium A in promoting production of IFN-γ, IL-10, IL-12p70, IL-1β, IL-6, IL-8 and TNF-α in hPBMC.

Statistics	Cytokines	Live Bacterium A	Live Bacterium
Mean	IFN-γ	867.16	12734.36
Mean	IL-10	68.35	412.51
Mean	IL-12p70	23.75	253.47
Mean	IL-1β	2300.86	6969.31
Mean	IL-2	44.24	65.17
Mean	IL-6	3431.05	9963.84
Mean	IL-8	65742.91	70357.25
Mean	TNF-α	3710.66	13825.49
Std Dev	IFN-γ	488.11	8200.13
Std Dev	IL-10	33.56	314.23

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Statistics	Cytokines	Live Bacterium A	Live Bacterium
Std Dev	IL-12p70	14.71	283.33
Std Dev	IL-1β	1569.72	7691.93
Std Dev	IL-2	32.97	36.29
Std Dev	IL-6	2212.62	6552.12
Std Dev	IL-8	18689.24	13669.28
Std Dev	TNF-α	2503.25	8302.26

The dimensions and values disclosed herein are not to be understood as being strictly limited to the exact numerical values recited. Instead, unless otherwise specified, each such dimension is intended to mean both the recited value and a functionally equivalent range surrounding that value. For example, a dimension disclosed as "40 mm" is intended to mean "about 40 mm."

Every document cited herein, including any cross referenced or related patent or application and any patent application or patent to which this application claims priority or benefit thereof, is hereby incorporated herein by reference in its entirety unless expressly excluded or otherwise limited. The citation of any document is not an admission that it is prior art with respect to any invention disclosed or claimed herein or that it alone, or in any combination with any other reference or references, teaches, suggests or discloses any such invention. Further, to the extent that any meaning or definition of a term in this document conflicts with any meaning or definition of the same term in a document incorporated by reference, the meaning or definition assigned to that term in this document shall govern.

While particular embodiments of the present invention have been illustrated and described, it would be obvious to those skilled in the art that various other changes and modifications can be made without departing from the spirit and scope of the invention. It is therefore intended to cover in the appended claims all such changes and modifications that are within the scope of this invention.

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Gly	Asp	Cys	Ser	Trp	Thr	Lys	Ser	Leu	Leu	Ser	Leu	Asn	Met	Ser	Ser
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Ile	Lys 210	Glu	Phe	Ser	Pro	Gly 215	Cys	Phe	His	Ala	Ile 220	Gly	Arg	Leu	Phe
Gly 225	Leu	Phe	Leu	Asn 230	Asn	Val	Gln	Leu	Gly	Pro 235	Ser	Leu	Thr	Glu	Lys 240
Leu	Cys	Leu	Glu	Leu 245	Ala	Asn	Thr	Ser	Ile	Arg 250	Asn	Leu	Ser	Leu	Ser 255
Asn	Ser	Gln	Leu	Ser 260	Thr	Thr	Ser	Asn 265	Thr	Thr	Phe	Leu	Gly	Leu	Lys 270
Trp	Thr 275	Asn	Leu	Thr	Met	Leu	Asp 280	Leu	Ser	Tyr	Asn 285	Asn	Leu	Asn	Val
Val	Gly 290	Asn	Asp	Ser	Phe	Ala 295	Trp	Leu	Pro	Gln	Leu 300	Glu	Tyr	Phe	Phe
Leu 305	Glu	Tyr	Asn	Asn 310	Ile	Gln	His	Leu	Phe	Ser 315	His	Ser	Leu	His	Gly 320
Leu	Phe	Asn	Val	Arg 325	Tyr	Leu	Asn	Leu	Lys 330	Arg	Ser	Phe	Thr	Lys	Gln 335
Ser	Ile	Ser	Leu 340	Ala	Ser	Leu	Pro	Lys 345	Ile	Asp	Asp	Phe	Ser	Phe	Gln
Trp	Leu 355	Lys	Cys	Leu	Glu	His	Leu 360	Asn	Met	Glu	Asp 365	Asn	Asp	Ile	Pro
Gly 370	Ile	Lys	Ser	Asn 375	Met	Phe	Thr	Gly	Leu	Ile 380	Asn	Leu	Lys	Tyr	Leu
Ser 385	Leu	Ser	Asn	Ser 390	Phe	Thr	Ser	Leu	Arg	Thr 395	Leu	Thr	Asn	Glu	Thr 400
Phe	Val	Ser	Leu 405	Ala	His	Ser	Pro	Leu	His 410	Ile	Leu	Asn	Leu	Thr	Lys 415
Asn	Lys	Ile	Ser 420	Lys	Ile	Glu	Ser	Asp 425	Ala	Phe	Ser	Trp	Leu	Gly	His 430
Leu	Glu 435	Val	Leu	Asp	Leu	Gly	Leu 440	Asn	Glu	Ile	Gly 445	Gln	Glu	Leu	Thr
Gly 450	Gln	Glu	Trp	Arg	Gly 455	Leu	Glu	Asn	Ile	Phe 460	Glu	Ile	Tyr	Leu	Ser
Tyr 465	Asn	Lys	Tyr	Leu 470	Gln	Leu	Thr	Arg	Asn 475	Ser	Phe	Ala	Leu	Val	Pro 480
Ser	Leu	Gln	Arg 485	Leu	Met	Leu	Arg	Arg	Val 490	Ala	Leu	Lys	Asn	Val	Asp 495
Ser	Ser	Pro	Ser 500	Pro	Phe	Gln	Pro	Leu 505	Arg	Asn	Leu	Thr	Ile	Leu	Asp 510
Leu	Ser 515	Asn	Asn	Asn	Ile	Ala	Asn 520	Ile	Asn	Asp	Asp	Met	Leu	Glu	Gly
Leu	Glu 530	Lys	Leu	Glu	Ile 535	Leu	Asp	Leu	Gln	His 540	Asn	Asn	Leu	Ala	Arg

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Leu Trp Lys His Ala Asn Pro Gly Gly Pro Ile Tyr Phe Leu Lys Gly
545                               550                               555                               560

Leu Ser His Leu His Ile Leu Asn Leu Glu Ser Asn Gly Phe Asp Glu
                               565                               570                               575

Ile Pro Val Glu Val Phe Lys Asp Leu Phe Glu Leu Lys Ile Ile Asp
                               580                               585                               590

Leu Gly Leu Asn Asn Leu Asn Thr Leu Pro Ala Ser Val Phe Asn Asn
                               595                               600                               605

Gln Val Ser Leu Lys Ser Leu Asn Leu Gln Lys Asn Leu Ile Thr Ser
610                               615                               620

Val Glu Lys Lys Val Phe Gly Pro Ala Phe Arg Asn Leu Thr Glu Leu
625                               630                               635                               640

Asp Met Arg Phe Asn Pro Phe Asp Cys Thr Cys Glu Ser Ile Ala Trp
                               645                               650                               655

Phe Val Asn Trp Ile Asn Glu Thr His Thr Asn Ile Pro Glu Leu Ser
                               660                               665                               670

Ser His Tyr Leu Cys Asn Thr Pro Pro His Tyr His Gly Phe Pro Val
                               675                               680                               685

Arg Leu Phe Asp Thr Ser Ser Cys Lys Asp Ser Ala Pro Phe Glu Leu
690                               695                               700

Phe Phe Met Ile Asn Thr Ser Ile Leu Leu Ile Phe Ile Phe Ile Val
705                               710                               715                               720

Leu Leu Ile His Phe Glu Gly Trp Arg Ile Ser Phe Tyr Trp Asn Val
                               725                               730                               735

Ser Val His Arg Val Leu Gly Phe Lys Glu Ile Asp Arg Gln Thr Glu
                               740                               745                               750

Gln Phe Glu Tyr Ala Ala Tyr Ile Ile His Ala Tyr Lys Asp Lys Asp
755                               760                               765

Trp Val Trp Glu His Phe Ser Ser Met Glu Lys Glu Asp Gln Ser Leu
770                               775                               780

Lys Phe Cys Leu Glu Glu Arg Asp Phe Glu Ala Gly Val Phe Glu Leu
785                               790                               795                               800

Glu Ala Ile Val Asn Ser Ile Lys Arg Ser Arg Lys Ile Ile Phe Val
                               805                               810                               815

Ile Thr His His Leu Leu Lys Asp Pro Leu Cys Lys Arg Phe Lys Val
                               820                               825                               830

His His Ala Val Gln Gln Ala Ile Glu Gln Asn Leu Asp Ser Ile Ile
                               835                               840                               845

Leu Val Phe Leu Glu Glu Ile Pro Asp Tyr Lys Leu Asn His Ala Leu
850                               855                               860

Cys Leu Arg Arg Gly Met Phe Lys Ser His Cys Ile Leu Asn Trp Pro
865                               870                               875                               880

Val Gln Lys Glu Arg Ile Gly Ala Phe Arg His Lys Leu Gln Val Ala
                               885                               890                               895

Leu Gly Ser Lys Asn Ser Val His
900

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<210> SEQ ID NO 4

<211> LENGTH: 798

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

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Met Glu Leu Asn Phe Tyr Lys Ile Pro Asp Asn Leu Pro Phe Ser Thr
1           5           10           15

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Lys Asn Leu Asp Leu Ser Phe Asn Pro Leu Arg His Leu Gly Ser Tyr
 20 25 30
 Ser Phe Phe Ser Phe Pro Glu Leu Gln Val Leu Asp Leu Ser Arg Cys
 35 40 45
 Glu Ile Gln Thr Ile Glu Asp Gly Ala Tyr Gln Ser Leu Ser His Leu
 50 55 60
 Ser Thr Leu Ile Leu Thr Gly Asn Pro Ile Gln Ser Leu Ala Leu Gly
 65 70 75 80
 Ala Phe Ser Gly Leu Ser Ser Leu Gln Lys Leu Val Ala Val Glu Thr
 85 90 95
 Asn Leu Ala Ser Leu Glu Asn Phe Pro Ile Gly His Leu Lys Thr Leu
 100 105 110
 Lys Glu Leu Asn Val Ala His Asn Leu Ile Gln Ser Phe Lys Leu Pro
 115 120 125
 Glu Tyr Phe Ser Asn Leu Thr Asn Leu Glu His Leu Asp Leu Ser Ser
 130 135 140
 Asn Lys Ile Gln Ser Ile Tyr Cys Thr Asp Leu Arg Val Leu His Gln
 145 150 155 160
 Met Pro Leu Leu Asn Leu Ser Leu Asp Leu Ser Leu Asn Pro Met Asn
 165 170 175
 Phe Ile Gln Pro Gly Ala Phe Lys Glu Ile Arg Leu His Lys Leu Thr
 180 185 190
 Leu Arg Asn Asn Phe Asp Ser Leu Asn Val Met Lys Thr Cys Ile Gln
 195 200 205
 Gly Leu Ala Gly Leu Glu Val His Arg Leu Val Leu Gly Glu Phe Arg
 210 215 220
 Asn Glu Gly Asn Leu Glu Lys Phe Asp Lys Ser Ala Leu Glu Gly Leu
 225 230 235 240
 Cys Asn Leu Thr Ile Glu Glu Phe Arg Leu Ala Tyr Leu Asp Tyr Tyr
 245 250 255
 Leu Asp Asp Ile Ile Asp Leu Phe Asn Cys Leu Thr Asn Val Ser Ser
 260 265 270
 Phe Ser Leu Val Ser Val Thr Ile Glu Arg Val Lys Asp Phe Ser Tyr
 275 280 285
 Asn Phe Gly Trp Gln His Leu Glu Leu Val Asn Cys Lys Phe Gly Gln
 290 295 300
 Phe Pro Thr Leu Lys Leu Lys Ser Leu Lys Arg Leu Thr Phe Thr Ser
 305 310 315 320
 Asn Lys Gly Gly Asn Ala Phe Ser Glu Val Asp Leu Pro Ser Leu Glu
 325 330 335
 Phe Leu Asp Leu Ser Arg Asn Gly Leu Ser Phe Lys Gly Cys Cys Ser
 340 345 350
 Gln Ser Asp Phe Gly Thr Thr Ser Leu Lys Tyr Leu Asp Leu Ser Phe
 355 360 365
 Asn Gly Val Ile Thr Met Ser Ser Asn Phe Leu Gly Leu Glu Gln Leu
 370 375 380
 Glu His Leu Asp Phe Gln His Ser Asn Leu Lys Gln Met Ser Glu Phe
 385 390 395 400
 Ser Val Phe Leu Ser Leu Arg Asn Leu Ile Tyr Leu Asp Ile Ser His
 405 410 415
 Thr His Thr Arg Val Ala Phe Asn Gly Ile Phe Asn Gly Leu Ser Ser
 420 425 430

Leu	Glu	Val	Leu	Lys	Met	Ala	Gly	Asn	Ser	Phe	Gln	Glu	Asn	Phe	Leu
435						440						445			
Pro	Asp	Ile	Phe	Thr	Glu	Leu	Arg	Asn	Leu	Thr	Phe	Leu	Asp	Leu	Ser
450						455						460			
Gln	Cys	Gln	Leu	Glu	Gln	Leu	Ser	Pro	Thr	Ala	Phe	Asn	Ser	Leu	Ser
465			470						475			480			
Ser	Leu	Gln	Val	Leu	Asn	Met	Ser	His	Asn	Asn	Phe	Phe	Ser	Leu	Asp
			485						490			495			
Thr	Phe	Pro	Tyr	Lys	Cys	Leu	Asn	Ser	Leu	Gln	Val	Leu	Asp	Tyr	Ser
			500						505			510			
Leu	Asn	His	Ile	Met	Thr	Ser	Lys	Lys	Gln	Glu	Leu	Gln	His	Phe	Pro
515						520						525			
Ser	Ser	Leu	Ala	Phe	Leu	Asn	Leu	Thr	Gln	Asn	Asp	Phe	Ala	Cys	Thr
530						535						540			
Cys	Glu	His	Gln	Ser	Phe	Leu	Gln	Trp	Ile	Lys	Asp	Gln	Arg	Gln	Leu
545			550						555			560			
Leu	Val	Glu	Val	Glu	Arg	Met	Glu	Cys	Ala	Thr	Pro	Ser	Asp	Lys	Gln
			565						570			575			
Gly	Met	Pro	Val	Leu	Ser	Leu	Asn	Ile	Thr	Cys	Gln	Met	Asn	Lys	Thr
			580						585			590			
Ile	Ile	Gly	Val	Ser	Val	Leu	Ser	Val	Leu	Val	Val	Ser	Val	Val	Ala
595						600						605			
Val	Leu	Val	Tyr	Lys	Phe	Tyr	Phe	His	Leu	Met	Leu	Leu	Ala	Gly	Cys
610						615						620			
Ile	Lys	Tyr	Gly	Arg	Gly	Glu	Asn	Ile	Tyr	Asp	Ala	Phe	Val	Ile	Tyr
625			630						635			640			
Ser	Ser	Gln	Asp	Glu	Asp	Trp	Val	Arg	Asn	Glu	Leu	Val	Lys	Asn	Leu
			645						650			655			
Glu	Glu	Gly	Val	Pro	Pro	Phe	Gln	Leu	Cys	Leu	His	Tyr	Arg	Asp	Phe
			660			665						670			
Ile	Pro	Gly	Val	Ala	Ile	Ala	Ala	Asn	Ile	Ile	His	Glu	Gly	Phe	His
675						680						685			
Lys	Ser	Arg	Lys	Val	Ile	Val	Val	Val	Ser	Gln	His	Phe	Ile	Gln	Ser
690						695						700			
Arg	Trp	Cys	Ile	Phe	Glu	Tyr	Glu	Ile	Ala	Gln	Thr	Trp	Gln	Phe	Leu
705			710			715						720			
Ser	Ser	Arg	Ala	Gly	Ile	Ile	Phe	Ile	Val	Leu	Gln	Lys	Val	Glu	Lys
			725						730			735			
Thr	Leu	Leu	Arg	Gln	Gln	Val	Glu	Leu	Tyr	Arg	Leu	Leu	Ser	Arg	Asn
			740			745						750			
Thr	Tyr	Leu	Glu	Trp	Glu	Asp	Ser	Val	Leu	Gly	Arg	His	Ile	Phe	Trp
755						760						765			
Arg	Arg	Leu	Arg	Lys	Ala	Leu	Leu	Asp	Gly	Lys	Ser	Trp	Asn	Pro	Glu
770						775						780			
Gly	Thr	Val	Gly	Thr	Gly	Cys	Asn	Trp	Gln	Glu	Ala	Thr	Ser		
785			790						795						

<400> SEQUENCE: 5

Met Gly Asp His Leu Asp Leu Leu Leu Gly Val Val Leu Met Ala Gly
1 5 10 15

Pro	Val	Phe	Gly	Ile	Pro	Ser	Cys	Ser	Phe	Asp	Gly	Arg	Ile	Ala	Phe
			20					25				30			
Tyr	Arg	Phe	Cys	Asn	Leu	Thr	Gln	Val	Pro	Gln	Val	Leu	Asn	Thr	Thr
			35				40					45			
Glu	Arg	Leu	Leu	Leu	Ser	Phe	Asn	Tyr	Ile	Arg	Thr	Val	Thr	Ala	Ser
			50			55				60					
Ser	Phe	Pro	Phe	Leu	Glu	Gln	Leu	Gln	Leu	Leu	Glu	Leu	Gly	Ser	Gln
65					70				75						80
Tyr	Thr	Pro	Leu	Thr	Ile	Asp	Lys	Glu	Ala	Phe	Arg	Asn	Leu	Pro	Asn
				85					90					95	
Leu	Arg	Ile	Leu	Asp	Leu	Gly	Ser	Ser	Lys	Ile	Tyr	Phe	Leu	His	Pro
				100				105					110		
Asp	Ala	Phe	Gln	Gly	Leu	Phe	His	Leu	Phe	Glu	Leu	Arg	Leu	Tyr	Phe
				115			120					125			
Cys	Gly	Leu	Ser	Asp	Ala	Val	Leu	Lys	Asp	Gly	Tyr	Phe	Arg	Asn	Leu
						135					140				
Lys	Ala	Leu	Thr	Arg	Leu	Asp	Leu	Ser	Lys	Asn	Gln	Ile	Arg	Ser	Leu
145					150					155					160
Tyr	Leu	His	Pro	Ser	Phe	Gly	Lys	Leu	Asn	Ser	Leu	Lys	Ser	Ile	Asp
				165					170					175	
Phe	Ser	Ser	Asn	Gln	Ile	Phe	Leu	Val	Cys	Glu	His	Glu	Leu	Glu	Pro
				180				185					190		
Leu	Gln	Gly	Lys	Thr	Leu	Ser	Phe	Phe	Ser	Leu	Ala	Ala	Asn	Ser	Leu
				195			200					205			
Tyr	Ser	Arg	Val	Ser	Val	Asp	Trp	Gly	Lys	Cys	Met	Asn	Pro	Phe	Arg
					215						220				
Asn	Met	Val	Leu	Glu	Ile	Leu	Asp	Val	Ser	Gly	Asn	Gly	Trp	Thr	Val
225					230					235					240
Asp	Ile	Thr	Gly	Asn	Phe	Ser	Asn	Ala	Ile	Ser	Lys	Ser	Gln	Ala	Phe
				245				250						255	
Ser	Leu	Ile	Leu	Ala	His	His	Ile	Met	Gly	Ala	Gly	Phe	Gly	Phe	His
				260				265				270			
Asn	Ile	Lys	Asp	Pro	Asp	Gln	Asn	Thr	Phe	Ala	Gly	Leu	Ala	Arg	Ser
				275			280					285			
Ser	Val	Arg	His	Leu	Asp	Leu	Ser	His	Gly	Phe	Val	Phe	Ser	Leu	Asn
					295					300					
Ser	Arg	Val	Phe	Glu	Thr	Leu	Lys	Asp	Leu	Lys	Val	Leu	Asn	Leu	Ala
305					310					315					320
Tyr	Asn	Lys	Ile	Asn	Lys	Ile	Ala	Asp	Glu	Ala	Phe	Tyr	Gly	Leu	Asp
				325				330						335	
Asn	Leu	Gln	Val	Leu	Asn	Leu	Ser	Tyr	Asn	Leu	Leu	Gly	Glu	Leu	Tyr
				340				345				350			
Ser	Ser	Asn	Phe	Tyr	Gly	Leu	Pro	Lys	Val	Ala	Tyr	Ile	Asp	Leu	Gln
				355			360					365			
Lys	Asn	His	Ile	Ala	Ile	Ile	Gln	Asp	Gln	Thr	Phe	Lys	Phe	Leu	Glu
					375										

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Asn	Arg	Leu	Glu	Asn	Leu	Asp	Ile	Leu	Tyr	Phe	Leu	Leu	Arg	Val	Pro
		435					440					445			
His	Leu	Gln	Ile	Leu	Ile	Leu	Asn	Gln	Asn	Arg	Phe	Ser	Ser	Cys	Ser
	450					455					460				
Gly	Asp	Gln	Thr	Pro	Ser	Glu	Asn	Pro	Ser	Leu	Glu	Gln	Leu	Phe	Leu
	465				470					475					480
Gly	Glu	Asn	Met	Leu	Gln	Leu	Ala	Trp	Glu	Thr	Glu	Leu	Cys	Trp	Asp
			485					490						495	
Val	Phe	Glu	Gly	Leu	Ser	His	Leu	Gln	Val	Leu	Tyr	Leu	Asn	His	Asn
			500					505					510		
Tyr	Leu	Asn	Ser	Leu	Pro	Pro	Gly	Val	Phe	Ser	His	Leu	Thr	Ala	Leu
		515					520					525			
Arg	Gly	Leu	Ser	Leu	Asn	Ser	Asn	Arg	Leu	Thr	Val	Leu	Ser	His	Asn
	530					535					540				
Asp	Leu	Pro	Ala	Asn	Leu	Glu	Ile	Leu	Asp	Ile	Ser	Arg	Asn	Gln	Leu
	545				550					555					560
Leu	Ala	Pro	Asn	Pro	Asp	Val	Phe	Val	Ser	Leu	Ser	Val	Leu	Asp	Ile
				565					570					575	
Thr	His	Asn	Lys	Phe	Ile	Cys	Glu	Cys	Glu	Leu	Ser	Thr	Phe	Ile	Asn
			580					585					590		
Trp	Leu	Asn	His	Thr	Asn	Val	Thr	Ile	Ala	Gly	Pro	Pro	Ala	Asp	Ile
		595					600					605			
Tyr	Cys	Val	Tyr	Pro	Asp	Ser	Phe	Ser	Gly	Val	Ser	Leu	Phe	Ser	Leu
	610					615					620				
Ser	Thr	Glu	Gly	Cys	Asp	Glu	Glu	Glu	Val	Leu	Lys	Ser	Leu	Lys	Phe
	625				630					635					640
Ser	Leu	Phe	Ile	Val	Cys	Thr	Val	Thr	Leu	Thr	Leu	Phe	Leu	Met	Thr
				645					650					655	
Ile	Leu	Thr	Val	Thr	Lys	Phe	Arg	Gly	Phe	Cys	Phe	Ile	Cys	Tyr	Lys
			660					665					670		
Thr	Ala	Gln	Arg	Leu	Val	Phe	Lys	Asp	His	Pro	Gln	Gly	Thr	Glu	Pro
		675					680					685			
Asp	Met	Tyr	Lys	Tyr	Asp	Ala	Tyr	Leu	Cys	Phe	Ser	Ser	Lys	Asp	Phe
	690					695					700				
Thr	Trp	Val	Gln	Asn	Ala	Leu	Leu	Lys	His	Leu	Asp	Thr	Gln	Tyr	Ser
	705				710					715					720
Asp	Gln	Asn	Arg	Phe	Asn	Leu	Cys	Phe	Glu	Glu	Arg	Asp	Phe	Val	Pro
				725					730					735	
Gly	Glu	Asn	Arg	Ile	Ala	Asn	Ile	Gln	Asp	Ala	Ile	Trp	Asn	Ser	Arg
			740					745					750		
Lys	Ile	Val	Cys	Leu	Val	Ser	Arg	His	Phe	Leu	Arg	Asp	Gly	Trp	Cys
		755					760					765			
Leu	Glu	Ala	Phe	Ser	Tyr	Ala	Gln	Gly	Arg	Cys	Leu	Ser	Asp	Leu	Asn
	770					775					780				
Ser	Ala	Leu	Ile	Met	Val	Val	Val	Gly	Ser	Leu	Ser	Gln	Tyr	Gln	Leu
	785				790					795					800
Met	Lys	His	Gln	Ser	Ile	Arg	Gly	Phe	Val	Gln	Lys	Gln	Gln	Tyr	Leu
				805					810					815	
Arg	Trp	Pro	Glu	Asp	Leu	Gln	Asp	Val	Gly	Trp	Phe	Leu	His	Lys	Leu
			820					825					830		
Ser	Gln	Gln	Ile	Leu	Lys	Lys	Glu	Lys	Glu	Lys	Lys	Lys	Asp	Asn	Asn
		835					840						845		
Ile	Pro	Leu	Gln	Thr	Val	Ala	Thr	Ile	Ser						

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850 855

<210> SEQ ID NO 6
 <211> LENGTH: 796
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

Met Thr Lys Asp Lys Glu Pro Ile Val Lys Ser Phe His Phe Val Cys
 1 5 10 15

Leu Met Ile Ile Ile Val Gly Thr Arg Ile Gln Phe Ser Asp Gly Asn
 20 25 30

Glu Phe Ala Val Asp Lys Ser Lys Arg Gly Leu Ile His Val Pro Lys
 35 40 45

Asp Leu Pro Leu Lys Thr Lys Val Leu Asp Met Ser Gln Asn Tyr Ile
 50 55 60

Ala Glu Leu Gln Val Ser Asp Met Ser Phe Leu Ser Glu Leu Thr Val
 65 70 75 80

Leu Arg Leu Ser His Asn Arg Ile Gln Leu Leu Asp Leu Ser Val Phe
 85 90 95

Lys Phe Asn Gln Asp Leu Glu Tyr Leu Asp Leu Ser His Asn Gln Leu
 100 105 110

Gln Lys Ile Ser Cys His Pro Ile Val Ser Phe Arg His Leu Asp Leu
 115 120 125

Ser Phe Asn Asp Phe Lys Ala Leu Pro Ile Cys Lys Glu Phe Gly Asn
 130 135 140

Leu Ser Gln Leu Asn Phe Leu Gly Leu Ser Ala Met Lys Leu Gln Lys
 145 150 155 160

Leu Asp Leu Leu Pro Ile Ala His Leu His Leu Ser Tyr Ile Leu Leu
 165 170 175

Asp Leu Arg Asn Tyr Tyr Ile Lys Glu Asn Glu Thr Glu Ser Leu Gln
 180 185 190

Ile Leu Asn Ala Lys Thr Leu His Leu Val Phe His Pro Thr Ser Leu
 195 200 205

Phe Ala Ile Gln Val Asn Ile Ser Val Asn Thr Leu Gly Cys Leu Gln
 210 215 220

Leu Thr Asn Ile Lys Leu Asn Asp Asp Asn Cys Gln Val Phe Ile Lys
 225 230 235 240

Phe Leu Ser Glu Leu Thr Arg Gly Pro Thr Leu Leu Asn Phe Thr Leu
 245 250 255

Asn His Ile Glu Thr Thr Trp Lys Cys Leu Val Arg Val Phe Gln Phe
 260 265 270

Leu Trp Pro Lys Pro Val Glu Tyr Leu Asn Ile Tyr Asn Leu Thr Ile
 275 280 285

Ile Glu Ser Ile Arg Glu Glu Asp Phe Thr Tyr Ser Lys Thr Thr Leu
 290 295 300

Lys Ala Leu Thr Ile Glu His Ile Thr Asn Gln Val Phe Leu Phe Ser
 305 310 315 320

Gln Thr Ala Leu Tyr Thr Val Phe Ser Glu Met Asn Ile Met Met Leu
 325 330 335

Thr Ile Ser Asp Thr Pro Phe Ile His Met Leu Cys Pro His Ala Pro
 340 345 350

Ser Thr Phe Lys Phe Leu Asn Phe Thr Gln Asn Val Phe Thr Asp Ser
 355 360 365

Ile 370	Phe	Glu	Lys	Cys	Ser	Thr 375	Leu	Val	Lys	Leu	Glu 380	Thr	Leu	Ile	Leu
Gln 385	Lys	Asn	Gly	Leu	Lys 390	Asp	Leu	Phe	Lys	Val 395	Gly	Leu	Met	Thr	Lys 400
Asp	Met	Pro	Ser	Leu 405	Glu	Ile	Leu	Asp	Val 410	Ser	Trp	Asn	Ser	Leu 415	Glu
Ser	Gly	Arg	His 420	Lys	Glu	Asn	Cys	Thr 425	Trp	Val	Glu	Ser	Ile 430	Val	Val
Leu	Asn 435	Leu	Ser	Ser	Asn	Met	Leu 440	Thr	Asp	Ser	Val	Phe 445	Arg	Cys	Leu
Pro 450	Pro	Arg	Ile	Lys	Val	Leu 455	Asp	Leu	His	Ser	Asn 460	Lys	Ile	Lys	Ser
Val 465	Pro	Lys	Gln	Val	Val 470	Lys	Leu	Glu	Ala	Leu 475	Gln	Glu	Leu	Asn	Val 480
Ala	Phe	Asn	Ser	Leu 485	Thr	Asp	Leu	Pro	Gly 490	Cys	Gly	Ser	Phe	Ser 495	Ser
Leu	Ser	Val	Leu 500	Ile	Ile	Asp	His	Asn 505	Ser	Val	Ser	His	Pro 510	Ser	Ala
Asp	Phe 515	Phe	Gln	Ser	Cys	Gln	Lys 520	Met	Arg	Ser	Ile	Lys 525	Ala	Gly	Asp
Asn 530	Pro	Phe	Gln	Cys	Thr	Cys 535	Glu	Leu	Arg	Glu	Phe 540	Val	Lys	Asn	Ile
Asp 545	Gln	Val	Ser	Ser	Glu 550	Val	Leu	Glu	Gly	Trp 555	Pro	Asp	Ser	Tyr	Lys 560
Cys	Asp	Tyr	Pro	Glu 565	Ser	Tyr	Arg	Gly	Ser 570	Pro	Leu	Lys	Asp	Phe 575	His
Met	Ser	Glu	Leu 580	Ser	Cys	Asn	Ile	Thr 585	Leu	Leu	Ile	Val 590	Thr	Ile	Gly
Ala	Thr 595	Met	Leu	Val	Leu	Ala	Val 600	Thr	Val	Thr	Ser	Leu 605	Cys	Ile	Tyr
Leu 610	Asp	Leu	Pro	Trp	Tyr	Leu 615	Arg	Met	Val	Cys	Gln 620	Trp	Thr	Gln	Thr
Arg 625	Arg	Arg	Ala	Arg	Asn 630	Ile	Pro	Leu	Glu	Glu 635	Leu	Gln	Arg	Asn	Leu 640
Gln	Phe	His	Ala 645	Phe	Ile	Ser	Tyr	Ser	Glu 650	His	Asp	Ser	Ala	Trp 655	Val
Lys	Ser	Glu	Leu 660	Val	Pro	Tyr	Leu	Glu 665	Lys	Glu	Asp	Ile	Gln 670	Ile	Cys
Leu	His 675	Glu	Arg	Asn	Phe	Val	Pro 680	Gly	Lys	Ser	Ile	Val 685	Glu	Asn	Ile
Ile 690	Asn	Cys	Ile	Glu	Lys 695	Ser	Tyr	Lys	Ser	Ile	Phe 700	Val	Leu	Ser	Pro
Asn 705	Phe	Val	Gln	Ser	Glu 710	Trp	Cys	His	Tyr	Glu 715	Leu	Tyr	Phe	Ala	His 720
His	Asn	Leu	Phe 725	His	Glu	Gly	Ser	Asn	Asn 730	Leu	Ile	Leu	Ile 735	Leu	Leu
Glu	Pro	Ile	Pro 740	Gln	Asn	Ser	Ile	Pro	Asn 745	Lys	Tyr	His	Lys 750	Leu	Lys
Ala	Leu 755	Met	Thr	Gln	Arg	Thr	Tyr 760	Leu	Gln	Trp	Pro	Lys 765	Glu	Lys	Ser
Lys 770	Arg	Gly	Leu	Phe	Trp	Ala 775	Asn	Ile	Arg	Ala	Ala 780	Phe	Asn	Met	Lys
Leu	Thr	Leu	Val	Thr	Glu	Asn	Asn	Asp	Val	Lys	Ser				

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Leu Lys Ser	Leu Lys Ile	Leu Arg Ile Arg	Gly Tyr Val	Phe Lys Glu
370	375		380	
Leu Lys Ser	Phe Asn Leu Ser	Pro Leu His Asn Leu	Gln Asn Leu Glu	
385	390	395	400	
Val Leu Asp	Leu Gly Thr	Asn Phe Ile Lys	Ile Ala Asn Leu Ser	Met
	405	410	415	
Phe Lys Gln	Phe Lys Arg	Leu Lys Val Ile	Asp Leu Ser Val	Asn Lys
	420	425	430	
Ile Ser Pro	Ser Gly Asp	Ser Ser Glu Val	Gly Phe Cys Ser	Asn Ala
	435	440	445	
Arg Thr Ser	Val Glu Ser	Tyr Glu Pro Gln	Val Leu Glu Gln	Leu His
	450	455	460	
Tyr Phe Arg	Tyr Asp Lys	Tyr Ala Arg Ser	Cys Arg Phe Lys	Asn Lys
	465	470	475	480
Glu Ala Ser	Phe Met Ser	Val Asn Glu Ser	Cys Tyr Lys Tyr	Gly Gln
	485	490	495	
Thr Leu Asp	Leu Ser Lys	Asn Ser Ile Phe	Phe Val Lys Ser	Ser Asp
	500	505	510	
Phe Gln His	Leu Ser Phe	Leu Lys Cys Leu	Asn Leu Ser Gly	Asn Leu
	515	520	525	
Ile Ser Gln	Thr Leu Asn	Gly Ser Glu Phe	Gln Pro Leu Ala	Glu Leu
	530	535	540	
Arg Tyr Leu	Asp Phe Ser	Asn Asn Arg Leu	Asp Leu Leu His	Ser Thr
	545	550	555	560
Ala Phe Glu	Glu Leu His	Lys Leu Glu Val	Leu Asp Ile Ser	Ser Asn
	565	570	575	
Ser His Tyr	Phe Gln Ser	Glu Gly Ile Thr	His Met Leu Asn	Phe Thr
	580	585	590	
Lys Asn Leu	Lys Val Leu	Gln Lys Leu Met	Met Asn Asp	Asn Asp Ile
	595	600	605	
Ser Ser Ser	Thr Ser Arg	Thr Met Glu Ser	Glu Ser Leu Arg	Thr Leu
	610	615	620	
Glu Phe Arg	Gly Asn His	Leu Asp Val Leu	Trp Arg Glu Gly	Asp Asn
	625	630	635	640
Arg Tyr Leu	Gln Leu Phe	Lys Asn Leu Leu	Lys Leu Glu Glu	Leu Asp
	645	650	655	
Ile Ser Lys	Asn Ser Leu	Ser Phe Leu Pro	Ser Gly Val Phe	Asp Gly
	660	665	670	
Met Pro Pro	Asn Leu Lys	Asn Leu Ser Leu	Ala Lys Asn Gly	Leu Lys
	675	680	685	
Ser Phe Ser	Trp Lys Lys	Leu Gln Cys Leu	Lys Asn Leu Glu	Thr Leu
	690	695	700	
Asp Leu Ser	His Asn Gln	Leu Thr Thr Val	Pro Glu Arg Leu	Ser Asn
	705	710	715	720
Cys Ser Arg	Ser Leu Lys	Asn Leu Ile Leu	Lys Asn Asn Gln	Ile Arg
	725	730	735	
Ser Leu Thr	Lys Tyr Phe	Leu Gln Asp Ala	Phe Gln Leu Arg	Tyr Leu
	740	745	750	
Asp Leu Ser	Ser Asn Lys	Ile Gln Met Ile	Gln Lys Thr Ser	Phe Pro
	755	760	765	
Glu Asn Val	Leu Asn Asn	Leu Lys Met Leu	Leu Leu Leu His	His Asn Arg
	770	775	780	
Phe Leu Cys	Thr Cys Asp	Ala Val Trp Phe	Val Trp Trp Val	Asn His

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785	790	795	800
Thr Glu Val Thr Ile Pro Tyr Leu Ala Thr Asp Val Thr Cys Val Gly	805	810	815
Pro Gly Ala His Lys Gly Gln Ser Val Ile Ser Leu Asp Leu Tyr Thr	820	825	830
Cys Glu Leu Asp Leu Thr Asn Leu Ile Leu Phe Ser Leu Ser Ile Ser	835	840	845
Val Ser Leu Phe Leu Met Val Met Met Thr Ala Ser His Leu Tyr Phe	850	855	860
Trp Asp Val Trp Tyr Ile Tyr His Phe Cys Lys Ala Lys Ile Lys Gly	865	870	875
Tyr Gln Arg Leu Ile Ser Pro Asp Cys Cys Tyr Asp Ala Phe Ile Val	885	890	895
Tyr Asp Thr Lys Asp Pro Ala Val Thr Glu Trp Val Leu Ala Glu Leu	900	905	910
Val Ala Lys Leu Glu Asp Pro Arg Glu Lys His Phe Asn Leu Cys Leu	915	920	925
Glu Glu Arg Asp Trp Leu Pro Gly Gln Pro Val Leu Glu Asn Leu Ser	930	935	940
Gln Ser Ile Gln Leu Ser Lys Lys Thr Val Phe Val Met Thr Asp Lys	945	950	955
Tyr Ala Lys Thr Glu Asn Phe Lys Ile Ala Phe Tyr Leu Ser His Gln	965	970	975
Arg Leu Met Asp Glu Lys Val Asp Val Ile Ile Leu Ile Phe Leu Glu	980	985	990
Lys Pro Phe Gln Lys Ser Lys Phe Leu Gln Leu Arg Lys Arg Leu Cys	995	1000	1005
Gly Ser Ser Val Leu Glu Trp Pro Thr Asn Pro Gln Ala His Pro	1010	1015	1020
Tyr Phe Trp Gln Cys Leu Lys Asn Ala Leu Ala Thr Asp Asn His	1025	1030	1035
Val Ala Tyr Ser Gln Val Phe Lys Glu Thr Val	1040	1045	

<210> SEQ ID NO 8

<211> LENGTH: 1041

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

Met Glu Asn Met Phe Leu Gln Ser Ser Met Leu Thr Cys Ile Phe Leu	1	5	10	15
Leu Ile Ser Gly Ser Cys Glu Leu Cys Ala Glu Glu Asn Phe Ser Arg	20	25	30	
Ser Tyr Pro Cys Asp Glu Lys Lys Gln Asn Asp Ser Val Ile Ala Glu	35	40	45	
Cys Ser Asn Arg Arg Leu Gln Glu Val Pro Gln Thr Val Gly Lys Tyr	50	55	60	
Val Thr Glu Leu Asp Leu Ser Asp Asn Phe Ile Thr His Ile Thr Asn	65	70	75	80
Glu Ser Phe Gln Gly Leu Gln Asn Leu Thr Lys Ile Asn Leu Asn His	85	90	95	
Asn Pro Asn Val Gln His Gln Asn Gly Asn Pro Gly Ile Gln Ser Asn	100	105	110	

Gly 115	Leu 130	Asn 145	Ile 160	Thr 175	Asp 190	Gly 205	Ala 220	Phe 235	Leu 250	Asn 265	Leu 280	Lys 295	Asn 310	Leu 325	Arg 340
Glu 135	Leu 150	Leu 165	Leu 180	Glu 195	Asp 210	Asn 225	Gln 240	Leu 255	Pro 270	Gln 285	Ile 300	Pro 315	Ser 330	Gly 345	Leu 360
Pro 140	Glu 155	Ser 170	Leu 185	Thr 200	Glu 215	Leu 230	Thr 245	Ile 260	Val 275	Asn 290	Leu 305	Asn 320	Ile 335	Tyr 350	Asn 365
Ile 145	Thr 160	Lys 175	Glu 190	Gly 205	Ile 220	Ser 235	Arg 250	Leu 265	Ile 280	Asn 295	Leu 310	Lys 325	Asn 340	Leu 355	Tyr 370
Leu 150	Ala 165	Trp 180	Asn 195	Cys 210	Tyr 225	Phe 240	Asn 255	Lys 270	Val 285	Cys 300	Glu 315	Lys 330	Thr 345	Asn 360	Ile 375
Glu 155	Asp 170	Gly 185	Val 200	Phe 215	Glu 230	Thr 245	Leu 260	Thr 275	Asn 290	Leu 305	Glu 320	Leu 335	Leu 350	Ser 365	Leu 380
Ser 160	Phe 175	Asn 190	Ser 205	Leu 220	Ser 235	His 250	Val 265	Pro 280	Pro 295	Lys 310	Leu 325	Pro 340	Ser 355	Ser 370	Leu 385
Arg 165	Lys 180	Leu 195	Phe 210	Leu 225	Ser 240	Asn 255	Thr 270	Gln 285	Ile 300	Lys 315	Tyr 330	Ile 345	Ser 360	Glu 375	Glu 390
Asp 170	Phe 185	Lys 200	Gly 215	Leu 230	Ile 245	Asn 260	Leu 275	Thr 290	Leu 305	Asp 320	Leu 335	Leu 350	Ser 365	Gly 380	Asn 395
Cys 175	Pro 190	Arg 205	Cys 220	Phe 235	Asn 250	Ala 265	Pro 280	Phe 295	Pro 310	Cys 325	Val 340	Pro 355	Cys 370	Asp 385	Gly 400
Gly 180	Ala 195	Ser 210	Ile 225	Asn 240	Ile 255	Asp 270	Arg 285	Phe 300	Ala 315	Phe 330	Gln 345	Asn 360	Leu 375	Thr 390	Gln 405
Leu 185	Arg 190	Tyr 205	Leu 220	Asn 235	Leu 250	Ser 265	Ser 280	Thr 295	Ser 310	Leu 325	Arg 340	Lys 355	Ile 370	Asn 385	Ala 400
Ala 190	Trp 205	Phe 220	Lys 235	Asn 250	Met 265	Pro 280	His 295	Leu 310	Lys 325	Val 340	Leu 355	Asp 370	Leu 385	Glu 400	Phe 415
Asn 195	Tyr 210	Leu 225	Val 240	Gly 255	Glu 270	Ile 285	Ala 300	Ser 315	Gly 330	Ala 345	Phe 360	Leu 375	Thr 390	Met 405	Leu 420
Pro 200	Arg 215	Leu 230	Glu 245	Ile 260	Leu 275	Asp 290	Leu 305	Phe 320	Asn 335	Tyr 350	Ile 365	Lys 380	Gly 395	Ser 410	Ser 425
Tyr 205	Pro 220	Gln 235	His 250	Ile 265	Asn 280	Ile 295	Ser 310	Arg 325	Asn 340	Phe 355	Ser 370	Lys 385	Leu 400	Leu 415	Ser 430
Leu 210	Arg 225	Ala 240	Leu 255	His 270	Leu 285	Arg 300	Gly 315	Tyr 330	Val 345	Phe 360	Gln 375	Glu 390	Leu 405	Arg 420	Glu 435
Asp 215	Asp 230	Phe 245	Gln 260	Pro 275	Leu 290	Met 305	Gln 320	Leu 335	Pro 350	Asn 365	Leu 380	Ser 395	Thr 410	Ile 425	Asn 440
Leu 220	Gly 235	Ile 250	Asn 265	Phe 280	Ile 295	Lys 310	Gln 325	Ile 340	Asp 355	Phe 370	Lys 385	Leu 400	Phe 415	Gln 430	Asn 445
Phe 225	Ser 240	Asn 255	Leu 270	Glu 285	Ile 300	Ile 315	Tyr 330	Leu 345	Ser 360	Glu 375	Asn 390	Arg 405	Ile 420	Ser 435	Pro 450
Leu 230	Val 245	Lys 260	Asp 275	Thr 290	Arg 305	Gln 320	Ser 335	Tyr 350	Ala 365	Asn 380	Ser 395	Ser 410	Ser 425	Phe 440	Gln 455
Arg 235	His 250	Ile 265	Arg 280	Lys 295	Arg 310	Arg 325	Ser 340	Thr 355	Asp 370	Phe 385	Glu 400	Phe 415	Asp 430	Pro 445	His 460
Ser 240	Asn 255	Phe 270	Tyr 285	His 300	Phe 315	Thr 330	Arg 345	Pro 360	Leu 375	Ile 390	Lys 405	Pro 420	Gln 435	Cys 450	Ala 465
Ala 245	Tyr 260	Gly 275	Lys 290	Ala 305	Leu 320	Asp 335	Leu 350	Ser 365	Leu 380	Asn 395	Ser 410	Ile 425	Phe 440	Phe 455	Ile 470
Gly 250	Pro 265	Asn													

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530	535	540
Asp Asn Ala Ser Ala Leu Thr Glu Leu Ser Asp Leu Glu Val Leu Asp		
545	550	555 560
Leu Ser Tyr Asn Ser His Tyr Phe Arg Ile Ala Gly Val Thr His His		
	565	570 575
Leu Glu Phe Ile Gln Asn Phe Thr Asn Leu Lys Val Leu Asn Leu Ser		
	580	585 590
His Asn Asn Ile Tyr Thr Leu Thr Asp Lys Tyr Asn Leu Glu Ser Lys		
	595	600 605
Ser Leu Val Glu Leu Val Phe Ser Gly Asn Arg Leu Asp Ile Leu Trp		
610	615	620
Asn Asp Asp Asp Asn Arg Tyr Ile Ser Ile Phe Lys Gly Leu Lys Asn		
625	630	635 640
Leu Thr Arg Leu Asp Leu Ser Leu Asn Arg Leu Lys His Ile Pro Asn		
	645	650 655
Glu Ala Phe Leu Asn Leu Pro Ala Ser Leu Thr Glu Leu His Ile Asn		
	660	665 670
Asp Asn Met Leu Lys Phe Phe Asn Trp Thr Leu Leu Gln Gln Phe Pro		
	675	680 685
Arg Leu Glu Leu Leu Asp Leu Arg Gly Asn Lys Leu Leu Phe Leu Thr		
690	695	700
Asp Ser Leu Ser Asp Phe Thr Ser Ser Leu Arg Thr Leu Leu Leu Ser		
705	710	715 720
His Asn Arg Ile Ser His Leu Pro Ser Gly Phe Leu Ser Glu Val Ser		
	725	730 735
Ser Leu Lys His Leu Asp Leu Ser Ser Asn Leu Leu Lys Thr Ile Asn		
	740	745 750
Lys Ser Ala Leu Glu Thr Lys Thr Thr Thr Lys Leu Ser Met Leu Glu		
	755	760 765
Leu His Gly Asn Pro Phe Glu Cys Thr Cys Asp Ile Gly Asp Phe Arg		
770	775	780
Arg Trp Met Asp Glu His Leu Asn Val Lys Ile Pro Arg Leu Val Asp		
785	790	795 800
Val Ile Cys Ala Ser Pro Gly Asp Gln Arg Gly Lys Ser Ile Val Ser		
	805	810 815
Leu Glu Leu Thr Thr Cys Val Ser Asp Val Thr Ala Val Ile Leu Phe		
	820	825 830
Phe Phe Thr Phe Phe Ile Thr Thr Met Val Met Leu Ala Ala Leu Ala		
	835	840 845
His His Leu Phe Tyr Trp Asp Val Trp Phe Ile Tyr Asn Val Cys Leu		
850	855	860
Ala Lys Val Lys Gly Tyr Arg Ser Leu Ser Thr Ser Gln Thr Phe Tyr		
865	870	875 880
Asp Ala Tyr Ile Ser Tyr Asp Thr Lys Asp Ala Ser Val Thr Asp Trp		
	885	890 895
Val Ile Asn Glu Leu Arg Tyr His Leu Glu Glu Ser Arg Asp Lys Asn		
	900	905 910
Val Leu Leu Cys Leu Glu Glu Arg Asp Trp Asp Pro Gly Leu Ala Ile		
	915	920 925
Ile Asp Asn Leu Met Gln Ser Ile Asn Gln Ser Lys Lys Thr Val Phe		
930	935	940
Val Leu Thr Lys Lys Tyr Ala Lys Ser Trp Asn Phe Lys Thr Ala Phe		
945	950	955 960

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Tyr Leu Ala Leu Gln Arg Leu Met Asp Glu Asn Met Asp Val Ile Ile
 965 970 975
 Phe Ile Leu Leu Glu Pro Val Leu Gln His Ser Gln Tyr Leu Arg Leu
 980 985 990
 Arg Gln Arg Ile Cys Lys Ser Ser Ile Leu Gln Trp Pro Asp Asn Pro
 995 1000 1005
 Lys Ala Glu Gly Leu Phe Trp Gln Thr Leu Arg Asn Val Val Leu
 1010 1015 1020
 Thr Glu Asn Asp Ser Arg Tyr Asn Asn Met Tyr Val Asp Ser Ile
 1025 1030 1035
 Lys Gln Tyr
 1040

<210> SEQ ID NO 9
 <211> LENGTH: 1032
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

Met Gly Phe Cys Arg Ser Ala Leu His Pro Leu Ser Leu Leu Val Gln
 1 5 10 15
 Ala Ile Met Leu Ala Met Thr Leu Ala Leu Gly Thr Leu Pro Ala Phe
 20 25 30
 Leu Pro Cys Glu Leu Gln Pro His Gly Leu Val Asn Cys Asn Trp Leu
 35 40 45
 Phe Leu Lys Ser Val Pro His Phe Ser Met Ala Ala Pro Arg Gly Asn
 50 55 60
 Val Thr Ser Leu Ser Leu Ser Ser Asn Arg Ile His His Leu His Asp
 65 70 75 80
 Ser Asp Phe Ala His Leu Pro Ser Leu Arg His Leu Asn Leu Lys Trp
 85 90 95
 Asn Cys Pro Pro Val Gly Leu Ser Pro Met His Phe Pro Cys His Met
 100 105 110
 Thr Ile Glu Pro Ser Thr Phe Leu Ala Val Pro Thr Leu Glu Glu Leu
 115 120 125
 Asn Leu Ser Tyr Asn Asn Ile Met Thr Val Pro Ala Leu Pro Lys Ser
 130 135 140
 Leu Ile Ser Leu Ser Leu Ser His Thr Asn Ile Leu Met Leu Asp Ser
 145 150 155 160
 Ala Ser Leu Ala Gly Leu His Ala Leu Arg Phe Leu Phe Met Asp Gly
 165 170 175
 Asn Cys Tyr Tyr Lys Asn Pro Cys Arg Gln Ala Leu Glu Val Ala Pro
 180 185 190
 Gly Ala Leu Leu Gly Leu Gly Asn Leu Thr His Leu Ser Leu Lys Tyr
 195 200 205
 Asn Asn Leu Thr Val Val Pro Arg Asn Leu Pro Ser Ser Leu Glu Tyr
 210 215 220
 Leu Leu Leu Ser Tyr Asn Arg Ile Val Lys Leu Ala Pro Glu Asp Leu
 225 230 235 240
 Ala Asn Leu Thr Ala Leu Arg Val Leu Asp Val Gly Gly Asn Cys Arg
 245 250 255
 Arg Cys Asp His Ala Pro Asn Pro Cys Met Glu Cys Pro Arg His Phe
 260 265 270
 Pro Gln Leu His Pro Asp Thr Phe Ser His Leu Ser Arg Leu Glu Gly

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275					280					285					
Leu	Val	Leu	Lys	Asp	Ser	Ser	Leu	Ser	Trp	Leu	Asn	Ala	Ser	Trp	Phe
290						295					300				
Arg	Gly	Leu	Gly	Asn	Leu	Arg	Val	Leu	Asp	Leu	Ser	Glu	Asn	Phe	Leu
305				310						315					320
Tyr	Lys	Cys	Ile	Thr	Lys	Thr	Lys	Ala	Phe	Gln	Gly	Leu	Thr	Gln	Leu
				325					330					335	
Arg	Lys	Leu	Asn	Leu	Ser	Phe	Asn	Tyr	Gln	Lys	Arg	Val	Ser	Phe	Ala
			340					345					350		
His	Leu	Ser	Leu	Ala	Pro	Ser	Phe	Gly	Ser	Leu	Val	Ala	Leu	Lys	Glu
		355					360					365			
Leu	Asp	Met	His	Gly	Ile	Phe	Phe	Arg	Ser	Leu	Asp	Glu	Thr	Thr	Leu
	370					375					380				
Arg	Pro	Leu	Ala	Arg	Leu	Pro	Met	Leu	Gln	Thr	Leu	Arg	Leu	Gln	Met
385					390					395					400
Asn	Phe	Ile	Asn	Gln	Ala	Gln	Leu	Gly	Ile	Phe	Arg	Ala	Phe	Pro	Gly
				405					410					415	
Leu	Arg	Tyr	Val	Asp	Leu	Ser	Asp	Asn	Arg	Ile	Ser	Gly	Ala	Ser	Glu
		420					425						430		
Leu	Thr	Ala	Thr	Met	Gly	Glu	Ala	Asp	Gly	Gly	Glu	Lys	Val	Trp	Leu
		435					440					445			
Gln	Pro	Gly	Asp	Leu	Ala	Pro	Ala	Pro	Val	Asp	Thr	Pro	Ser	Ser	Glu
	450					455					460				
Asp	Phe	Arg	Pro	Asn	Cys	Ser	Thr	Leu	Asn	Phe	Thr	Leu	Asp	Leu	Ser
465				470					475						480
Arg	Asn	Asn	Leu	Val	Thr	Val	Gln	Pro	Glu	Met	Phe	Ala	Gln	Leu	Ser
			485					490						495	
His	Leu	Gln	Cys	Leu	Arg	Leu	Ser	His	Asn	Cys	Ile	Ser	Gln	Ala	Val
		500						505					510		
Asn	Gly	Ser	Gln	Phe	Leu	Pro	Leu	Thr	Gly	Leu	Gln	Val	Leu	Asp	Leu
	515						520					525			
Ser	His	Asn	Lys	Leu	Asp	Leu	Tyr	His	Glu	His	Ser	Phe	Thr	Glu	Leu
	530					535					540				
Pro	Arg	Leu	Glu	Ala	Leu	Asp	Leu	Ser	Tyr	Asn	Ser	Gln	Pro	Phe	Gly
545					550					555					560
Met	Gln	Gly	Val	Gly	His	Asn	Phe	Ser	Phe	Val	Ala	His	Leu	Arg	Thr
			565					570						575	
Leu	Arg	His	Leu	Ser	Leu	Ala	His	Asn	Asn	Ile	His	Ser	Gln	Val	Ser
		580						585					590		
Gln	Gln	Leu	Cys	Ser	Thr	Ser	Leu	Arg	Ala	Leu	Asp	Phe	Ser	Gly	Asn
		595						600				605			
Ala	Leu	Gly	His	Met	Trp	Ala	Glu	Gly	Asp	Leu	Tyr	Leu	His	Phe	Phe
	610					615					620				
Gln	Gly	Leu	Ser	Gly	Leu	Ile	Trp	Leu	Asp	Leu	Ser	Gln	Asn	Arg	Leu
625					630					635					640
His	Thr	Leu	Leu	Pro	Gln	Thr	Leu	Arg	Asn	Leu	Pro	Lys	Ser	Leu	Gln
			645						650					655	
Val	Leu	Arg	Leu	Arg	Asp	Asn	Tyr	Leu	Ala	Phe	Phe	Lys	Trp	Trp	Ser
		660						665					670		
Leu	His	Phe	Leu	Pro	Lys	Leu	Glu	Val	Leu	Asp	Leu	Ala	Gly	Asn	Gln
		675					680					685			
Leu	Lys	Ala	Leu	Thr	Asn	Gly	Ser	Leu	Pro	Ala	Gly	Thr	Arg	Leu	Arg
	690					695						700			

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Arg Leu Asp Val Ser Cys Asn Ser Ile Ser Phe Val Ala Pro Gly Phe
 705 710 715 720
 Phe Ser Lys Ala Lys Glu Leu Arg Glu Leu Asn Leu Ser Ala Asn Ala
 725 730 735
 Leu Lys Thr Val Asp His Ser Trp Phe Gly Pro Leu Ala Ser Ala Leu
 740 745 750
 Gln Ile Leu Asp Val Ser Ala Asn Pro Leu His Cys Ala Cys Gly Ala
 755 760 765
 Ala Phe Met Asp Phe Leu Leu Glu Val Gln Ala Ala Val Pro Gly Leu
 770 775 780
 Pro Ser Arg Val Lys Cys Gly Ser Pro Gly Gln Leu Gln Gly Leu Ser
 785 790 795 800
 Ile Phe Ala Gln Asp Leu Arg Leu Cys Leu Asp Glu Ala Leu Ser Trp
 805 810 815
 Asp Cys Phe Ala Leu Ser Leu Leu Ala Val Ala Leu Gly Leu Gly Val
 820 825 830
 Pro Met Leu His His Leu Cys Gly Trp Asp Leu Trp Tyr Cys Phe His
 835 840 845
 Leu Cys Leu Ala Trp Leu Pro Trp Arg Gly Arg Gln Ser Gly Arg Asp
 850 855 860
 Glu Asp Ala Leu Pro Tyr Asp Ala Phe Val Val Phe Asp Lys Thr Gln
 865 870 875 880
 Ser Ala Val Ala Asp Trp Val Tyr Asn Glu Leu Arg Gly Gln Leu Glu
 885 890 895
 Glu Cys Arg Gly Arg Trp Ala Leu Arg Leu Cys Leu Glu Glu Arg Asp
 900 905 910
 Trp Leu Pro Gly Lys Thr Leu Phe Glu Asn Leu Trp Ala Ser Val Tyr
 915 920 925
 Gly Ser Arg Lys Thr Leu Phe Val Leu Ala His Thr Asp Arg Val Ser
 930 935 940
 Gly Leu Leu Arg Ala Ser Phe Leu Leu Ala Gln Gln Arg Leu Leu Glu
 945 950 955 960
 Asp Arg Lys Asp Val Val Val Leu Val Ile Leu Ser Pro Asp Gly Arg
 965 970 975
 Arg Ser Arg Tyr Val Arg Leu Arg Gln Arg Leu Cys Arg Gln Ser Val
 980 985 990
 Leu Leu Trp Pro His Gln Pro Ser Gly Gln Arg Ser Phe Trp Ala Gln
 995 1000 1005
 Leu Gly Met Ala Leu Thr Arg Asp Asn His His Phe Tyr Asn Arg
 1010 1015 1020
 Asn Phe Cys Gln Gly Pro Thr Ala Glu
 1025 1030

What is claimed is:

1. A method of determining bacterial virulence in an oral cavity comprising:

- a. providing a reporter cell expressing at least one toll-like receptor;
- b. providing a sample of oral matter;
- c. combining the sample of oral matter and the reporter cell;
- d. measuring the toll-like receptor activation;
- e. providing another sample of oral matter from an individual who used an oral care composition prior to providing another sample of oral matter;

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60

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f. combining the another sample of oral matter and the reporter cell;

g. measuring the toll-like receptor response;

h. comparing the toll-like receptor response from the sample of oral matter and another sample of oral matter.

2. The method of claim 1, wherein the toll-like receptor is at least one of TLR1 to TLR9.

3. The method of claim 1, wherein the reporter cell expresses at least one TLR2 or TLR4.

4. The method of claim 1, wherein the toll-like receptor activates a reporter gene.

5. The method of claim 4, wherein the reporter gene is a secreted embryonic alkaline phosphatase (SEAP) reporter gene.

6. The method of claim 1, wherein the sample of oral matter is at least one of gum-line plaque, subgingival plaque, 5 supragingival plaque, interstitial plaque, gingival crevicular fluid (GCF), gingival biopsy, saliva, or tongue swab.

7. The method of claim 1, wherein the sample of oral matter comprises virulence factor that is at least one of Gram positive cell wall components, Gram negative cell wall 10 components, bacterial DNA, flagellin, peptidoglycan, bacterial metabolites, fimbriae; outer membrane vesicles bacterial proteins, or bacterial enzymes.

8. The method of claim 7, wherein the sample of oral matter comprises virulence factor that is at least one of 15 lipopolysaccharide or lipoteichoic acid.

9. The method of claim 1, where the sample of oral matter and the another sample of oral matter are from the same individual.

* * * * *