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(12) **United States Patent**  
**Haught et al.**

(10) **Patent No.:** **US 10,156,566 B2**  
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(54) **DETOXIFICATION OF MICROBIAL  
VIRULENCE FACTORS IN ORAL CAVITY**

(71) Applicant: **The Procter & Gamble Company**,  
Cincinnati, OH (US)

(72) Inventors: **John Christian Haught**, West Chester,  
OH (US); **Sancai Xie**, Liberty  
Township, OH (US); **Cheryl Sue  
Tansky**, Forest Park, OH (US); **Yogita  
Vinod Mantri**, Cincinnati, OH (US);  
**Thomas Glenn Huggins**, Mason, OH  
(US); **Donald James White**, Fairfield  
Township, OH (US)

(73) Assignee: **The Procter & Gamble Company**,  
Cincinnati, OH (US)

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patent is extended or adjusted under 35  
U.S.C. 154(b) by 36 days.

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**Related U.S. Application Data**

(60) Provisional application No. 62/309,110, filed on Mar.  
16, 2016, provisional application No. 62/157,671,  
filed on May 6, 2015, provisional application No.  
62/157,659, filed on May 6, 2015.

(51) **Int. Cl.**

**G01N 33/50** (2006.01)  
**G01N 33/566** (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);  
**C12Q 2600/136** (2013.01); **G01N 2333/195**  
(2013.01); **G01N 2333/705** (2013.01); **G01N**  
**2333/70596** (2013.01); **G01N 2400/50**  
(2013.01)

(58) **Field of Classification Search**

CPC ..... **G01N 33/5041**; **G01N 2333/195**; **G01N**  
**2333/70596**; **G01N 33/5044**; **G01N**  
**33/5091**; **G01N 2400/50**; **C12Q 1/6897**;  
**C12Q 2600/136**

See application file for complete search history.

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*Primary Examiner* — Christopher Adam Hixson

(74) *Attorney, Agent, or Firm* — Parker D. McCrary;  
James E. Oehlenschlager

(57) **ABSTRACT**

A method for identifying constituents for detoxifying oral  
virulence factors and a method for determining the effec-  
tiveness of detoxifying agents.

**7 Claims, 44 Drawing Sheets**

**Specification includes a Sequence Listing.**

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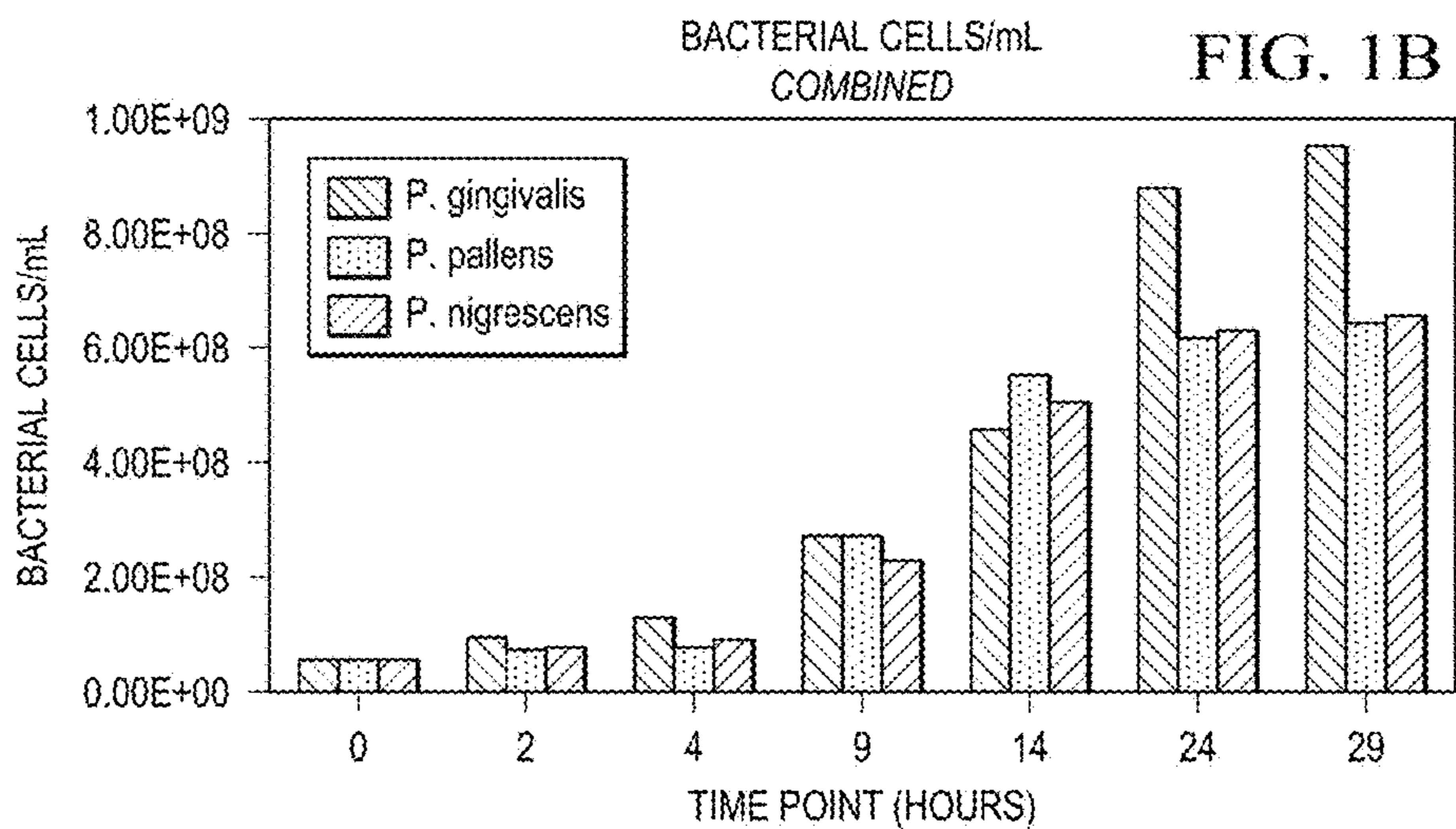
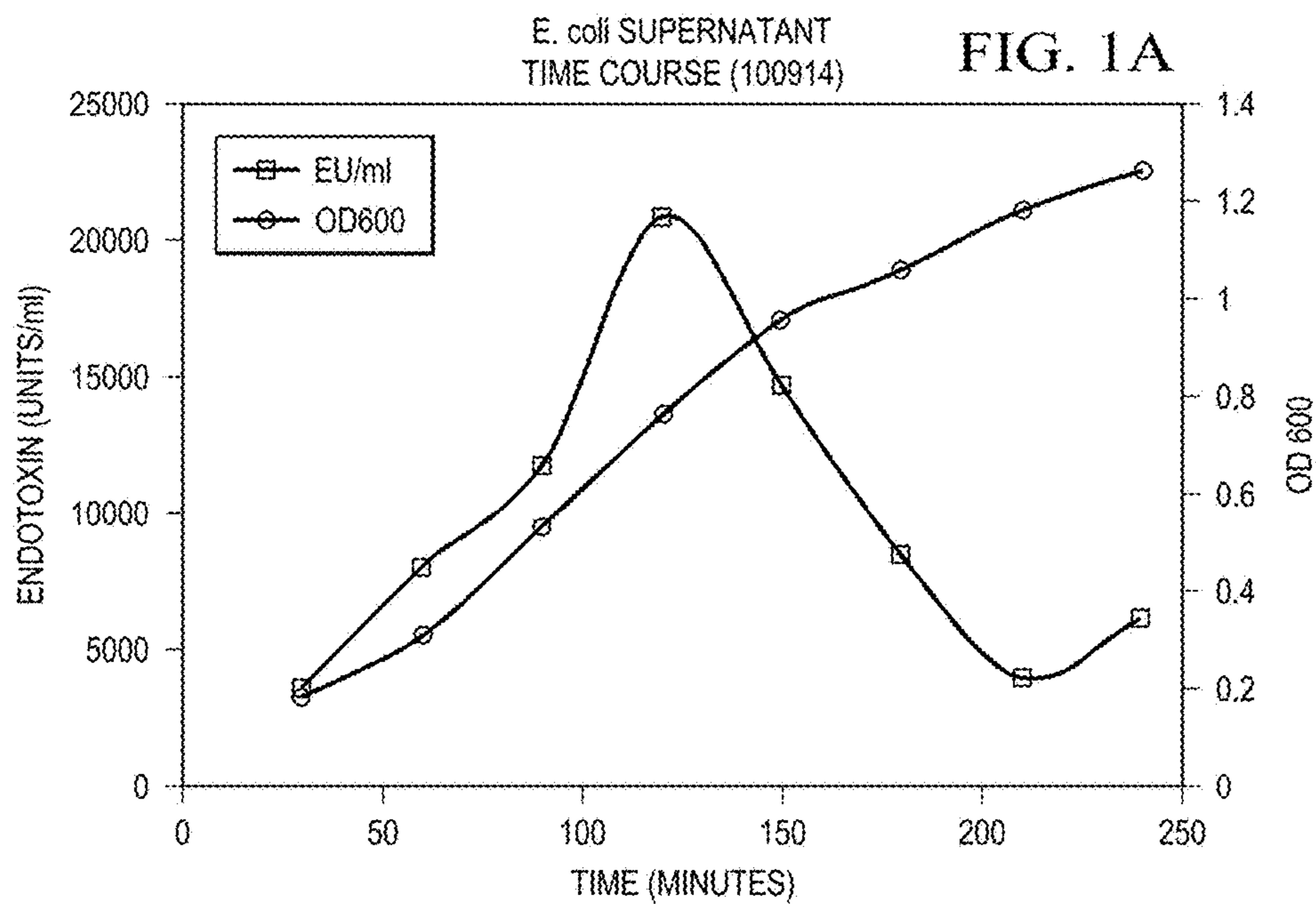
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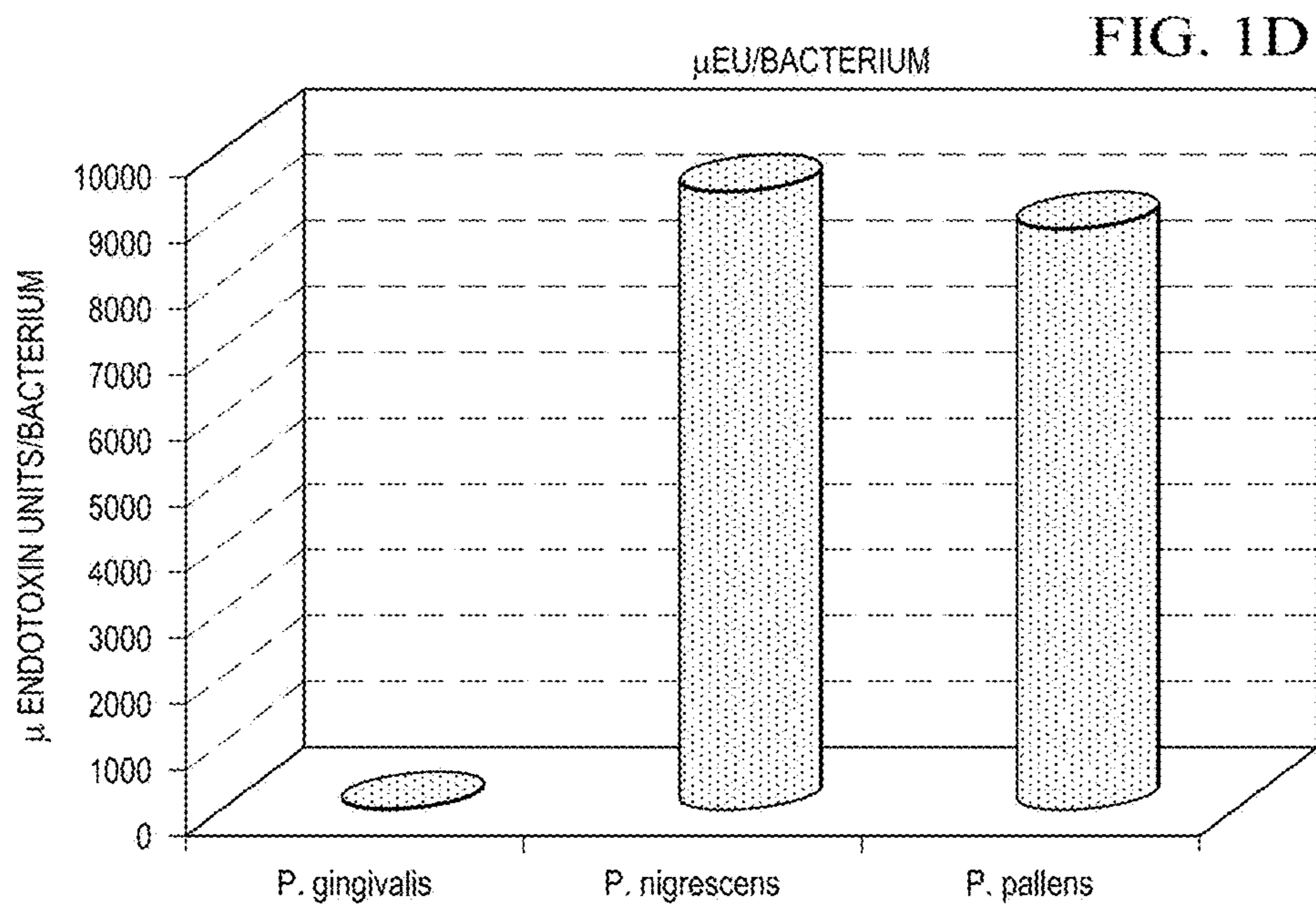
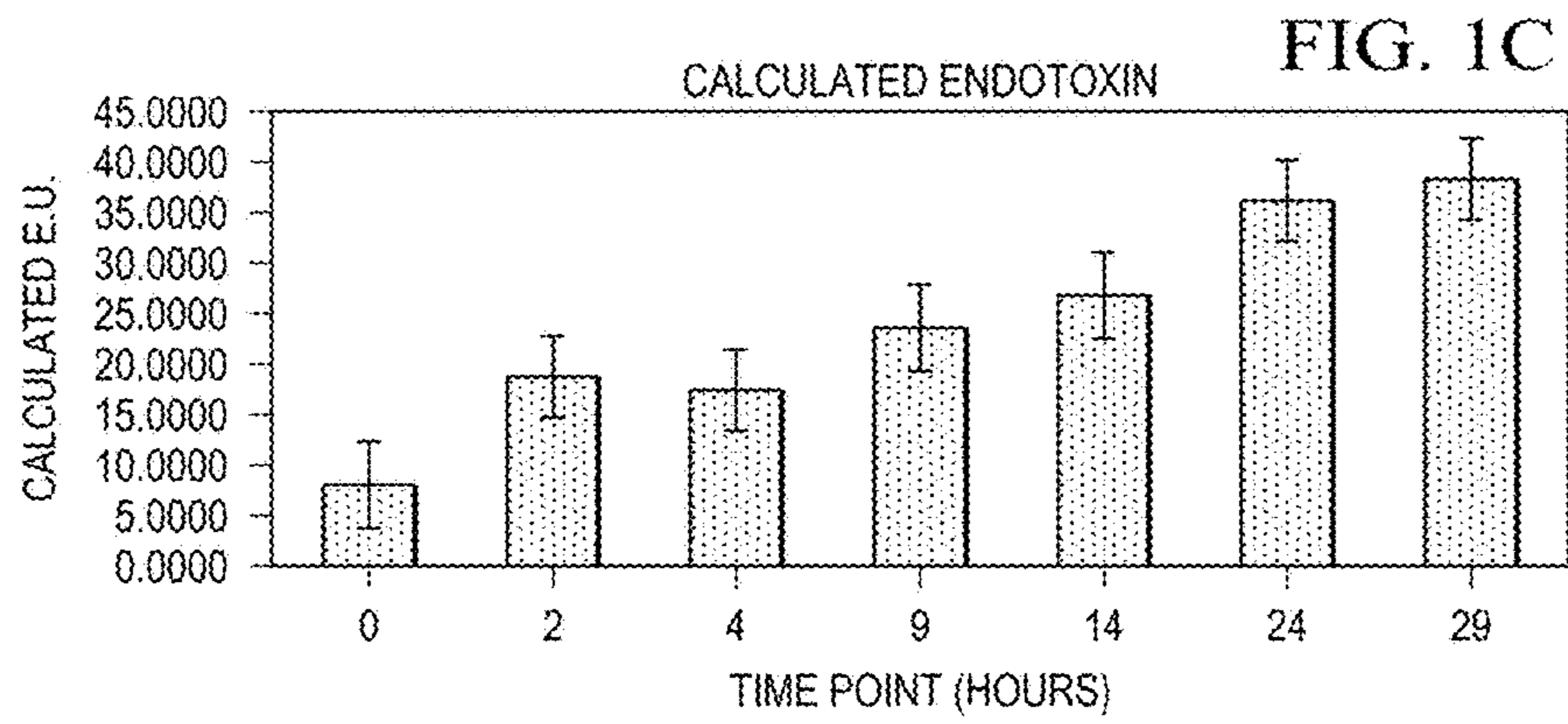
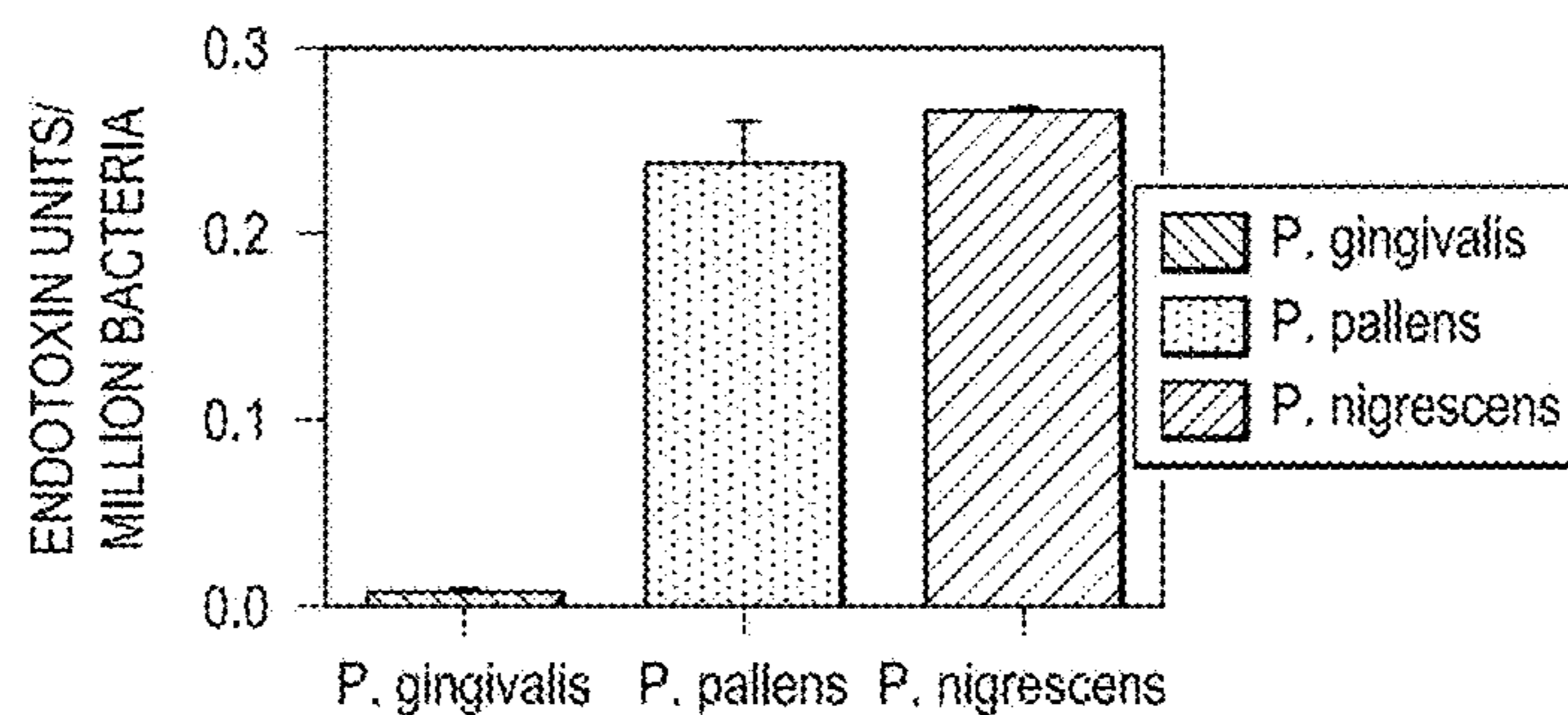
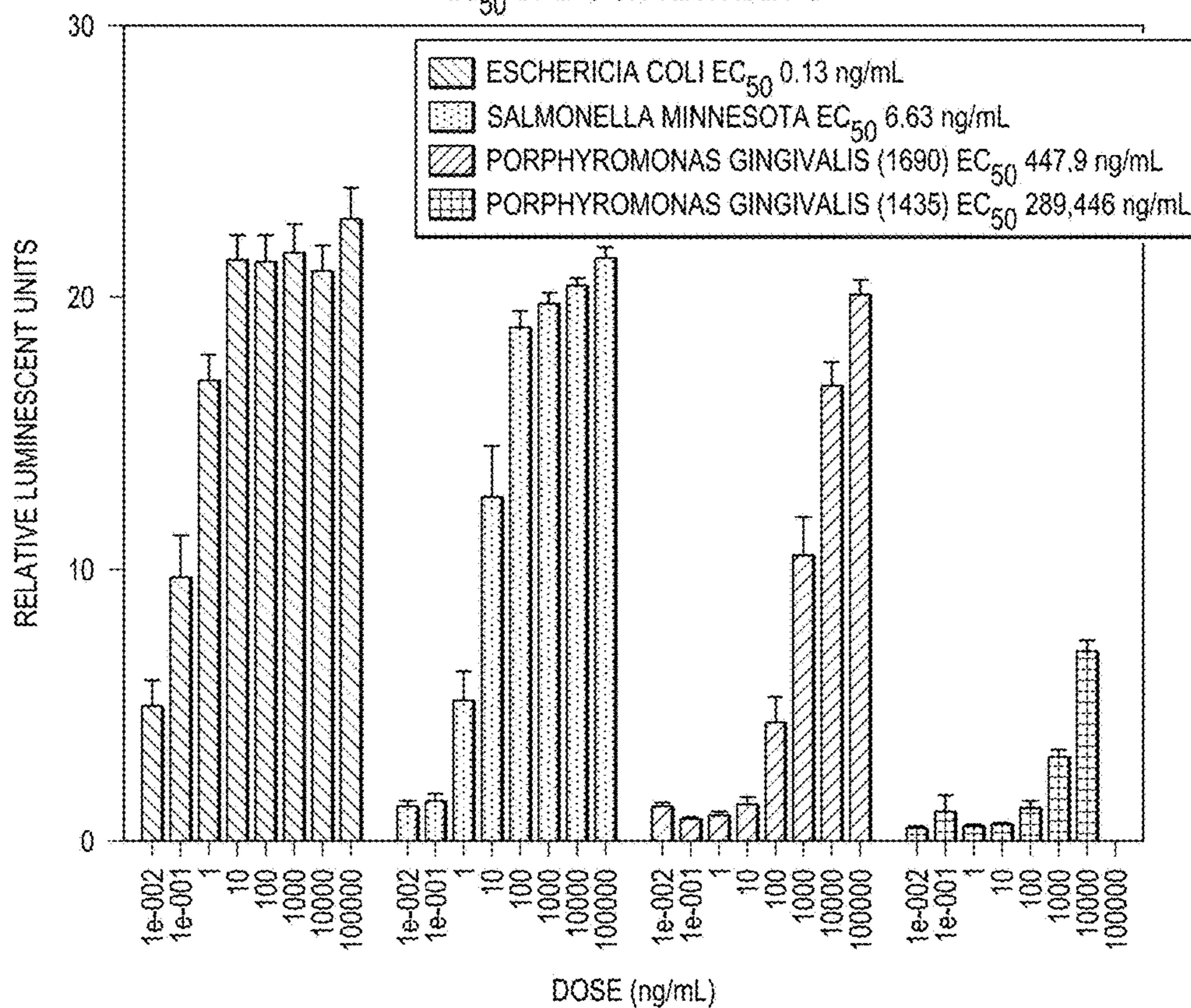


FIG. 1E



EC<sub>50</sub> OF LPS ON TLR4 ASSAYS

FIG. 2A



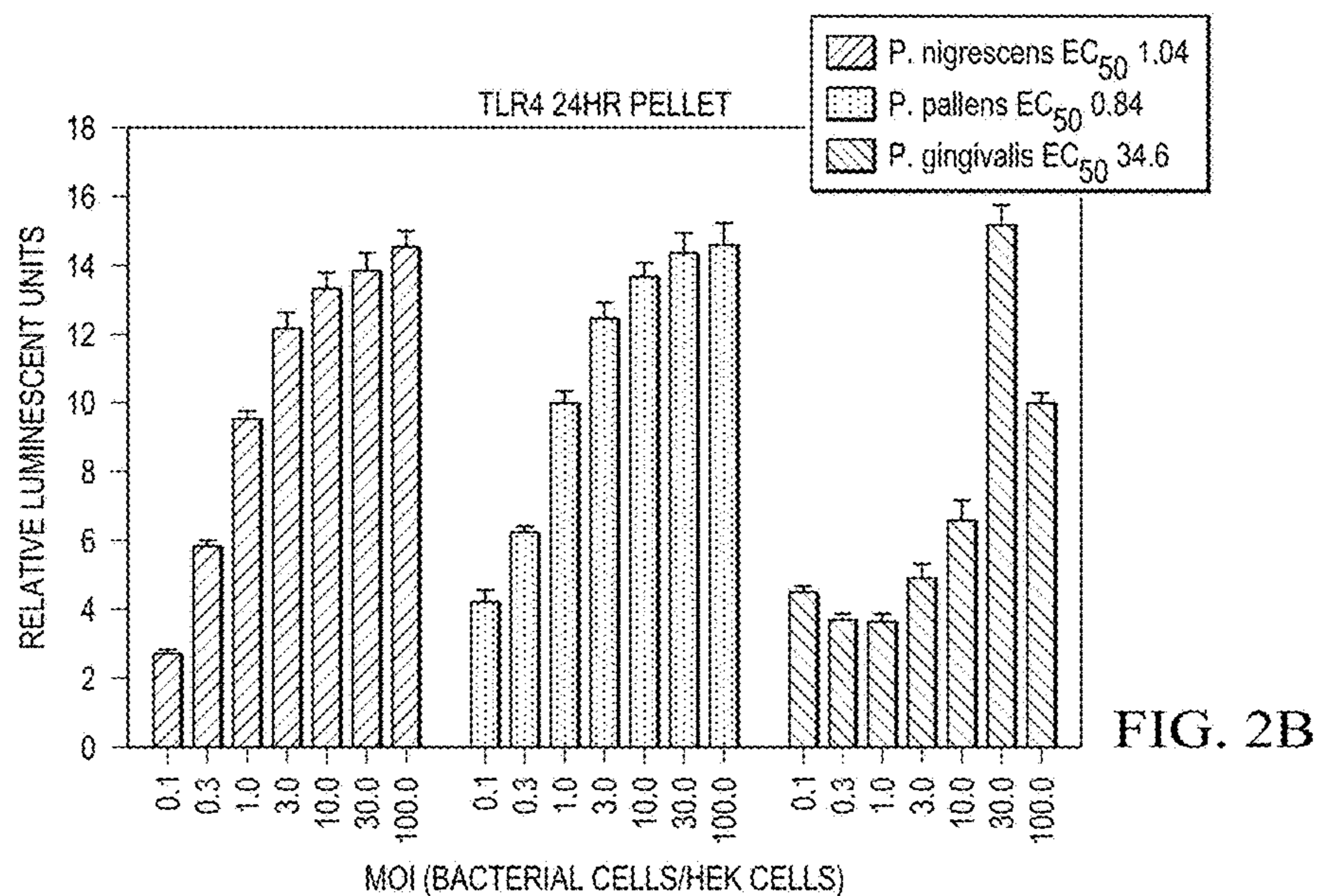


FIG. 2B

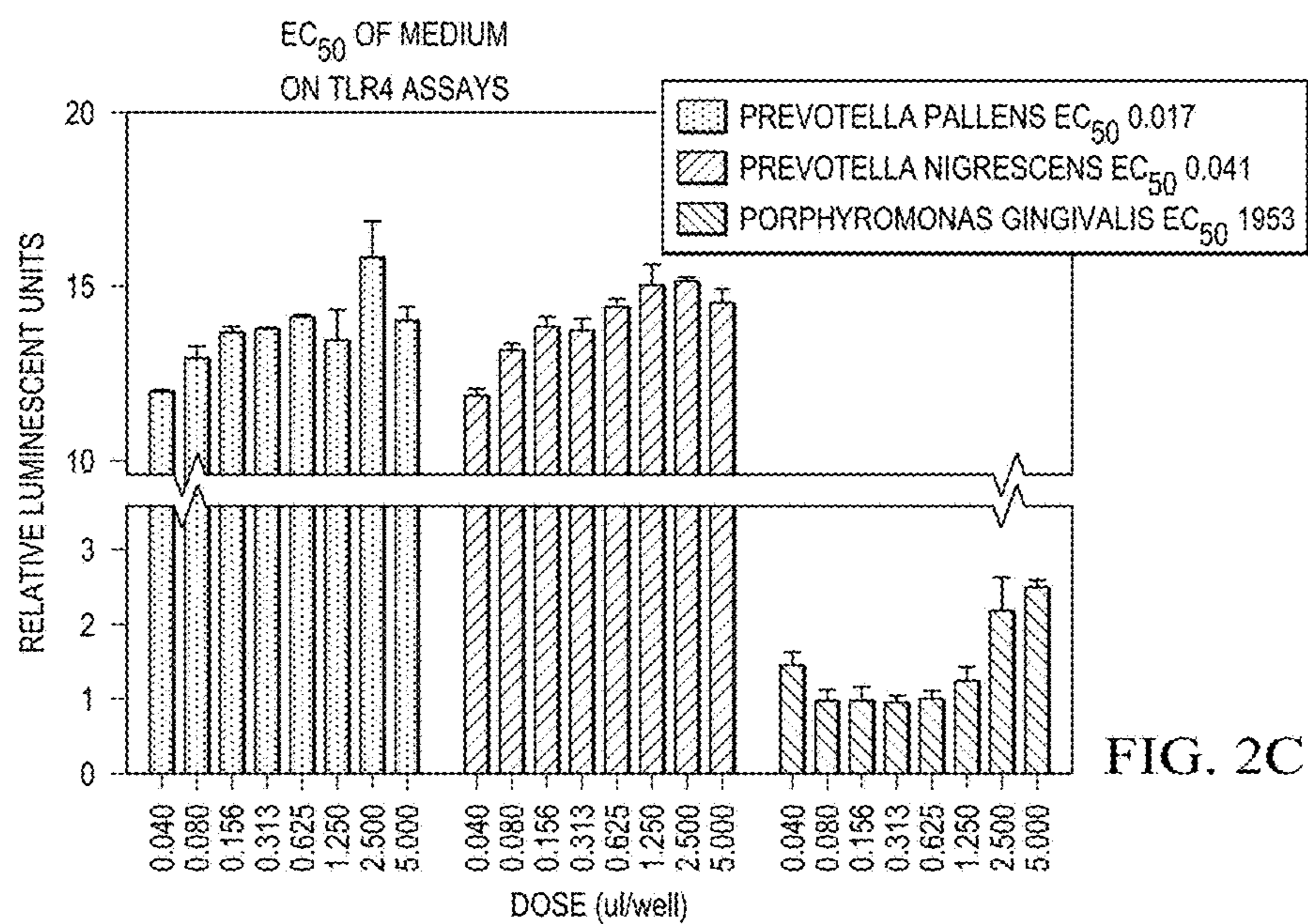
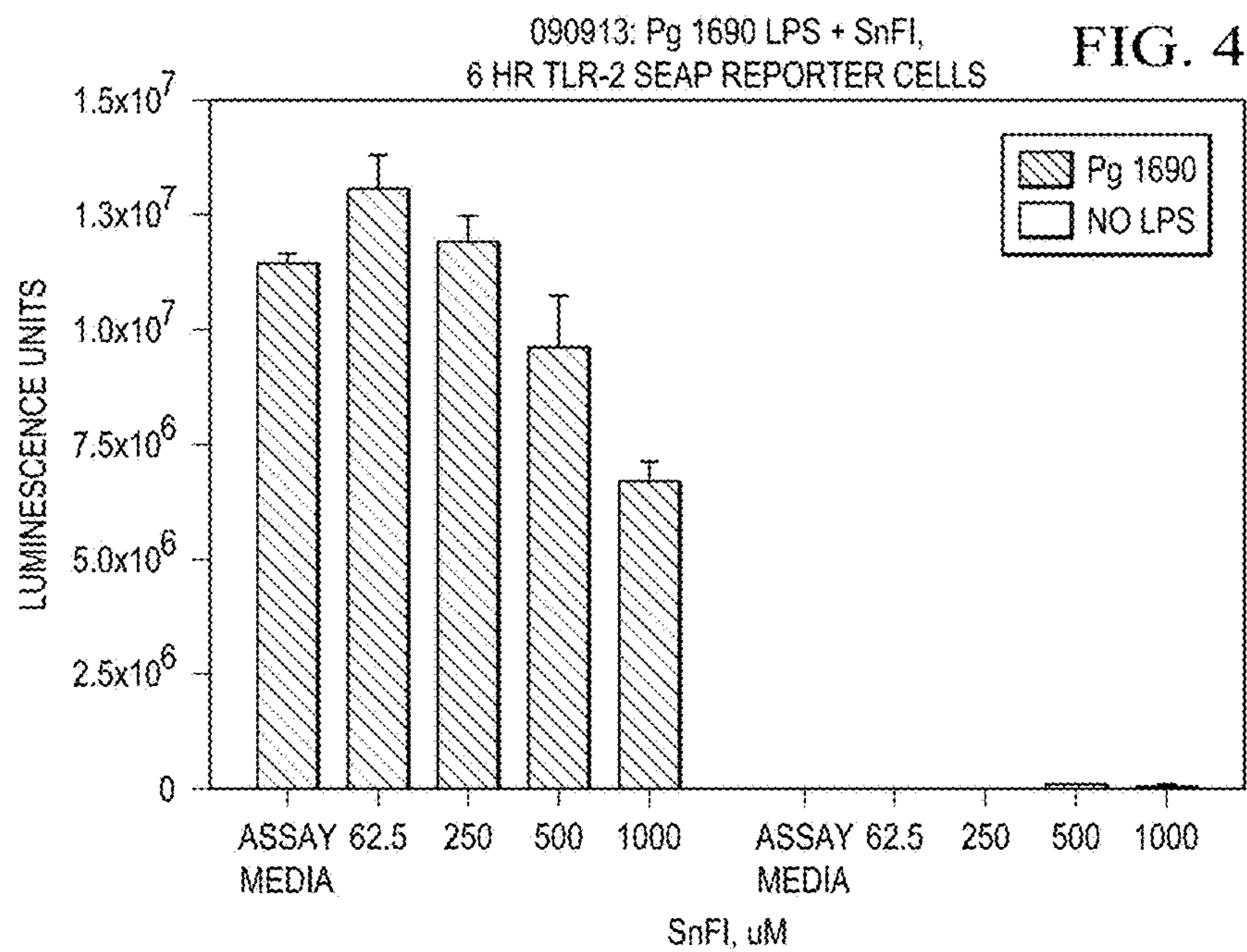
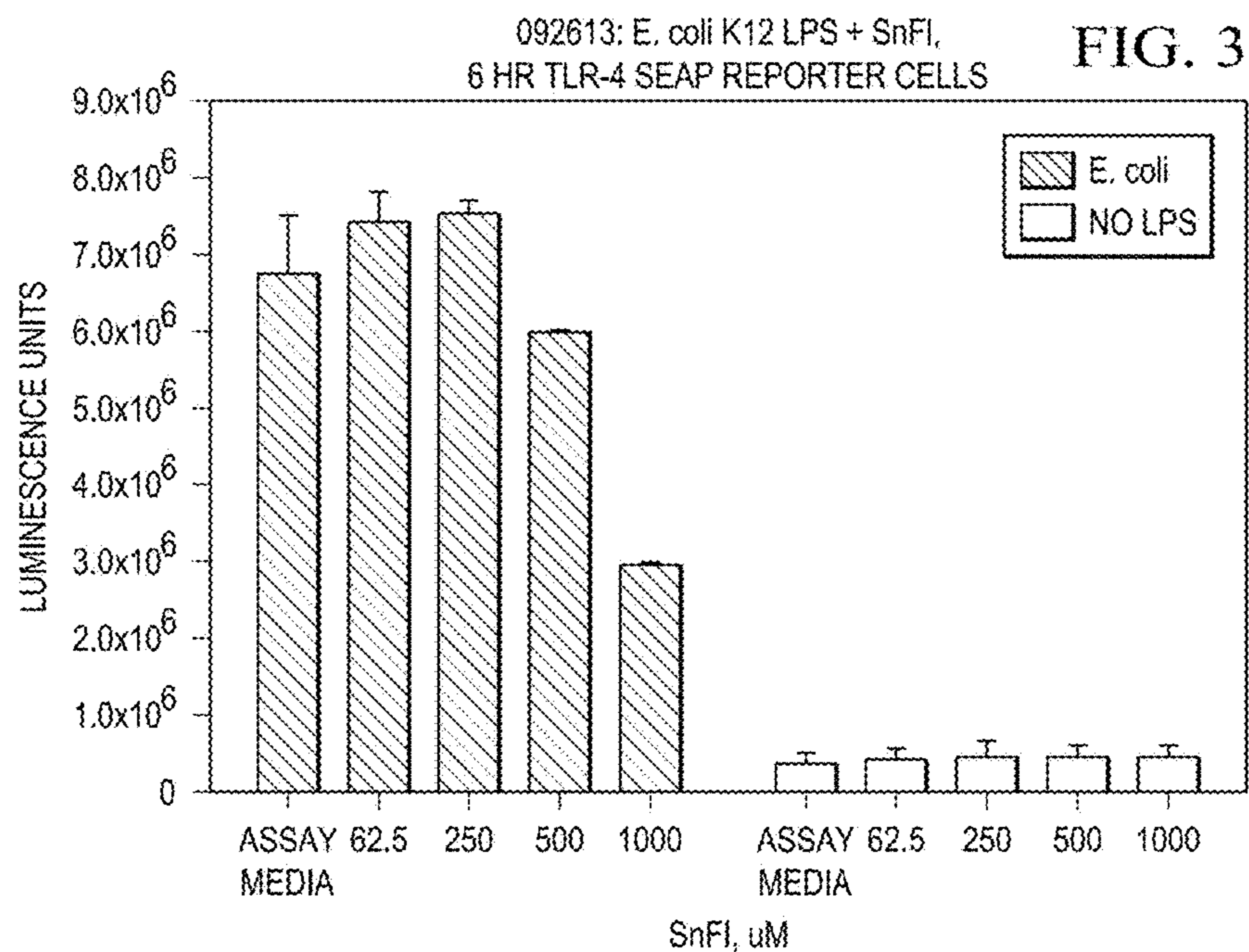


FIG. 2C



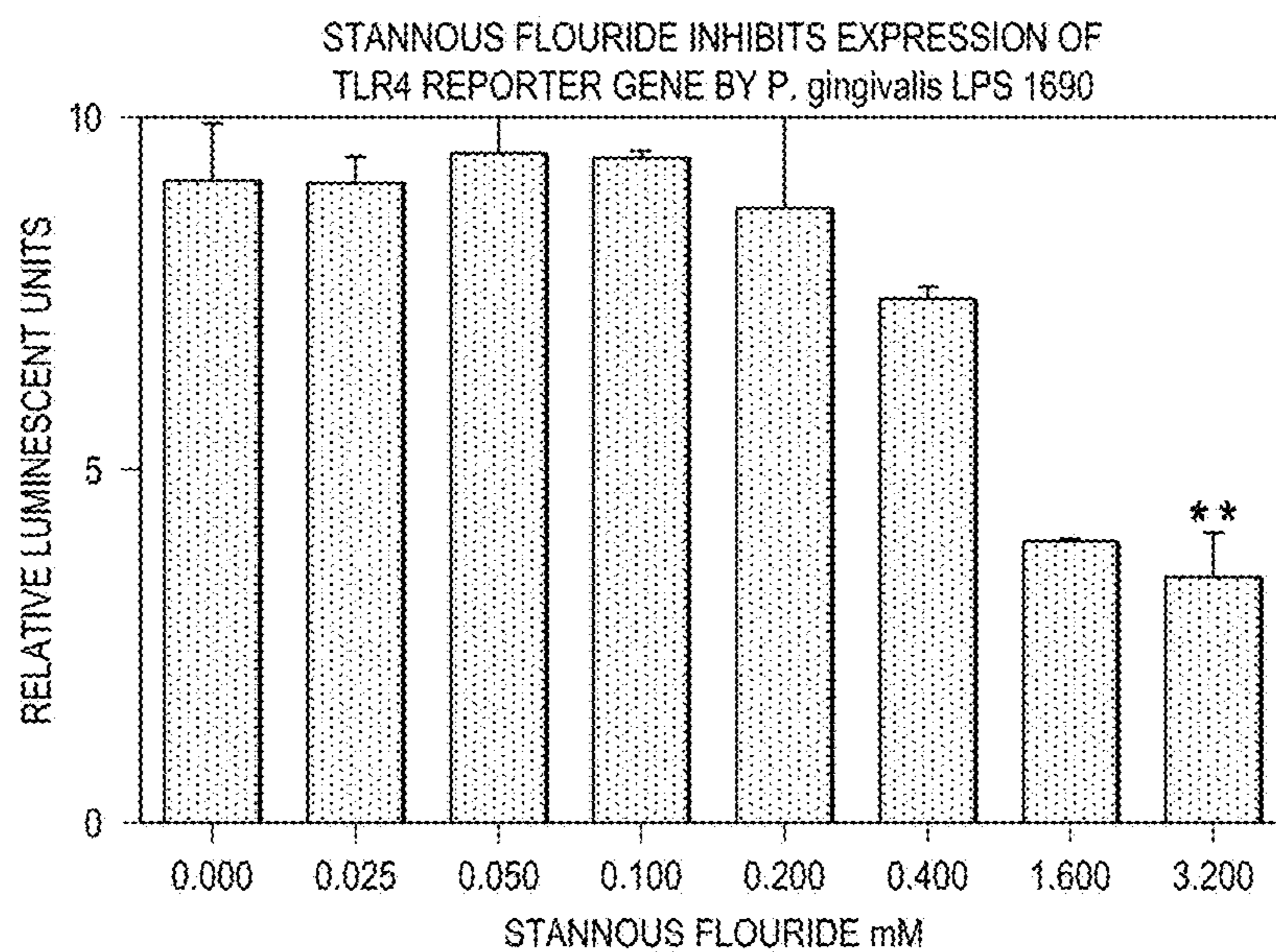


FIG. 5

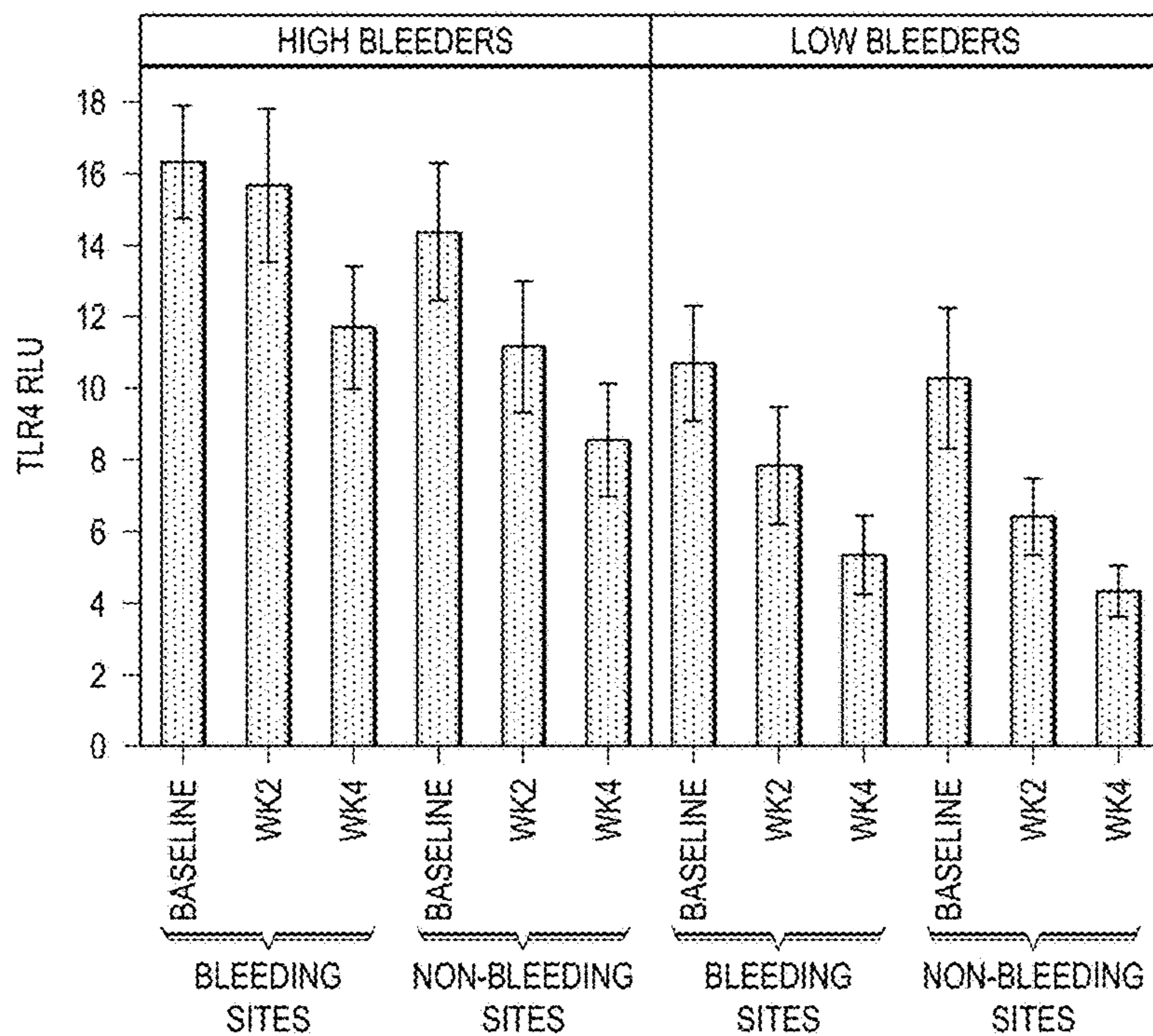


FIG. 7



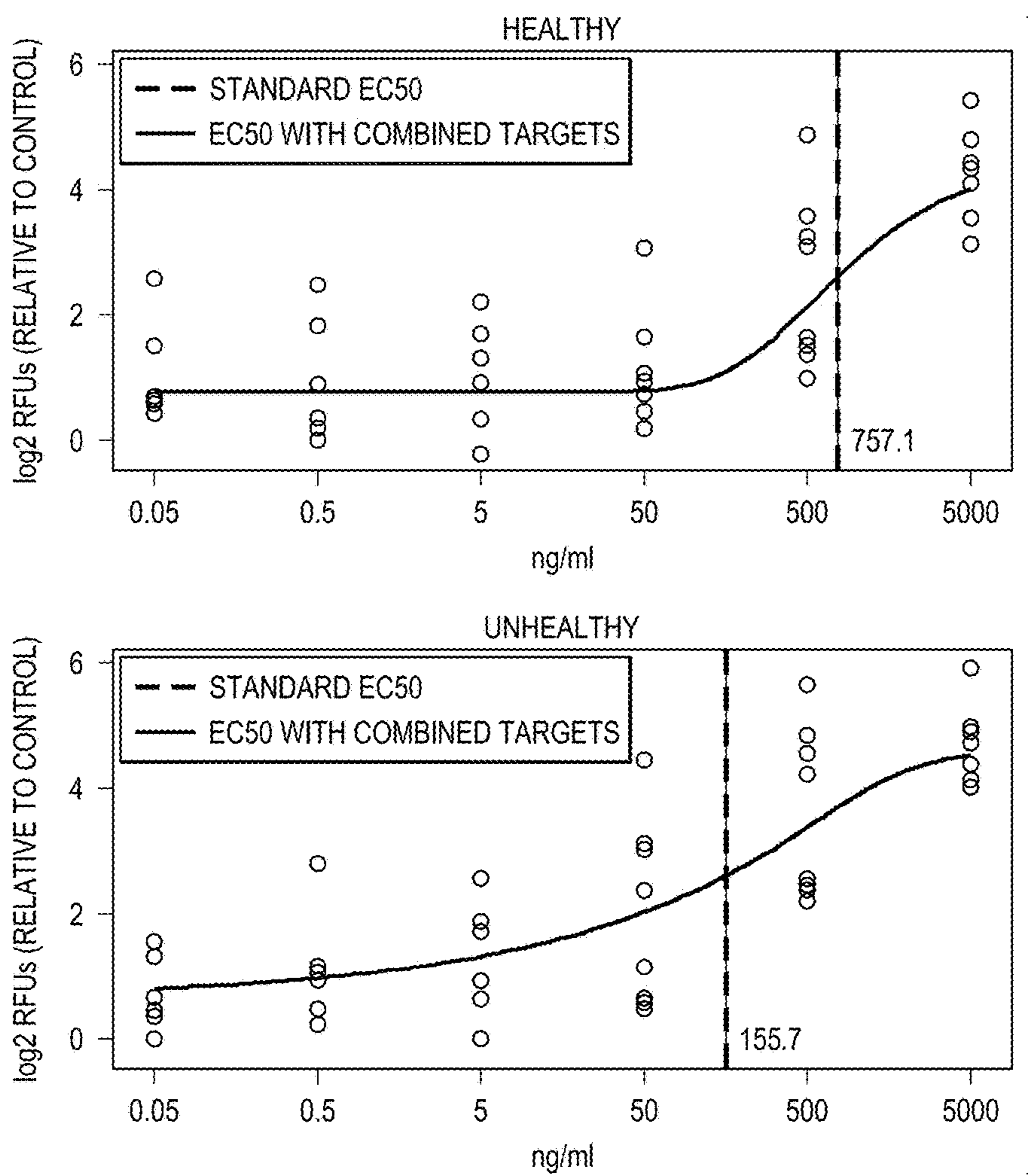


FIG. 6

FIG. 8A

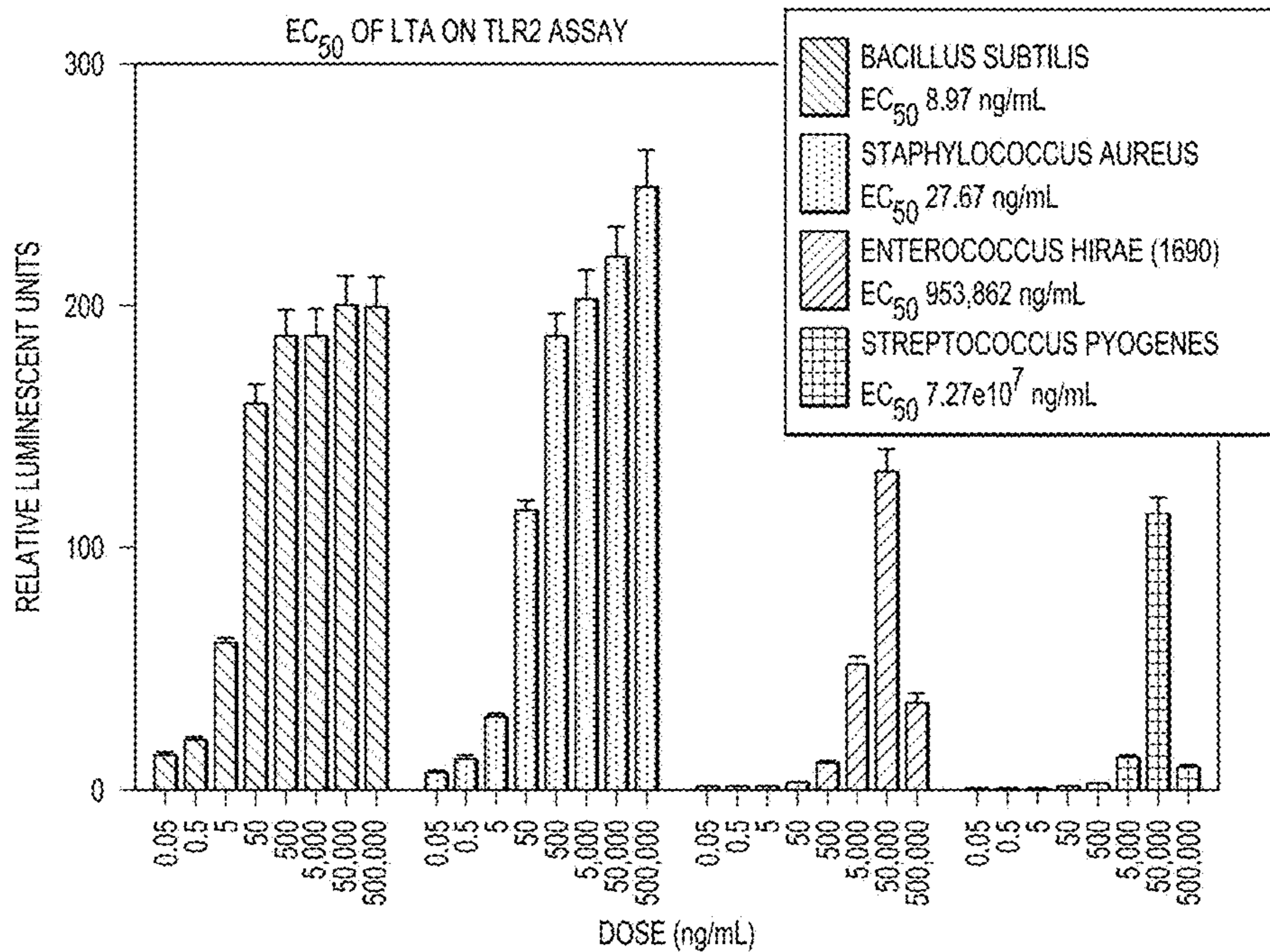
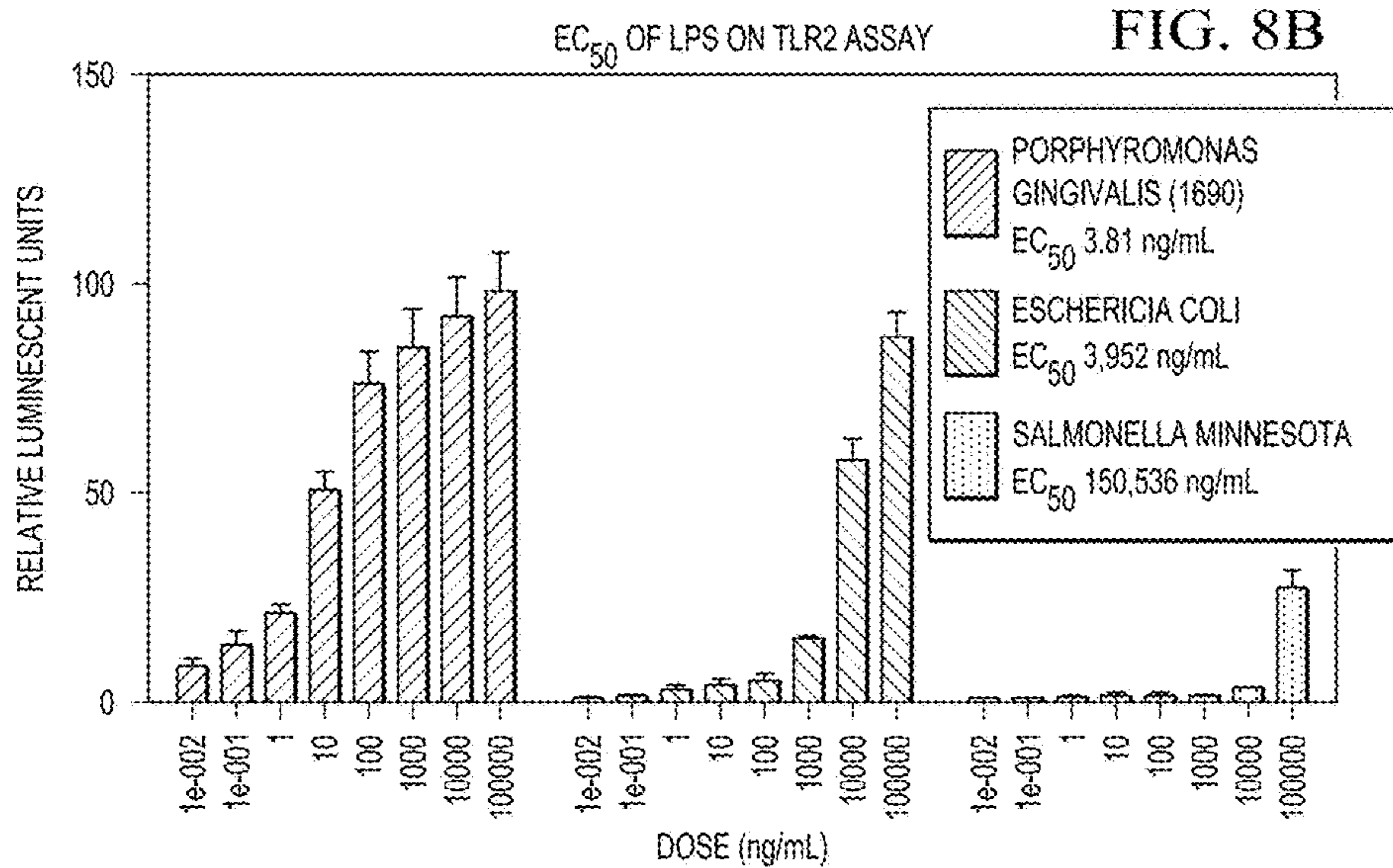


FIG. 8B



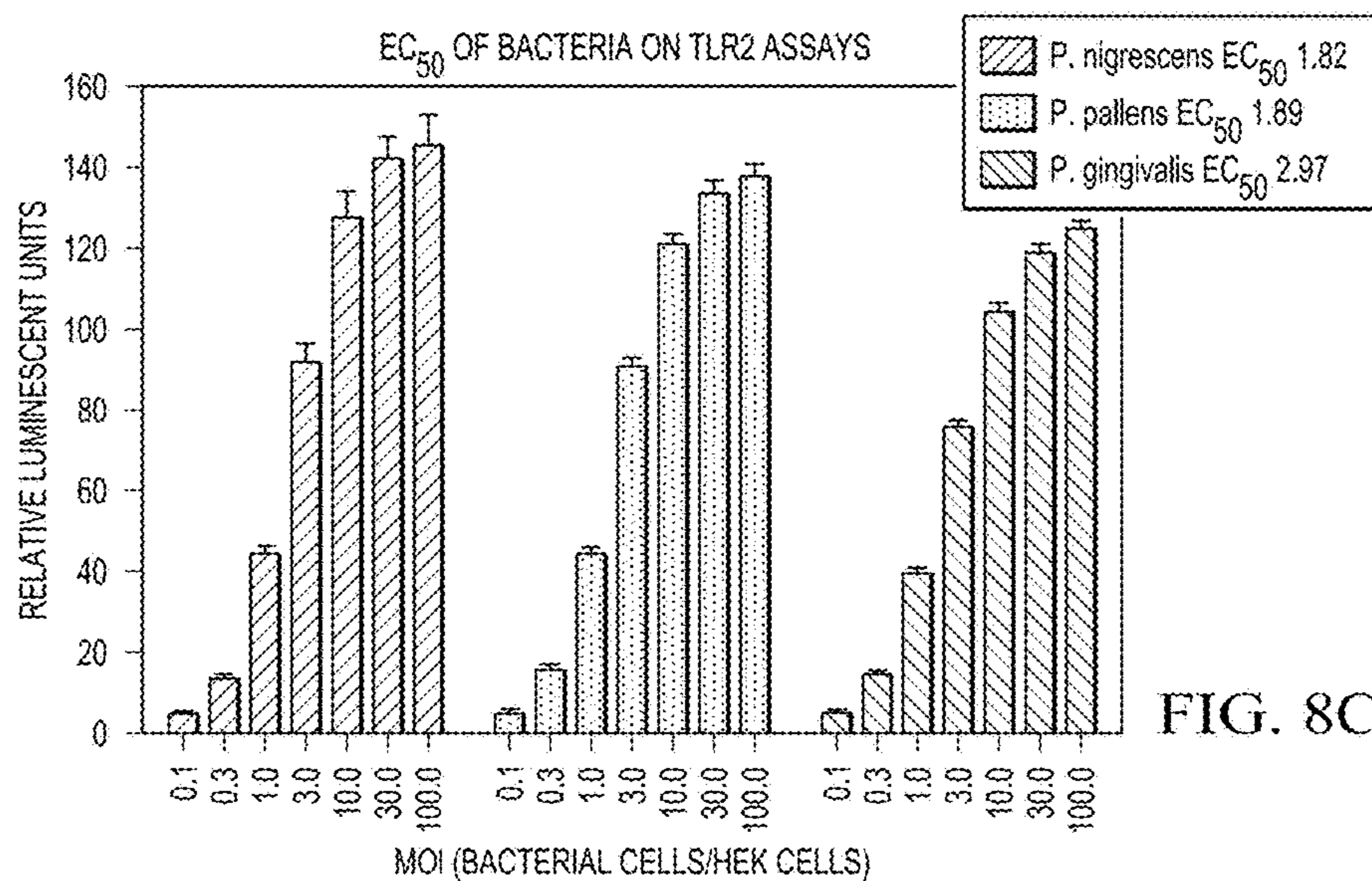


FIG. 8C

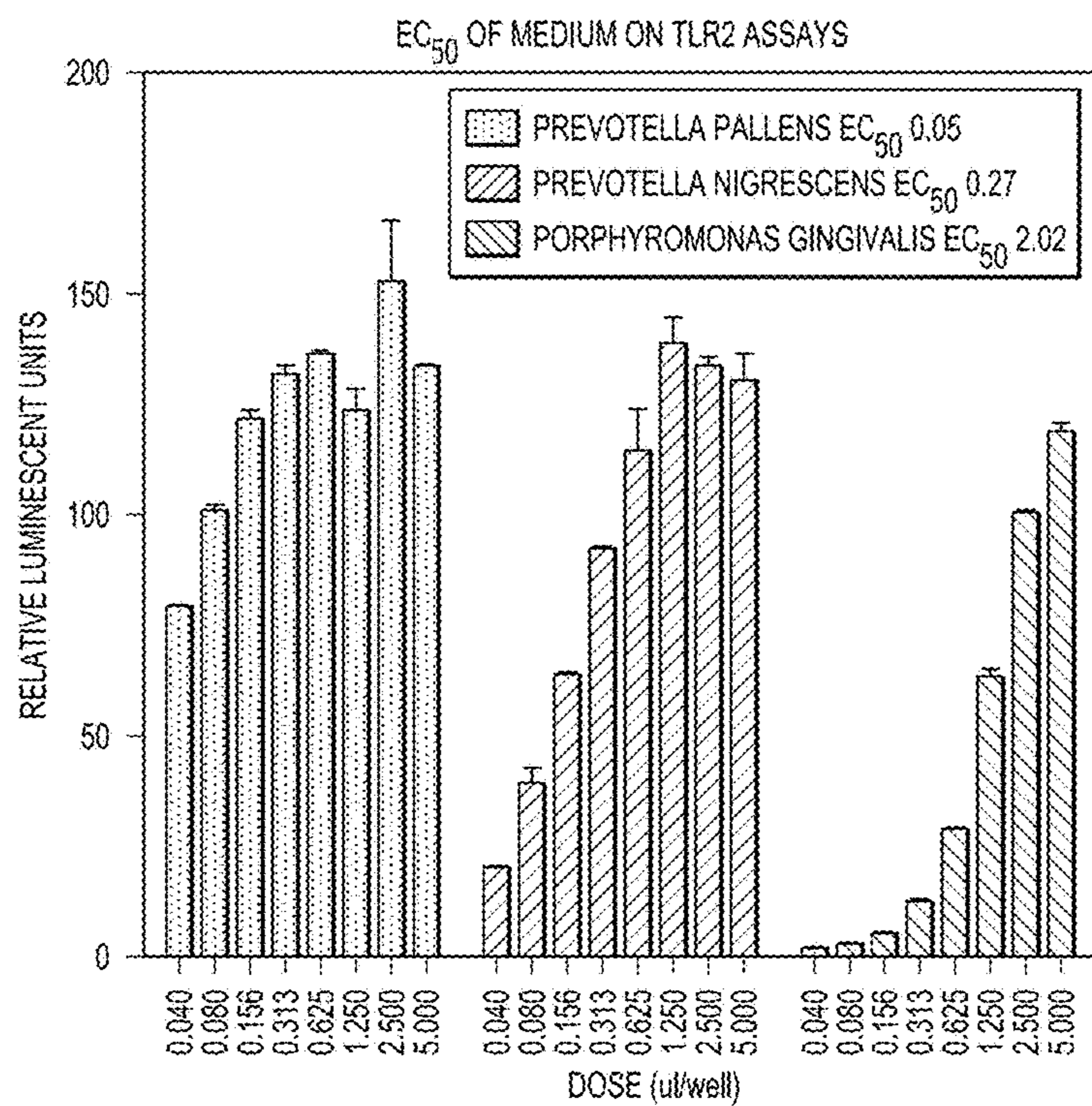


FIG. 8D

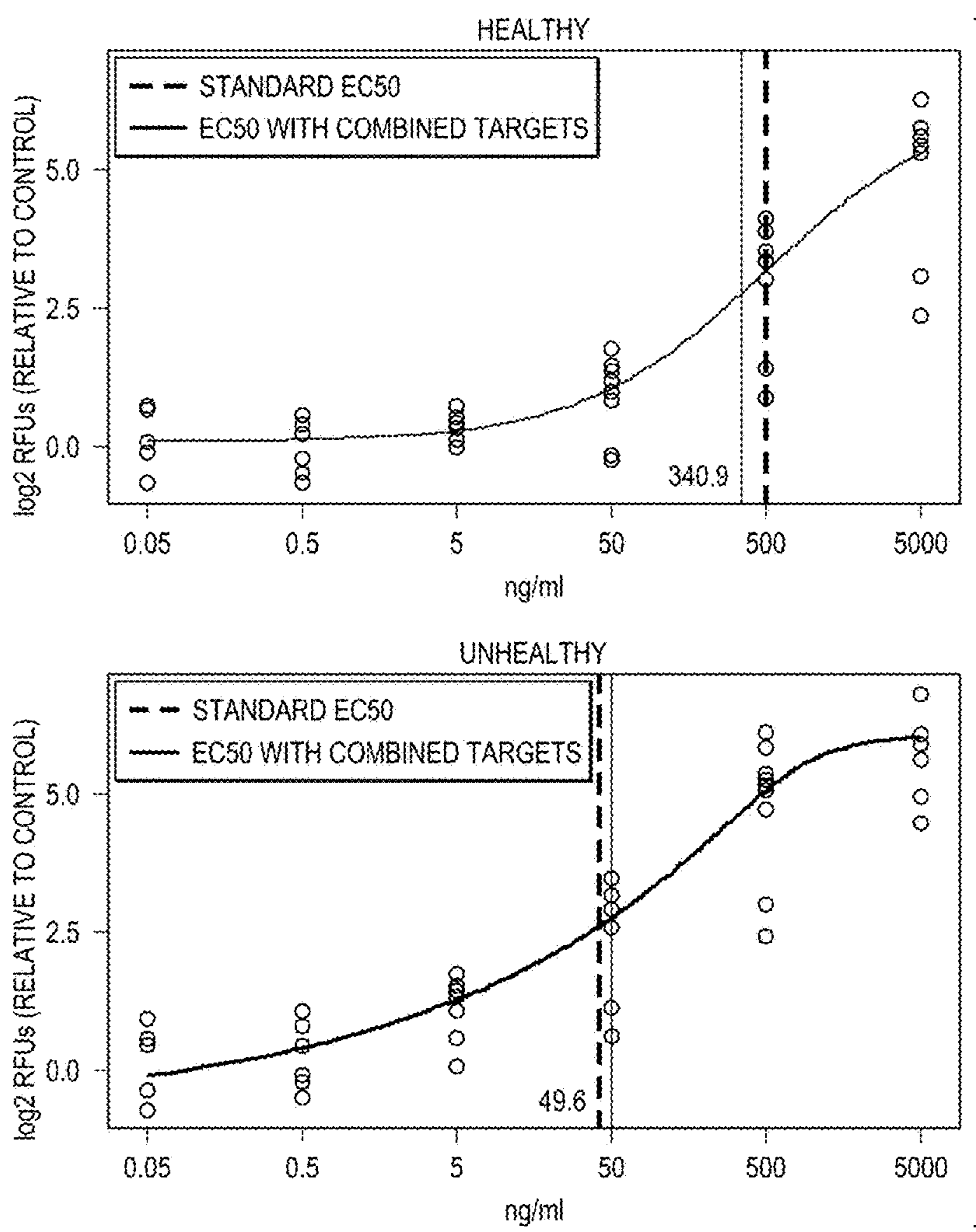


FIG. 9

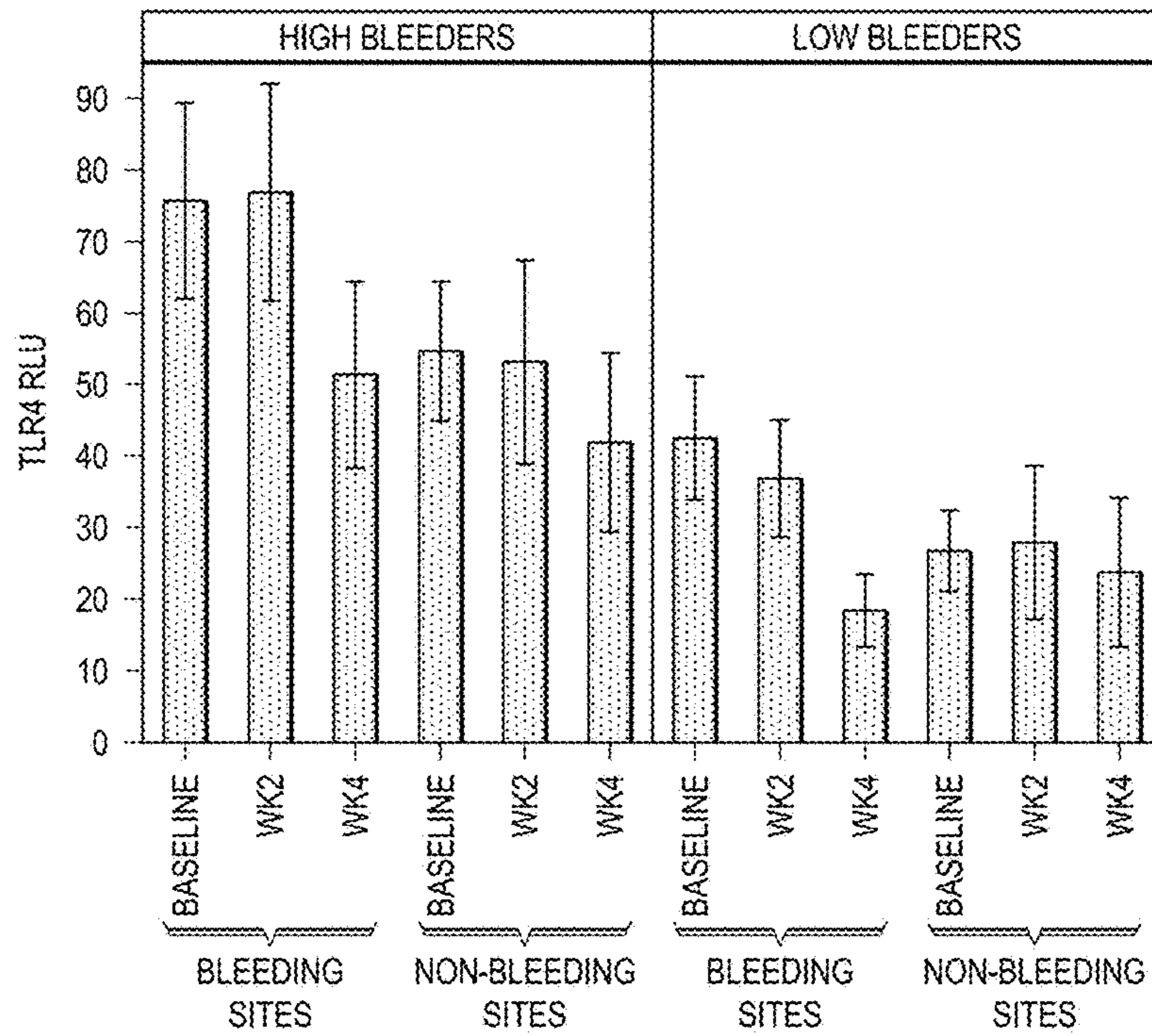


FIG. 10

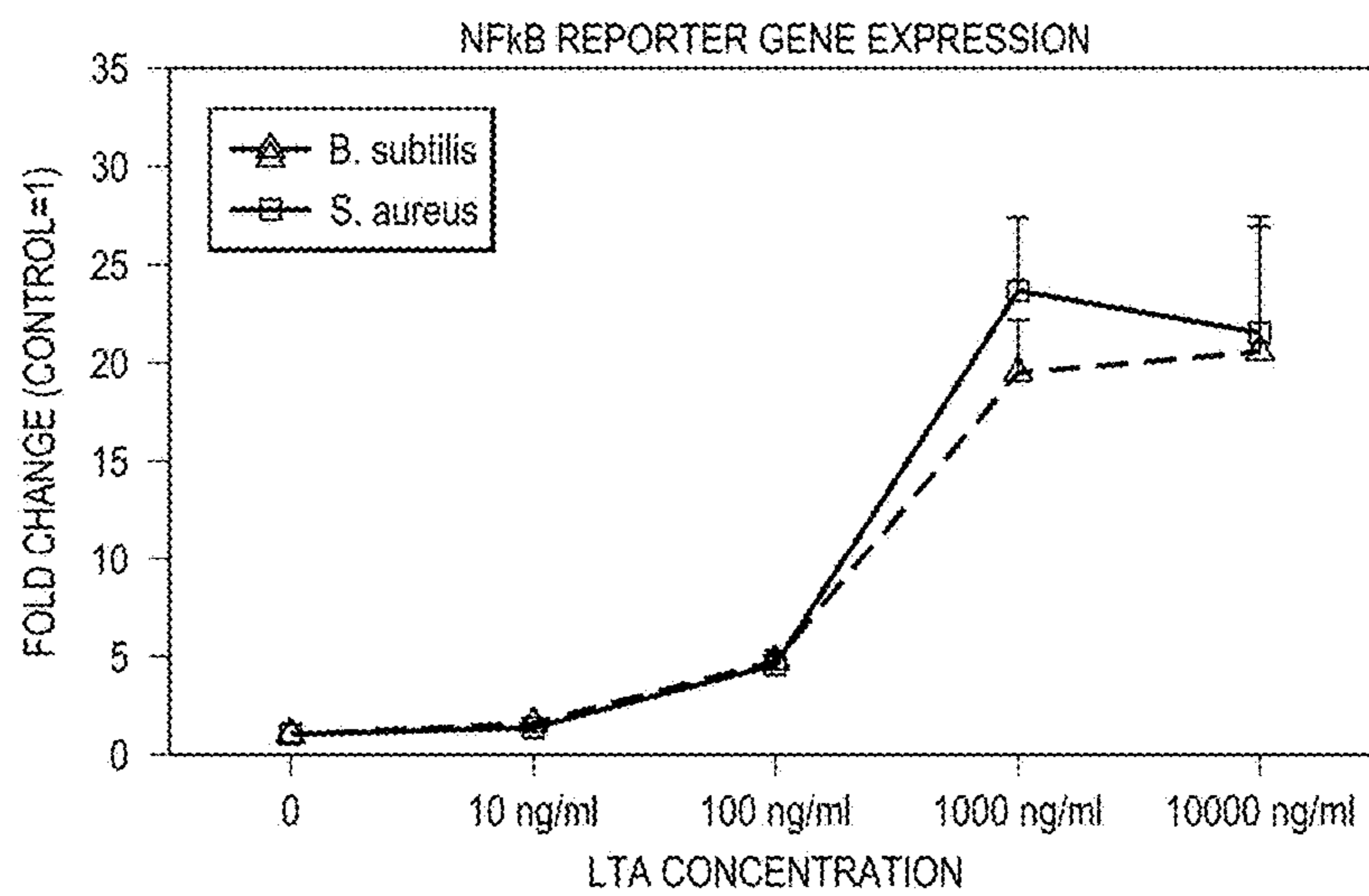


FIG. 13

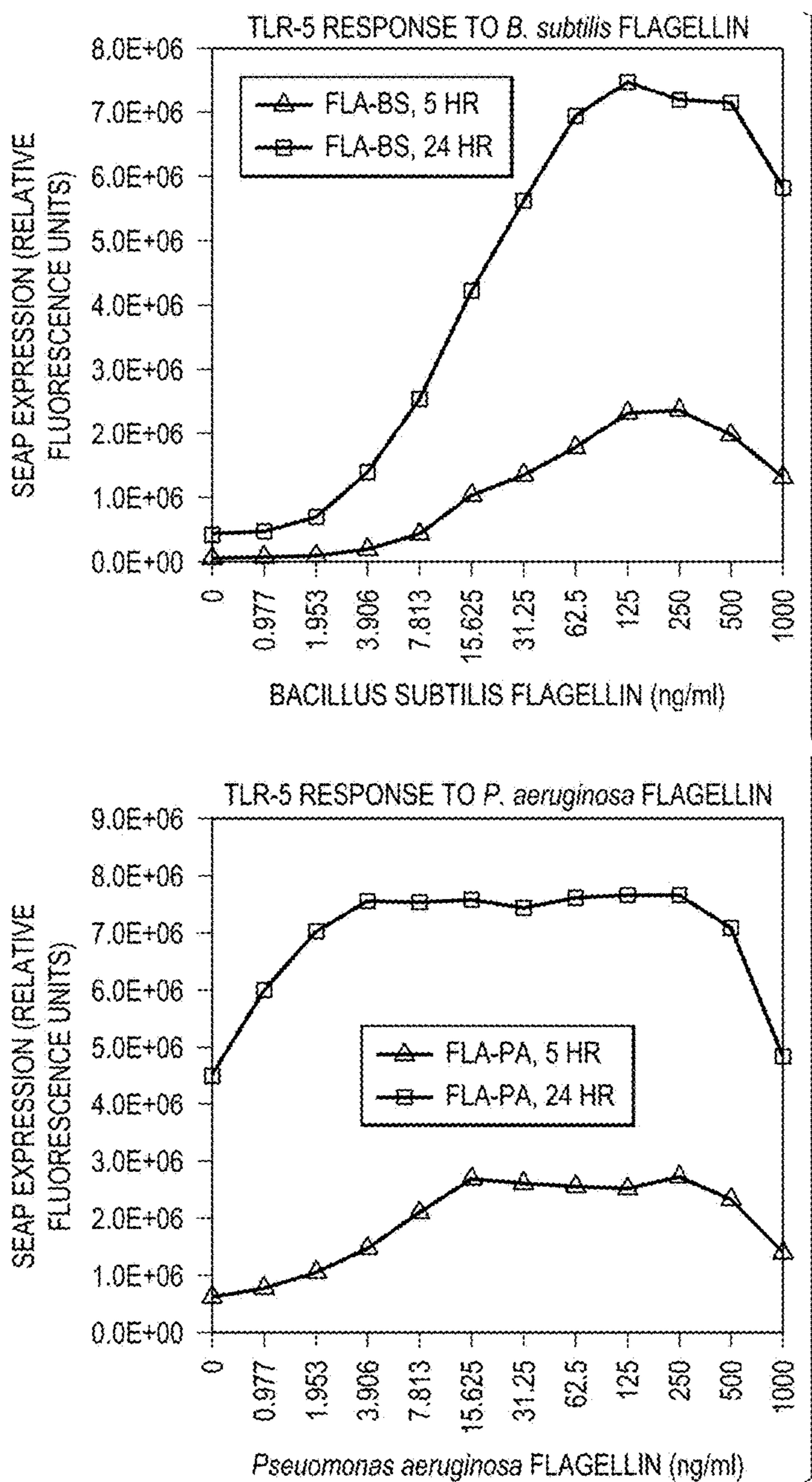


FIG. 11

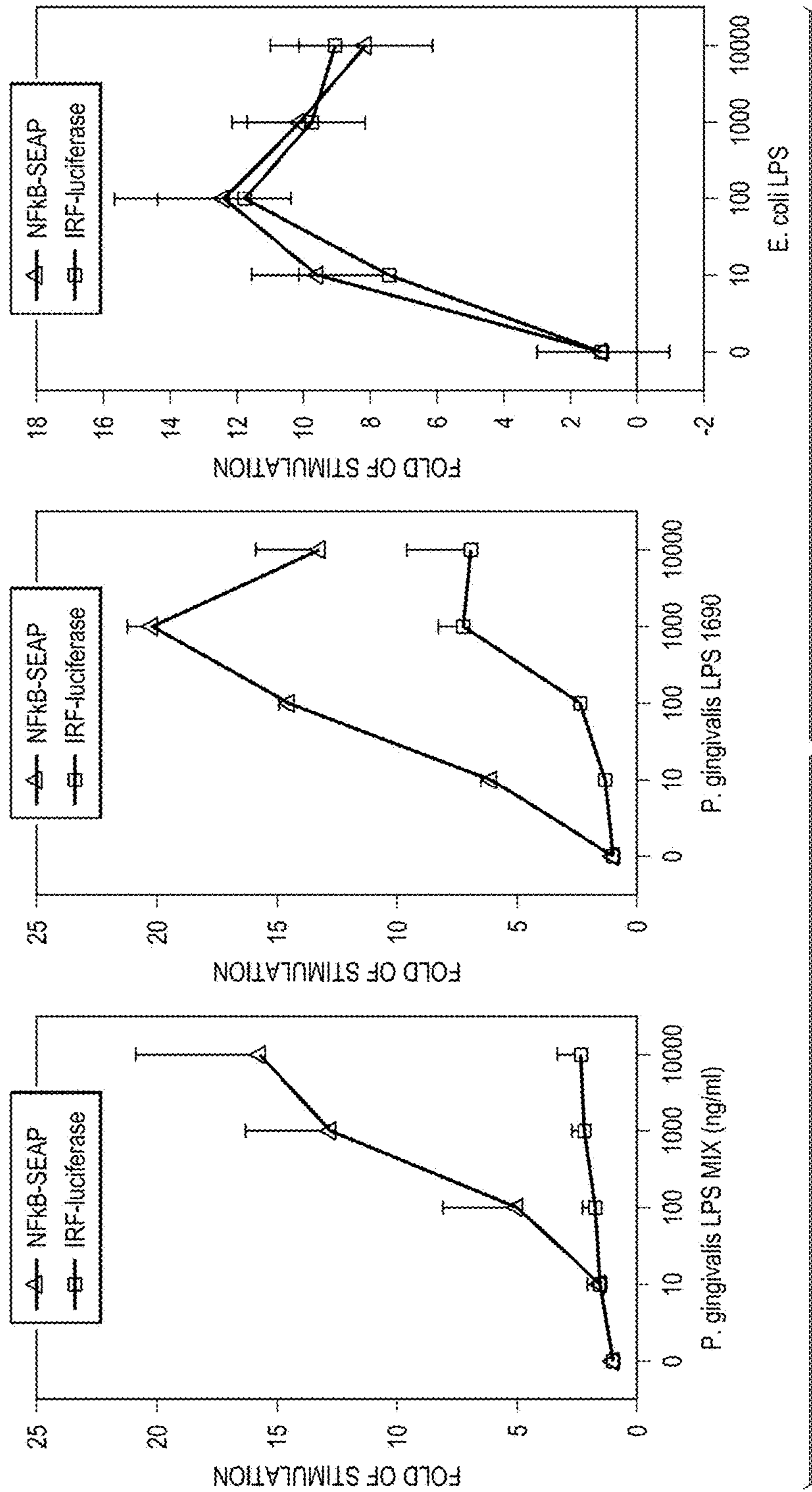


FIG. 12

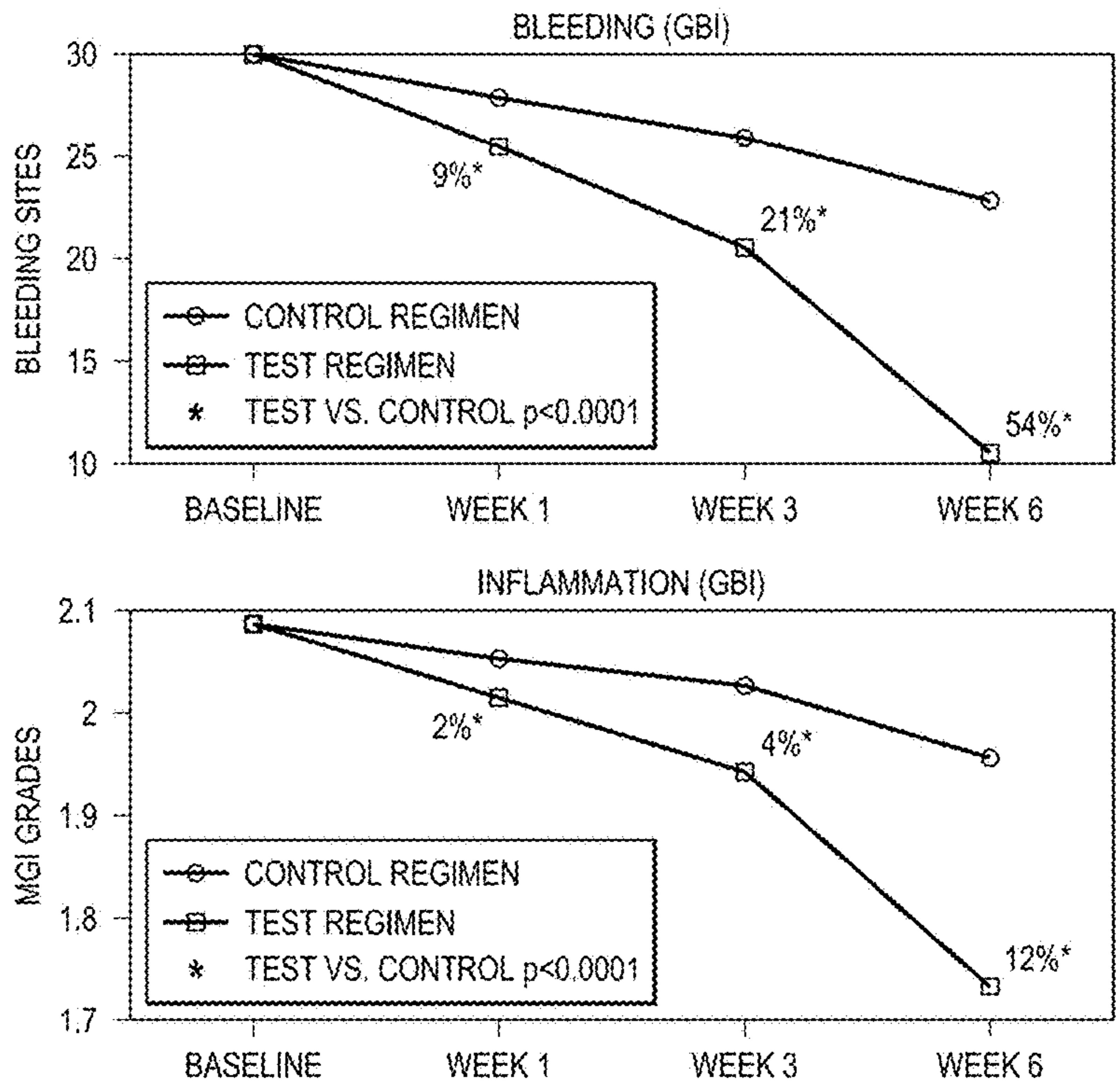


FIG. 14

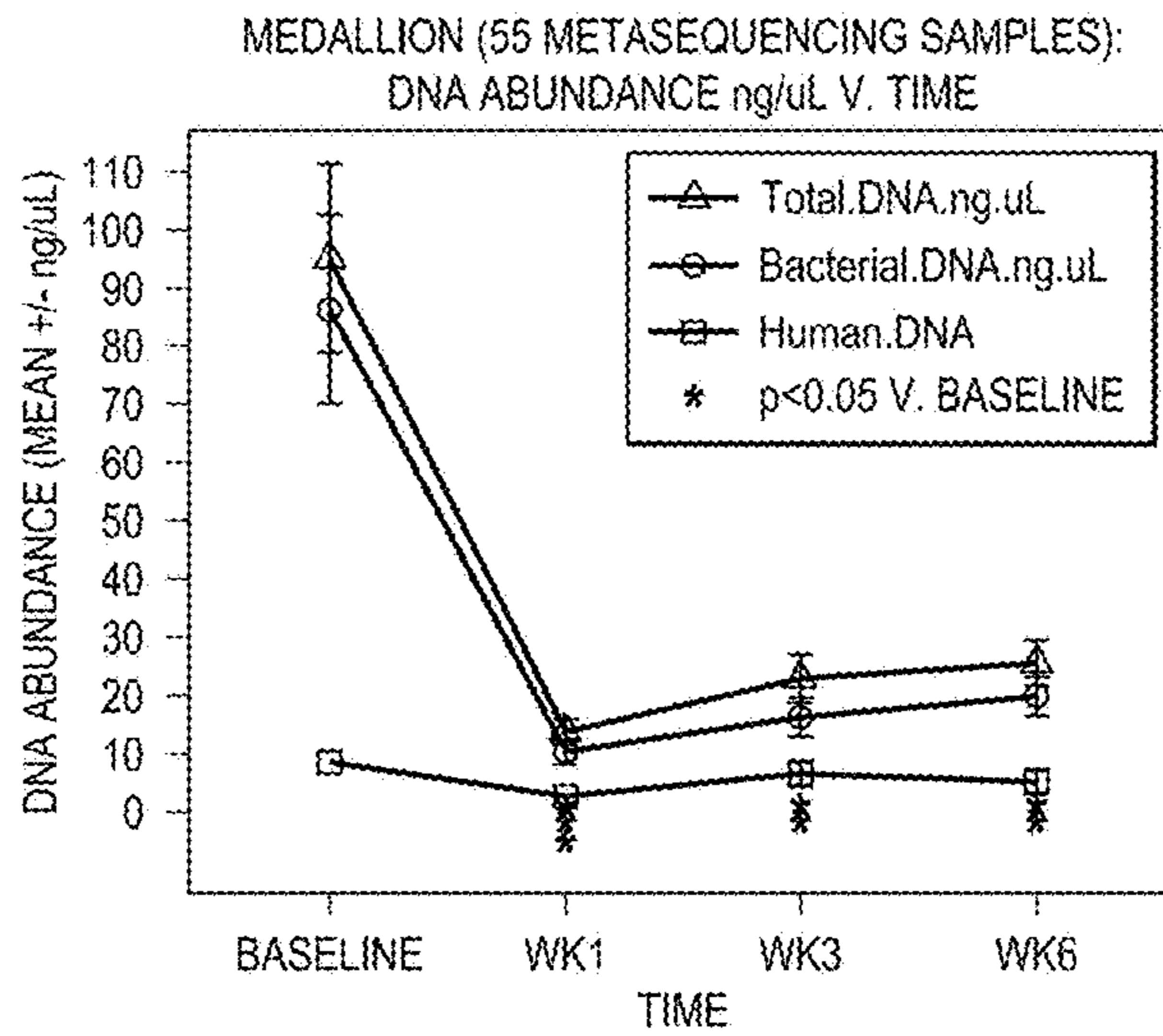


FIG. 15

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

FIG. 16A



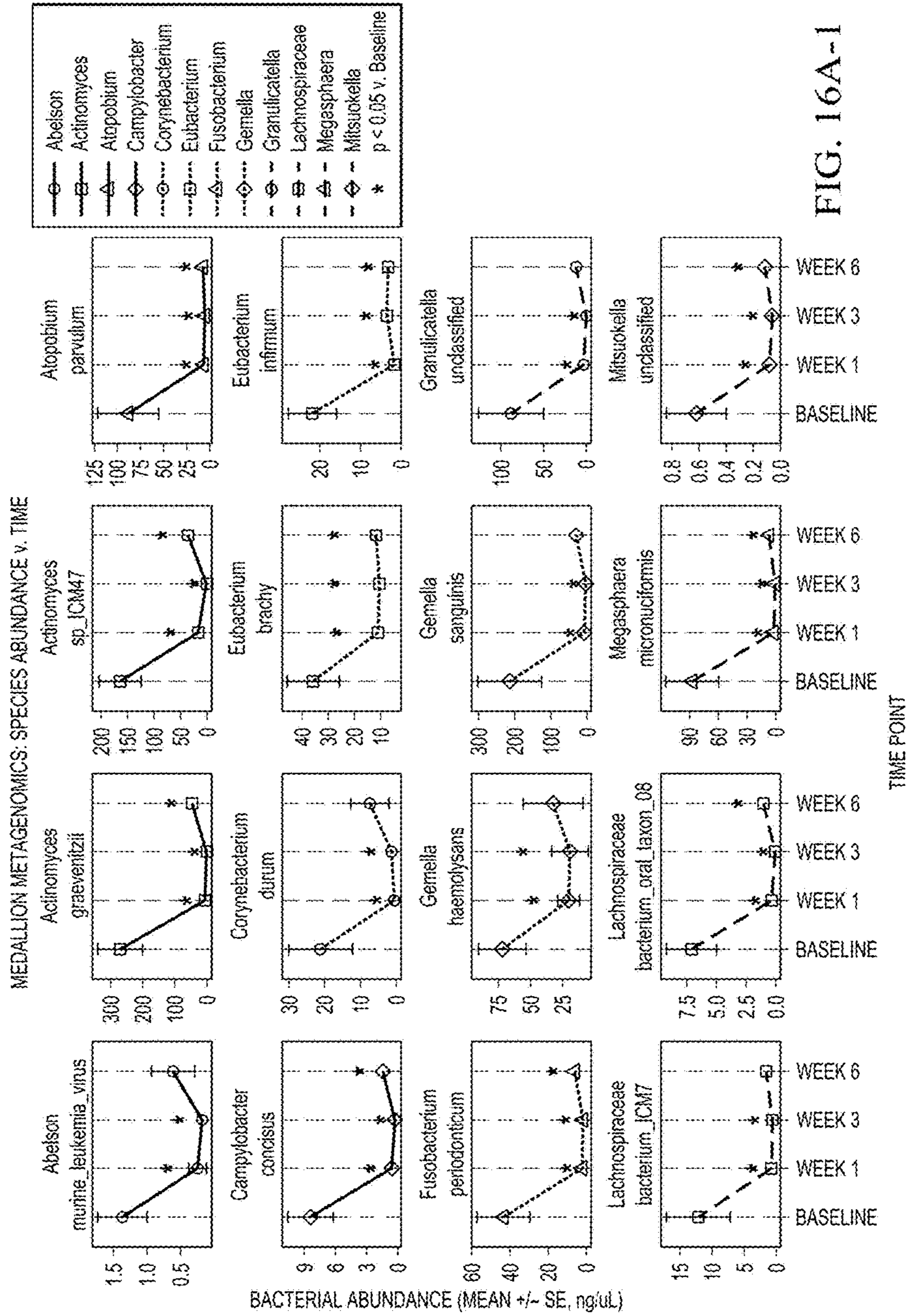


FIG. 16A-1

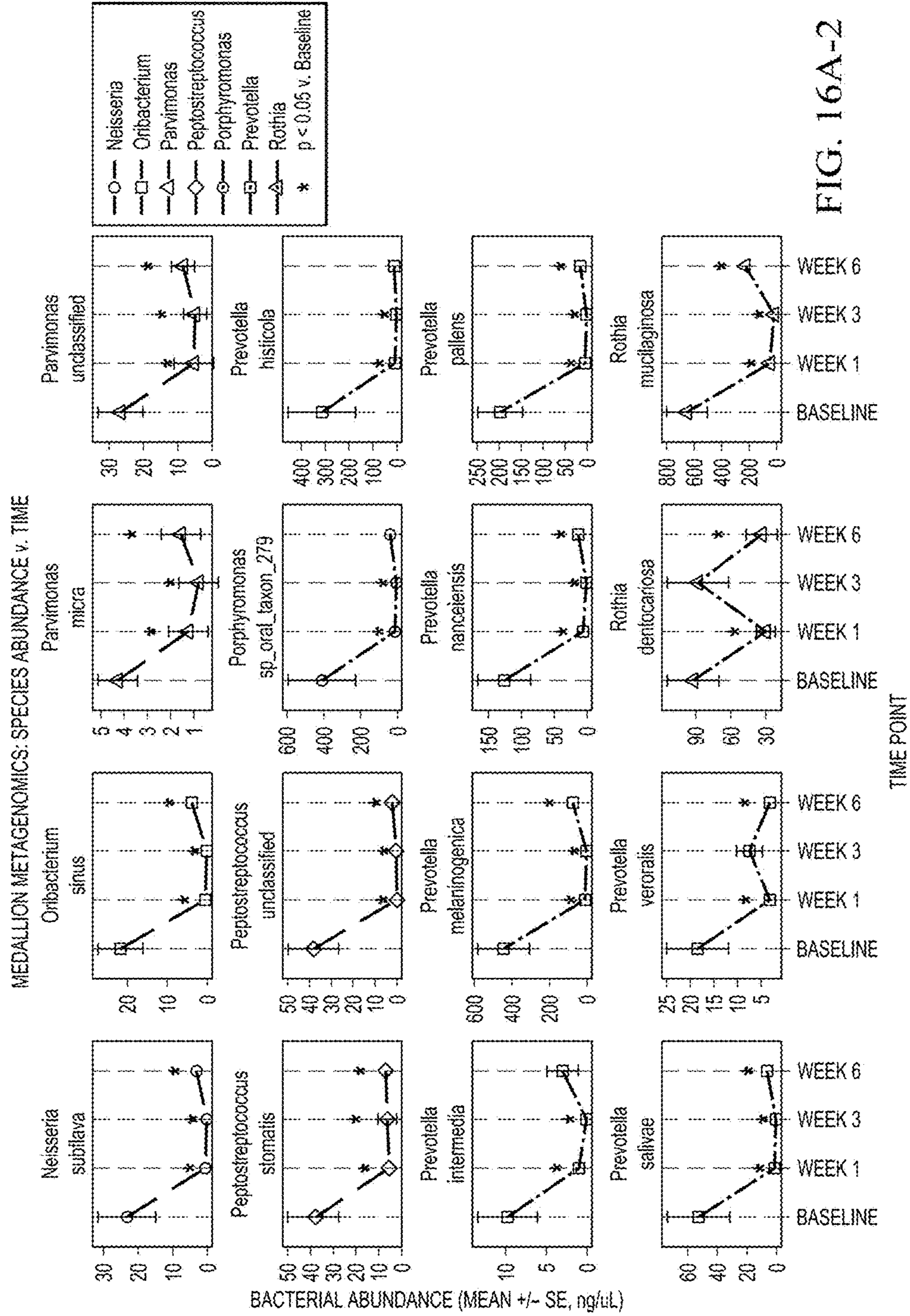


FIG. 16A-2

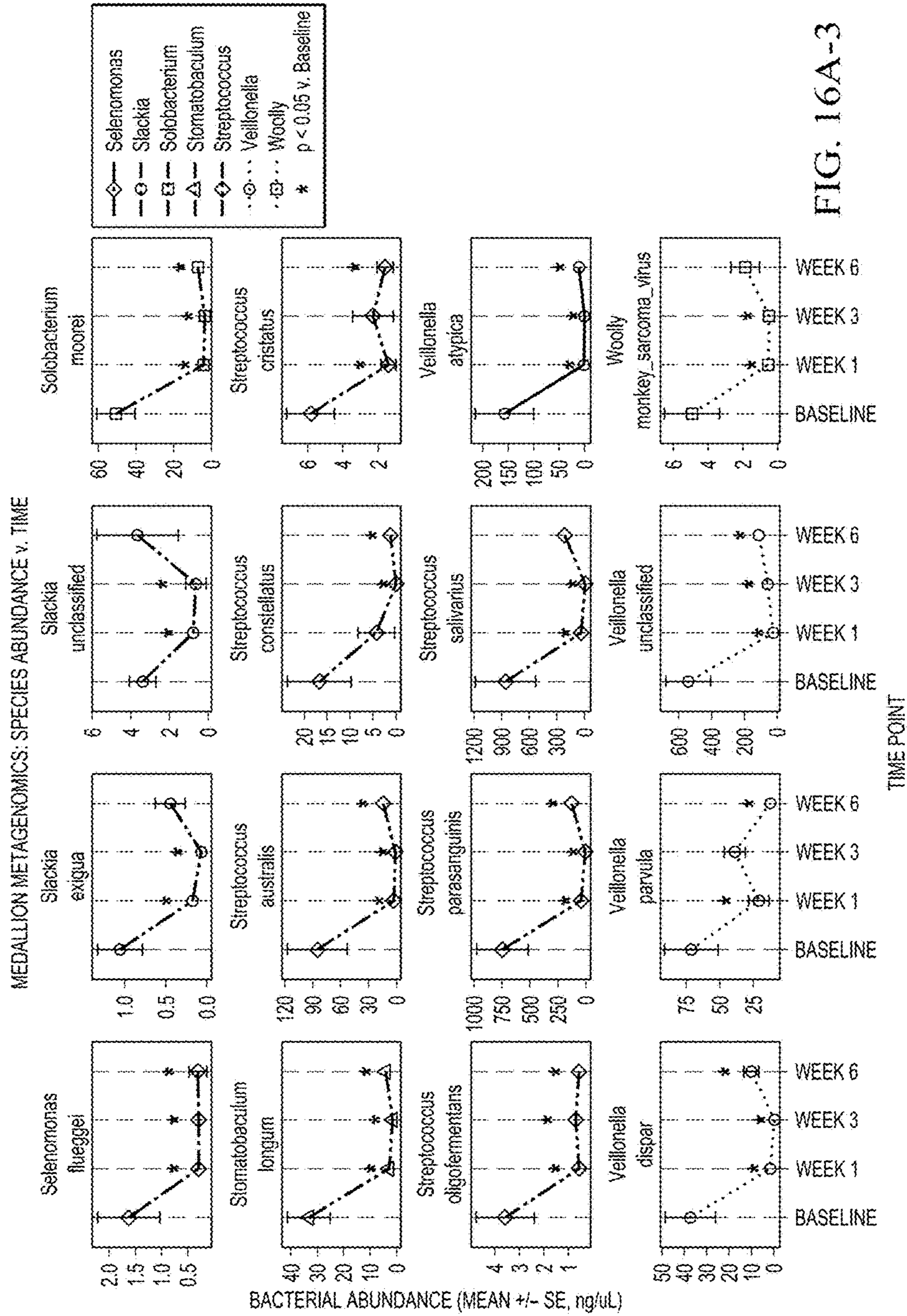


FIG. 16A-3

FIG. 16B-1  
FIG. 16B-2

FIG. 16B

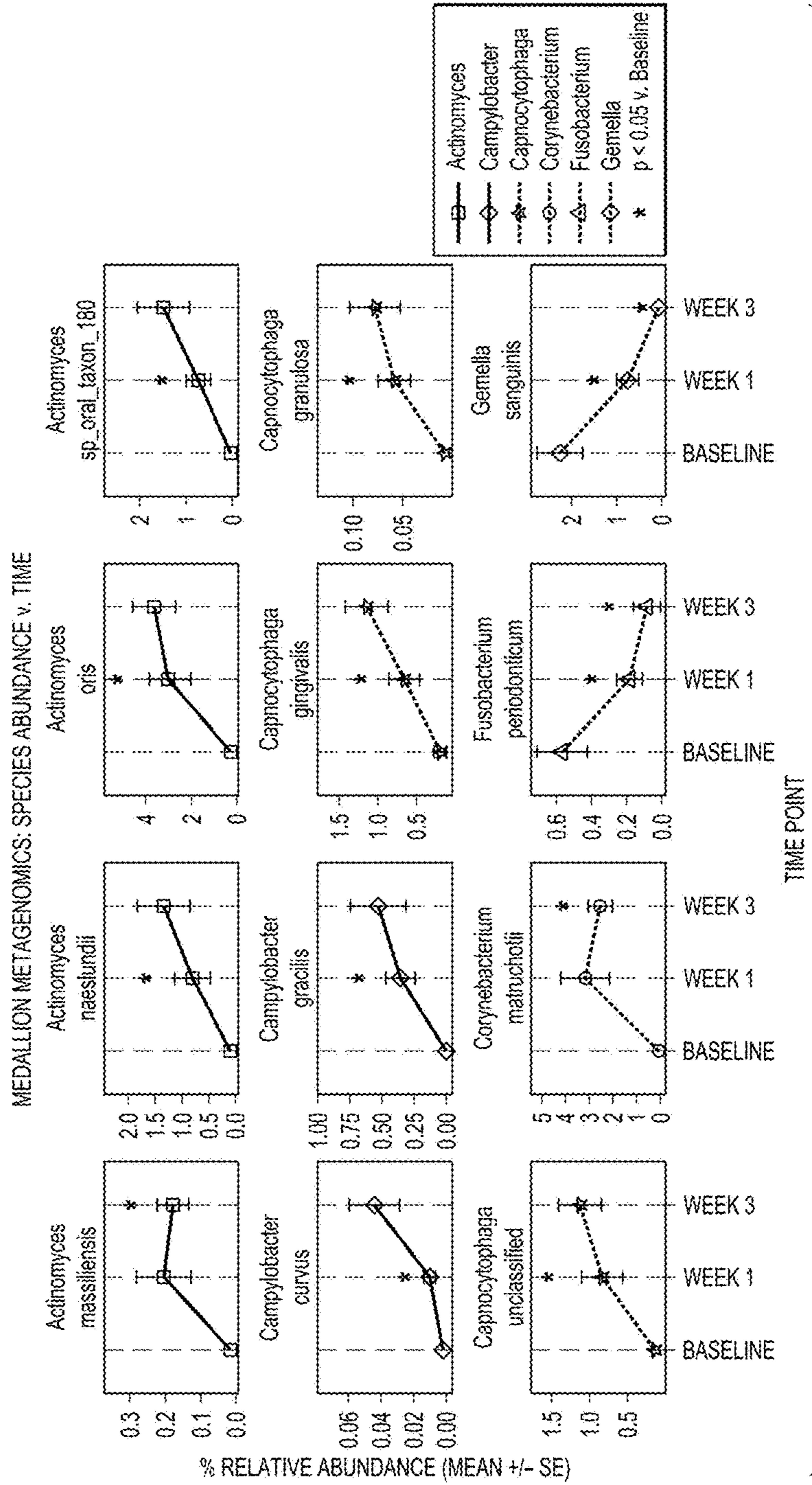


FIG. 16B-1

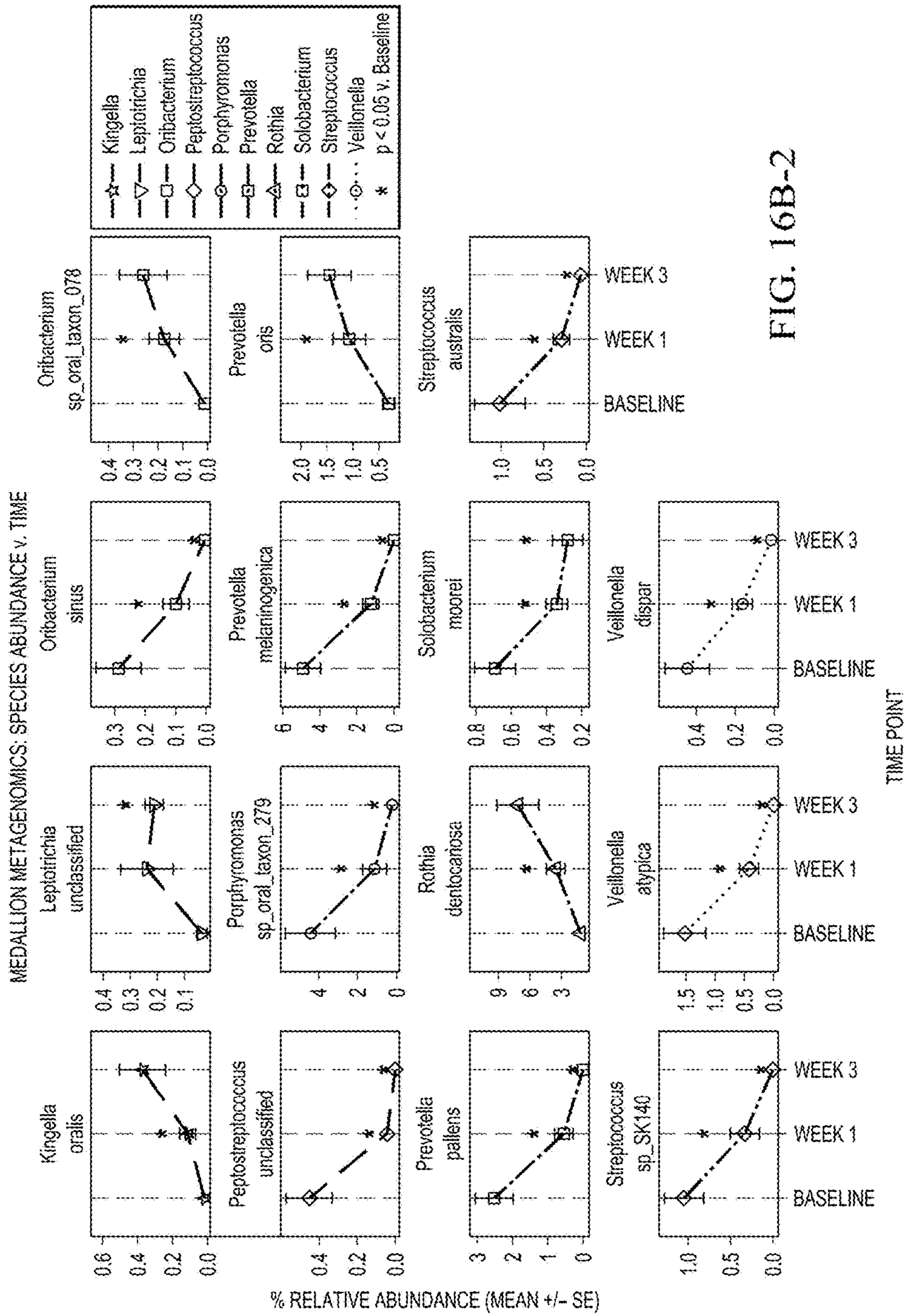


FIG. 16B-2

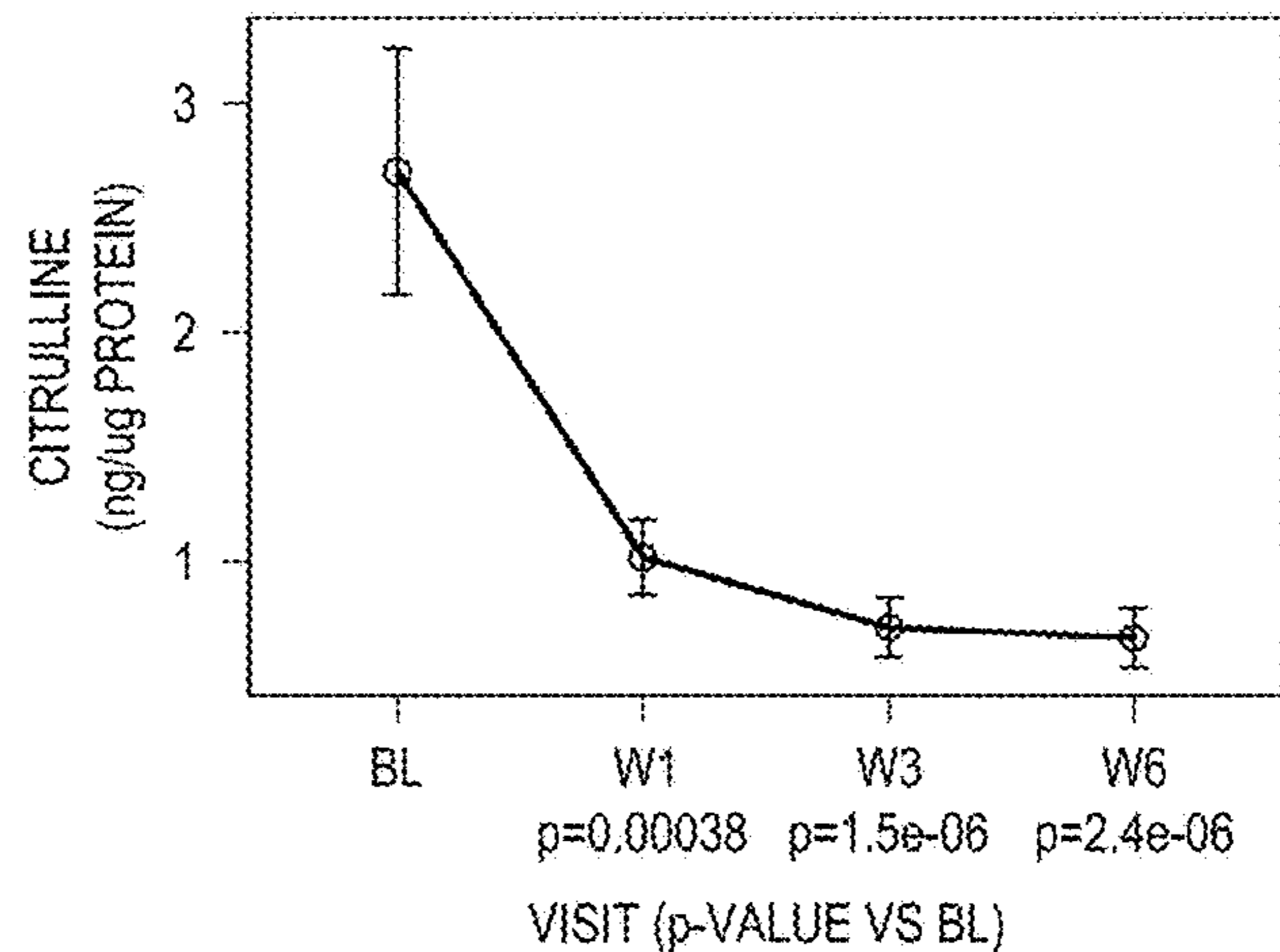


FIG. 17

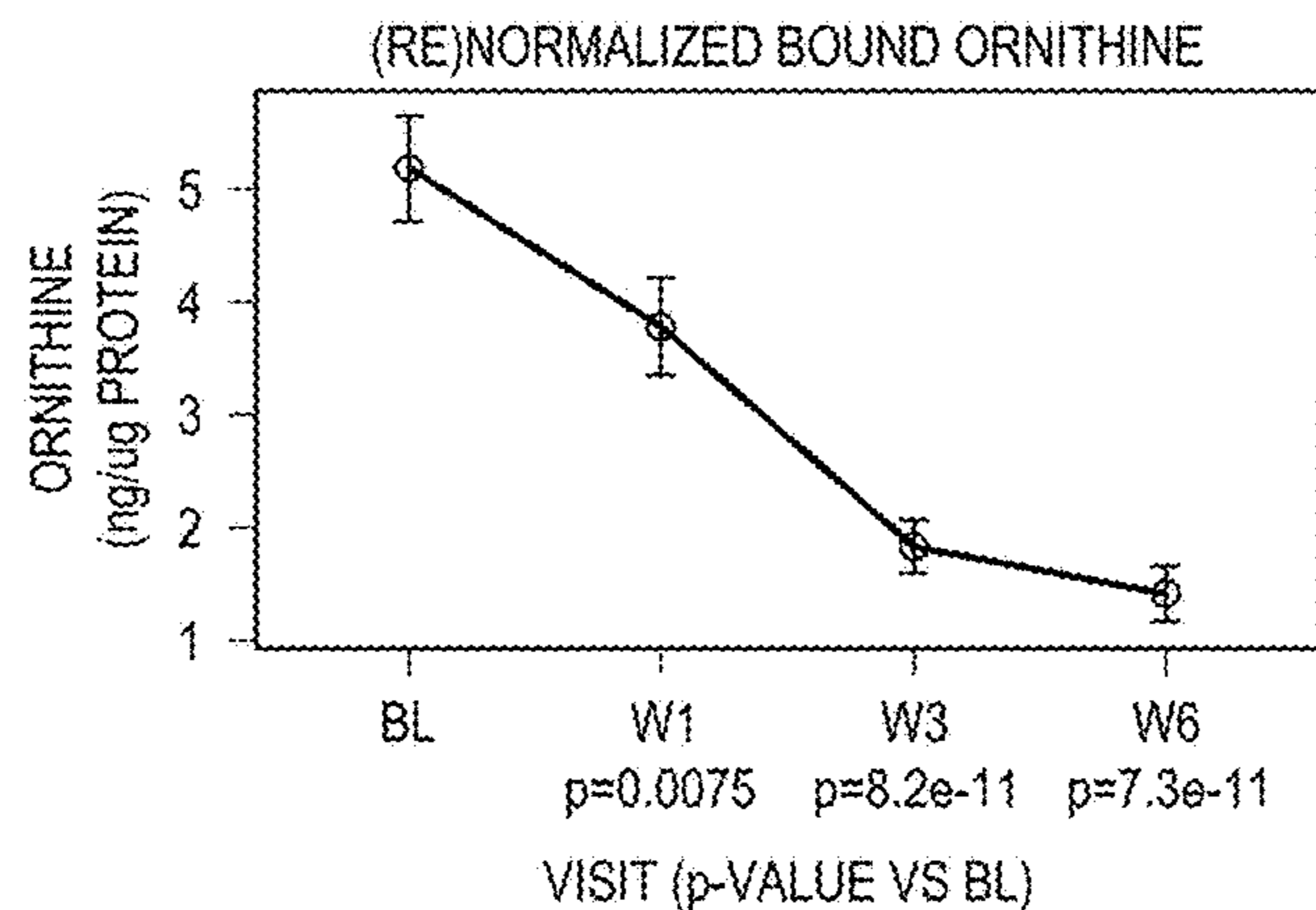


FIG. 18

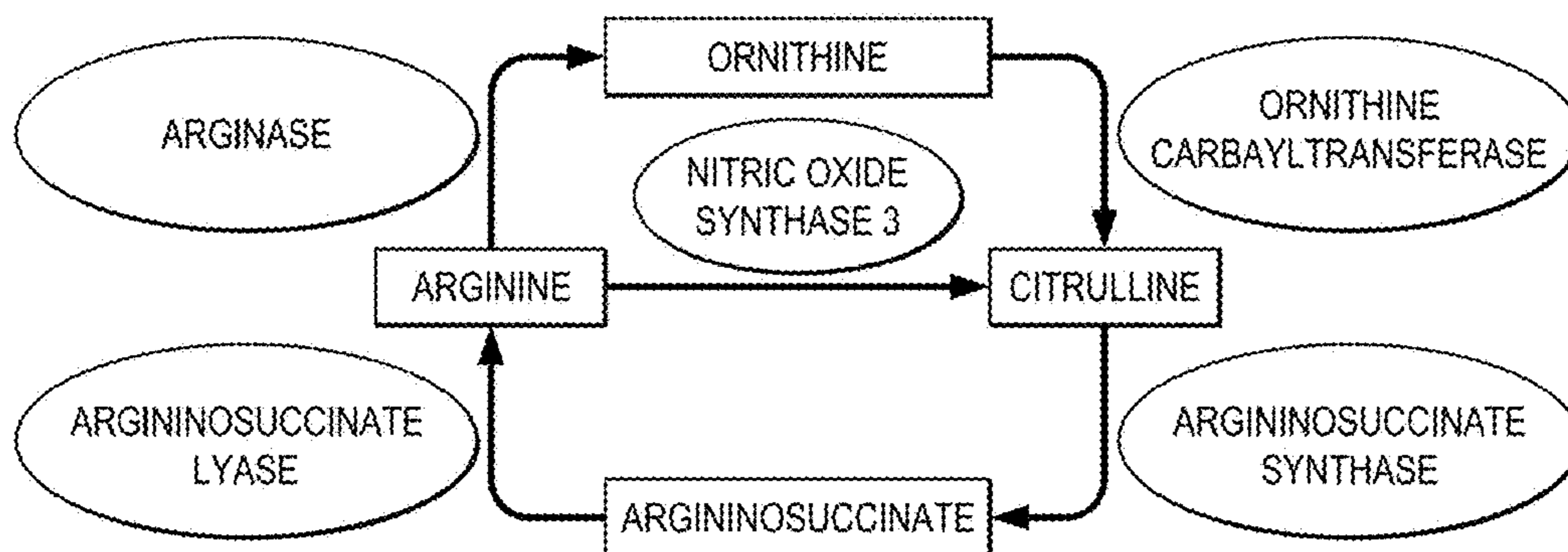
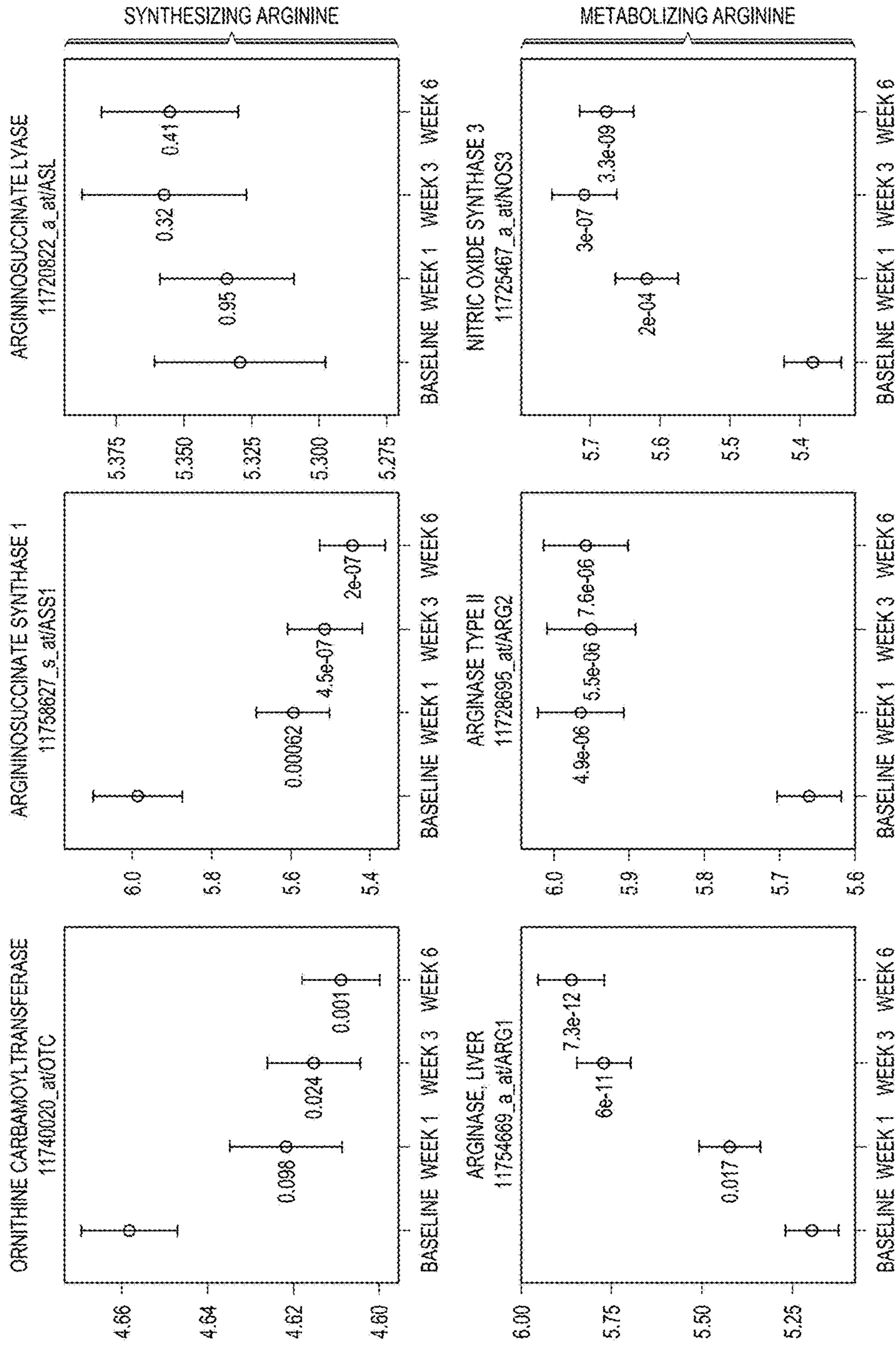
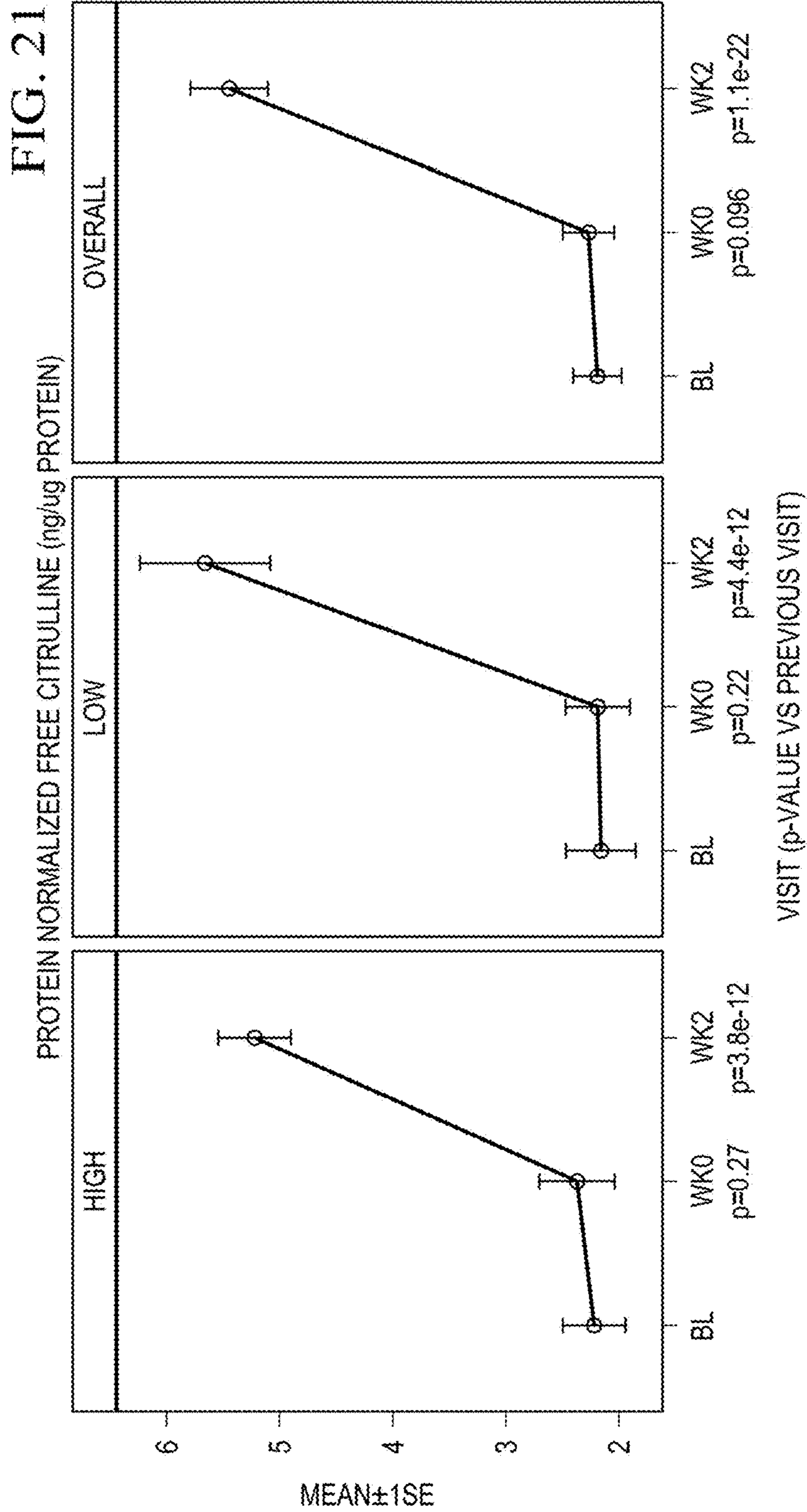


FIG. 19

FIG. 20







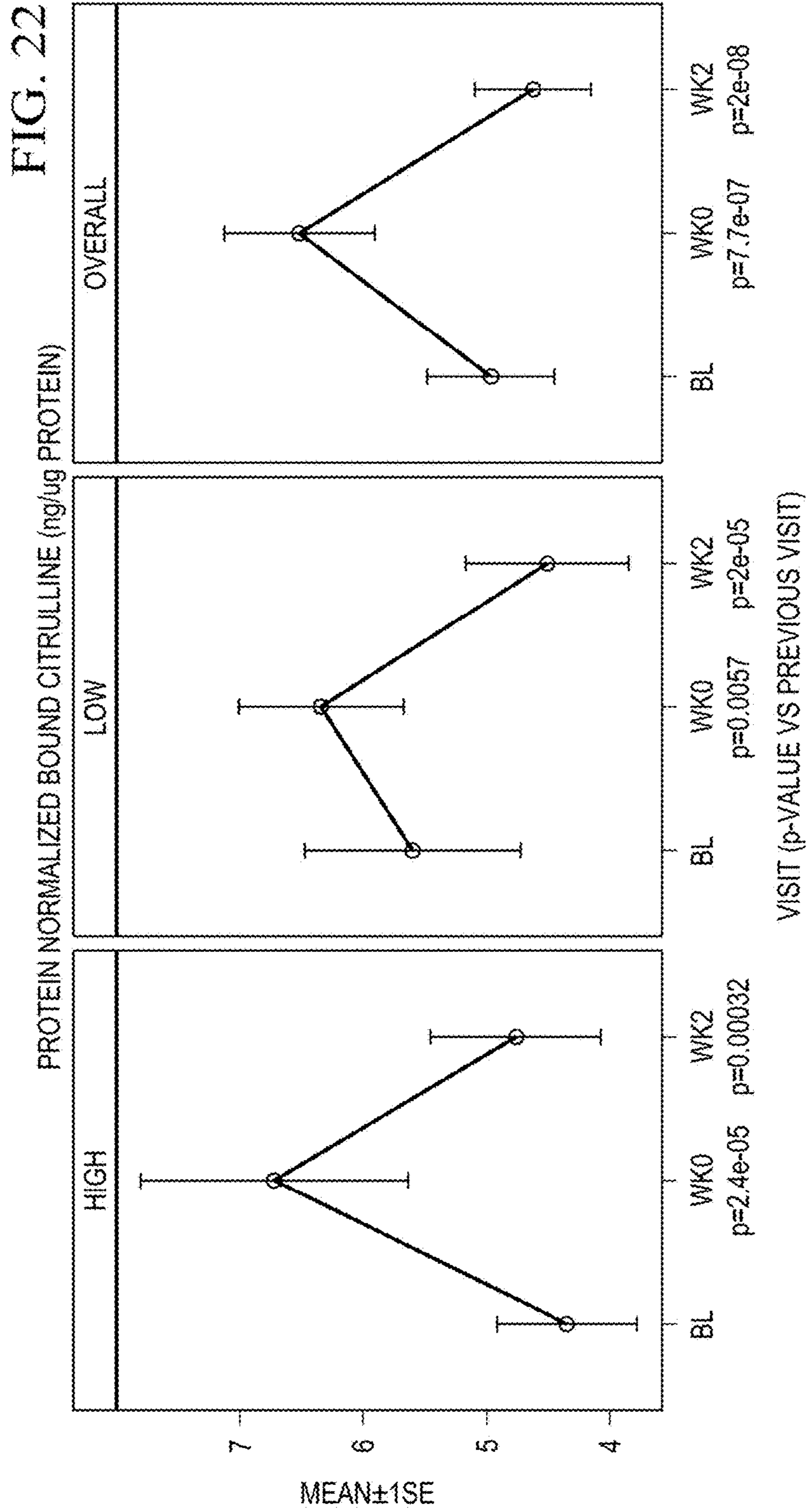


FIG. 23

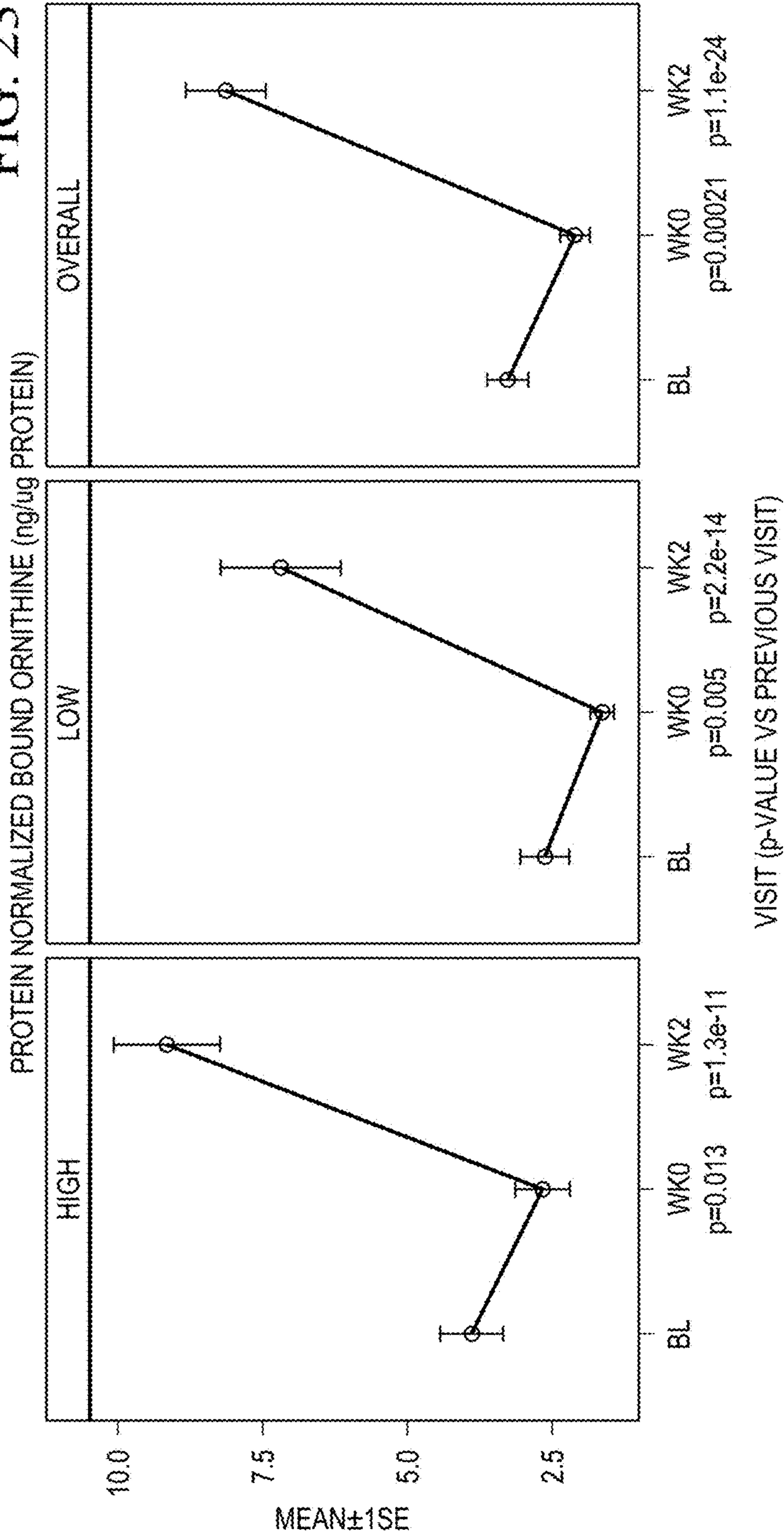




FIG. 25

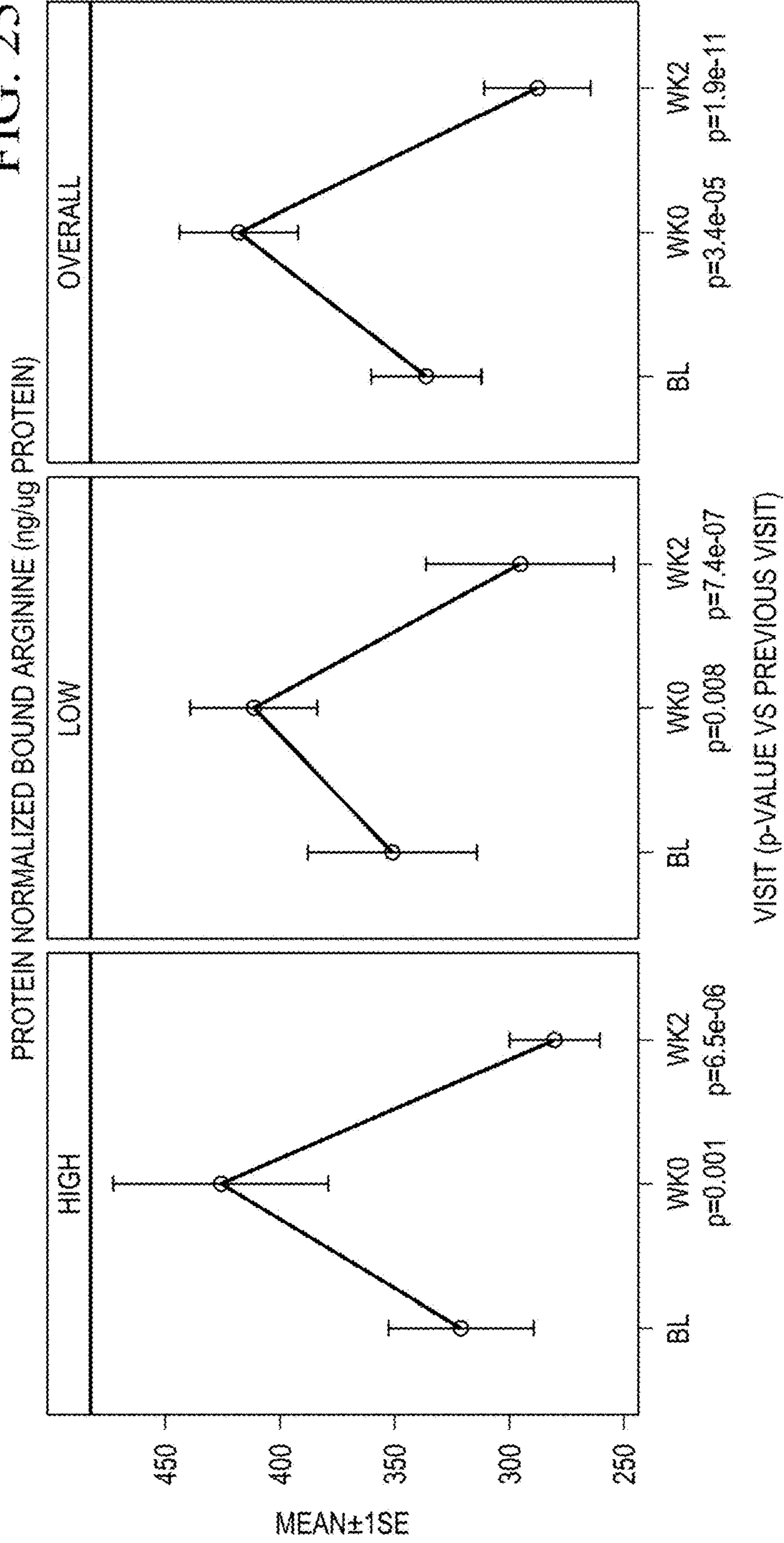
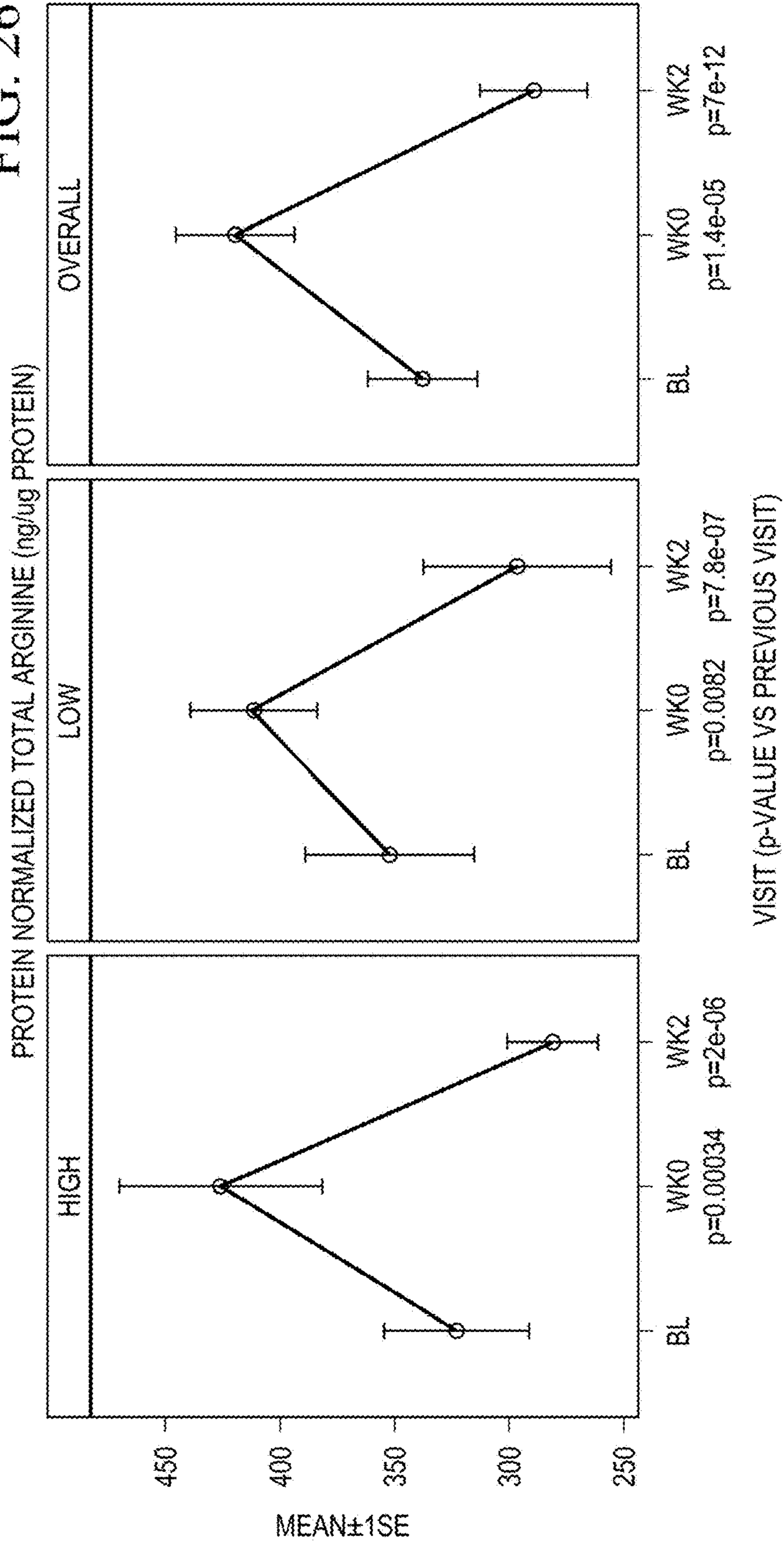


FIG. 26



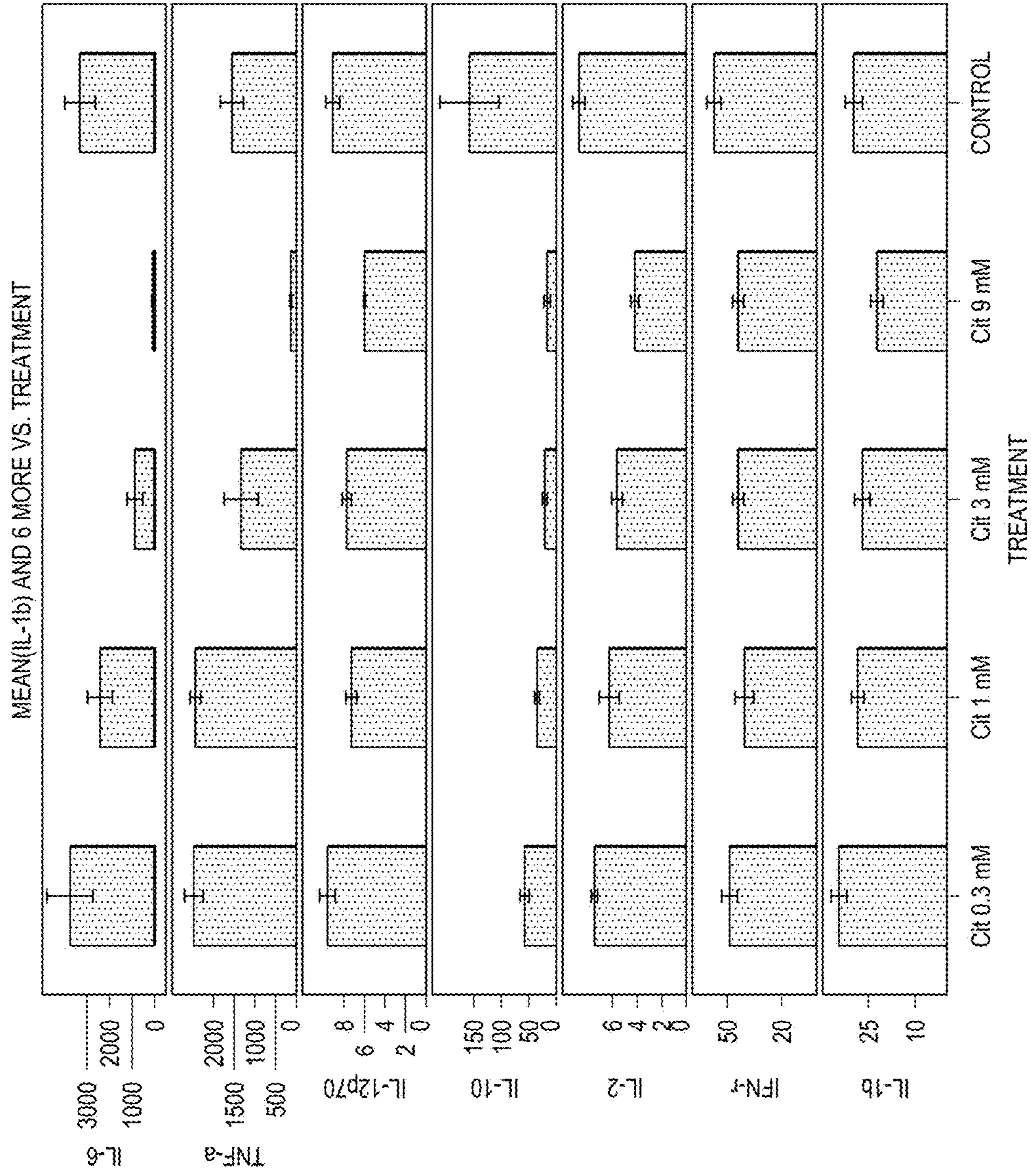
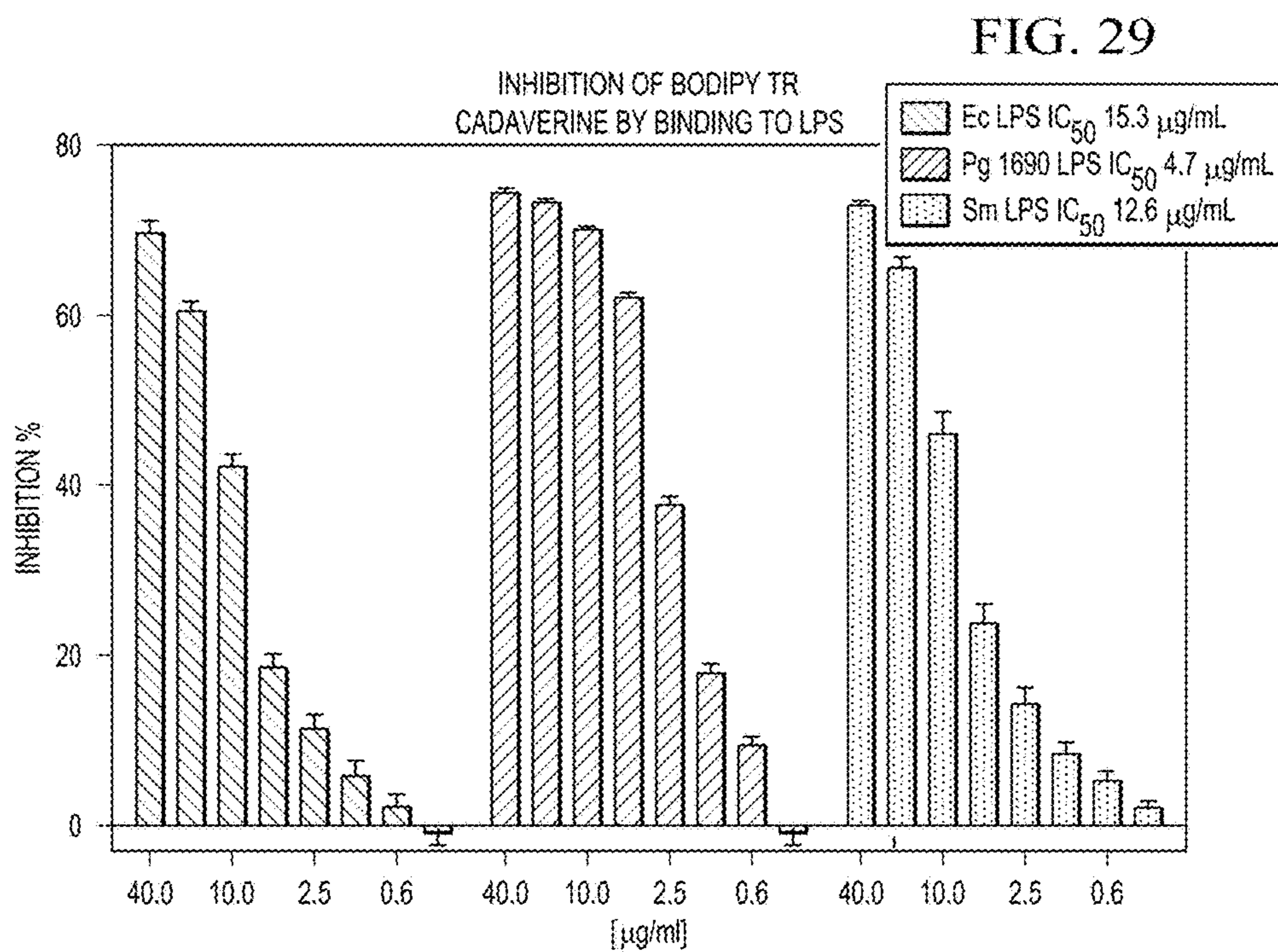
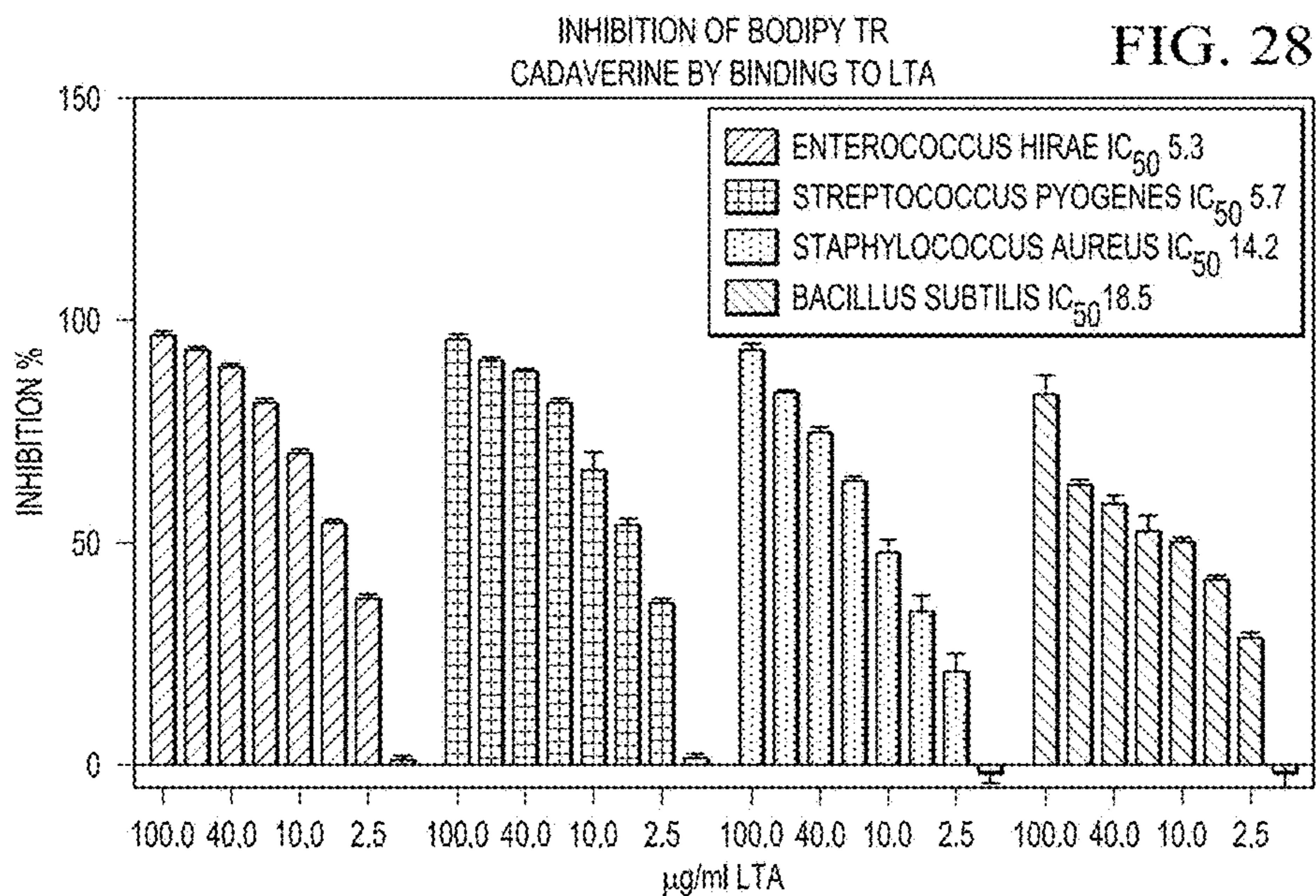
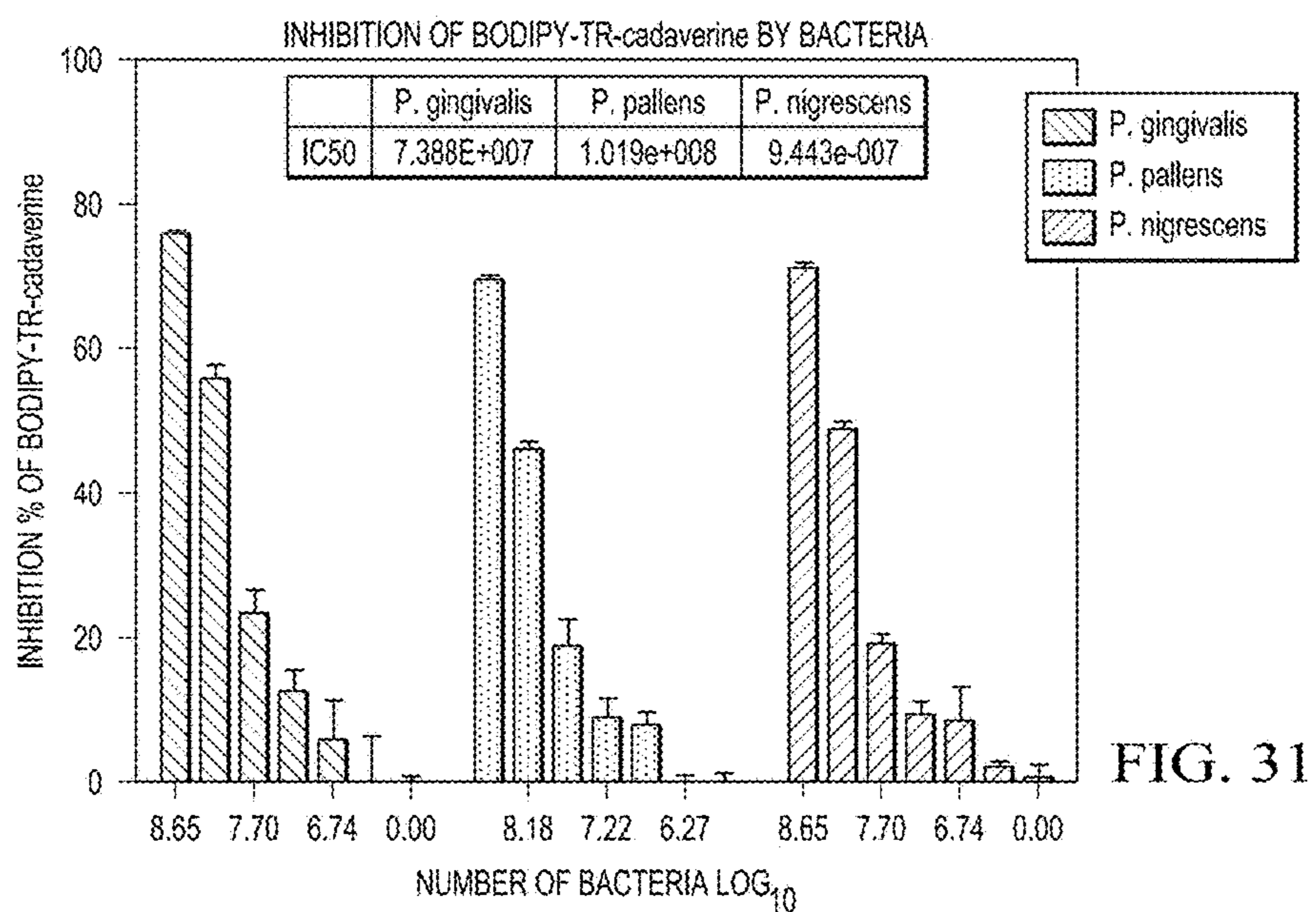
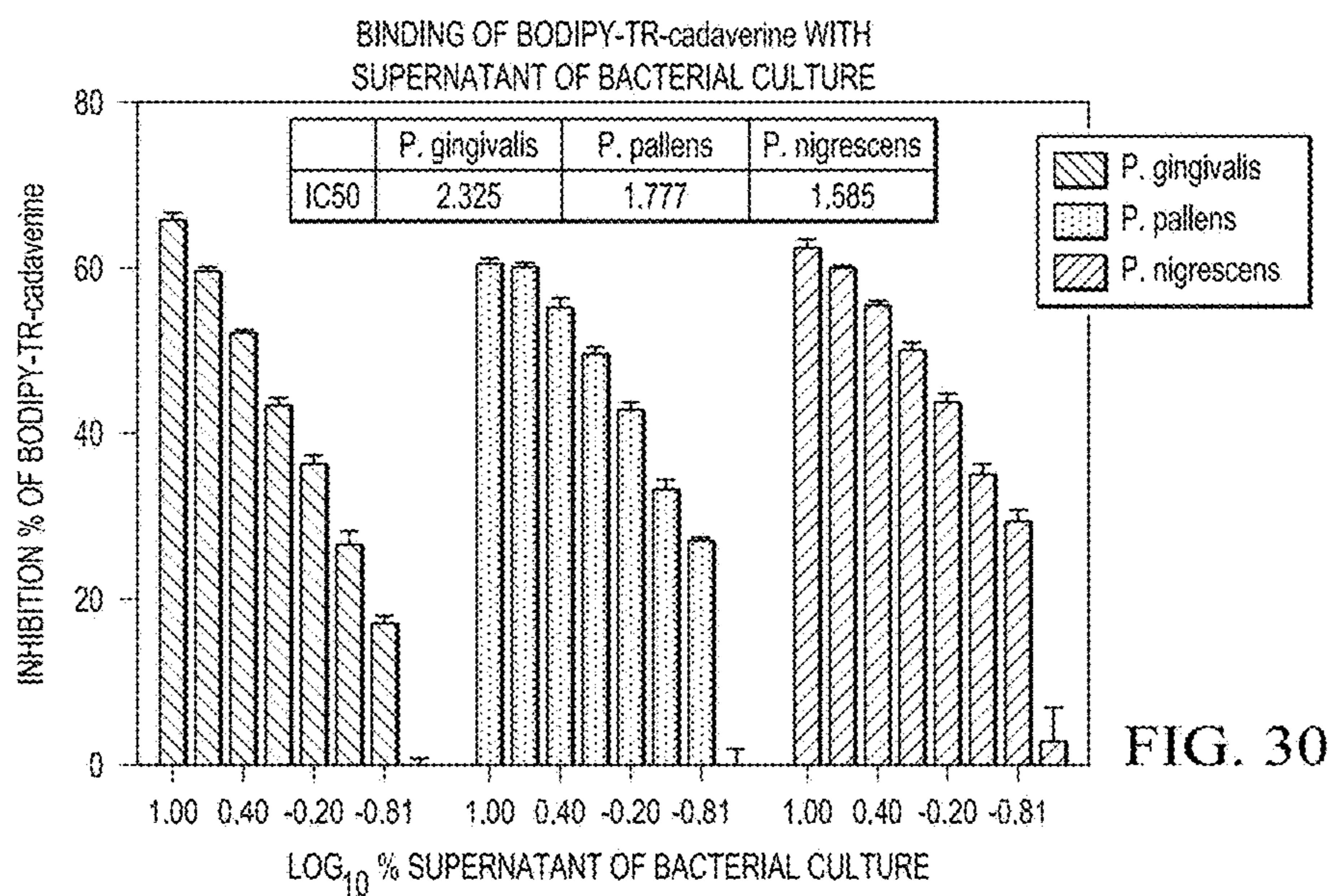


FIG. 27







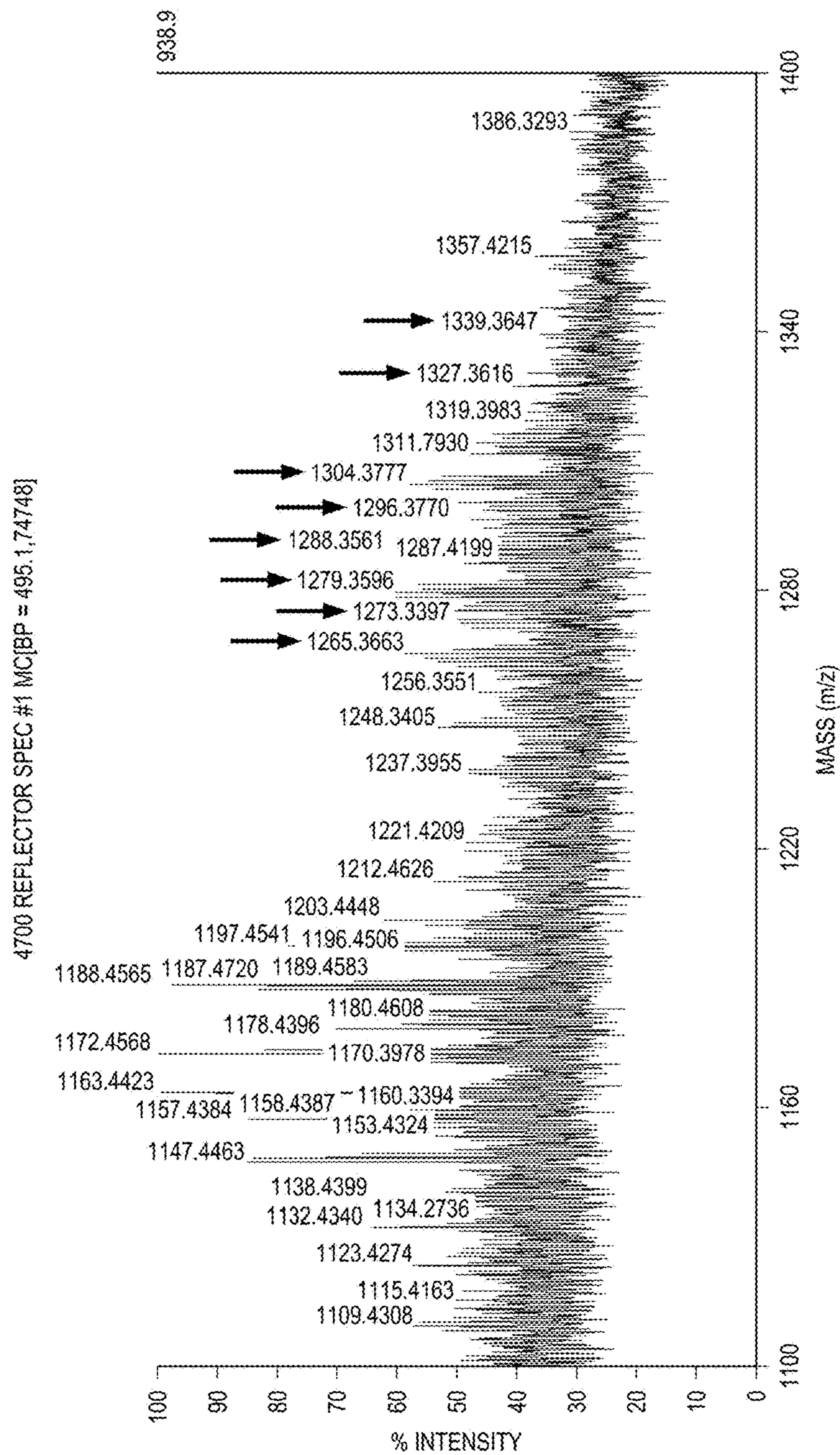


FIG. 32

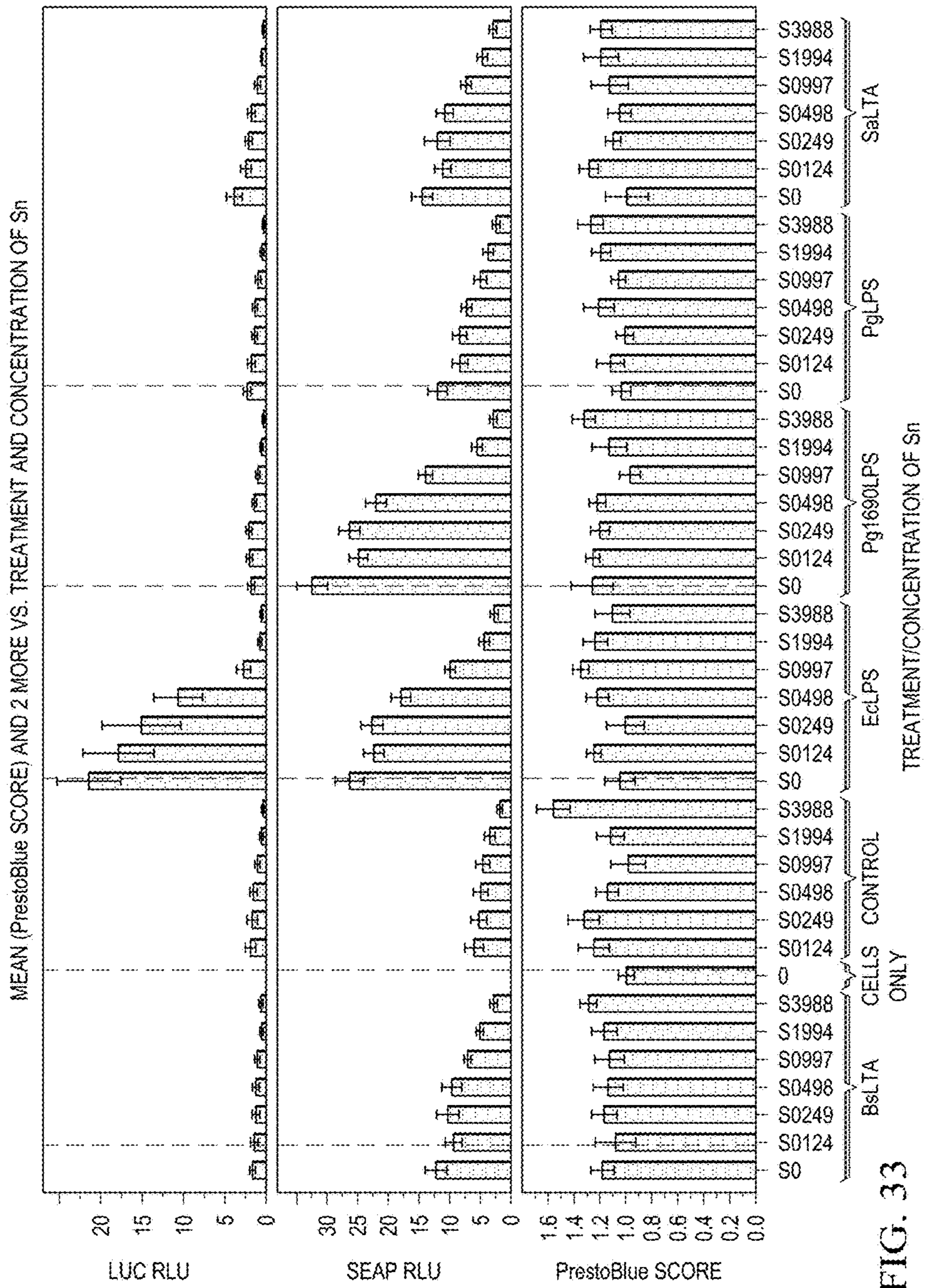
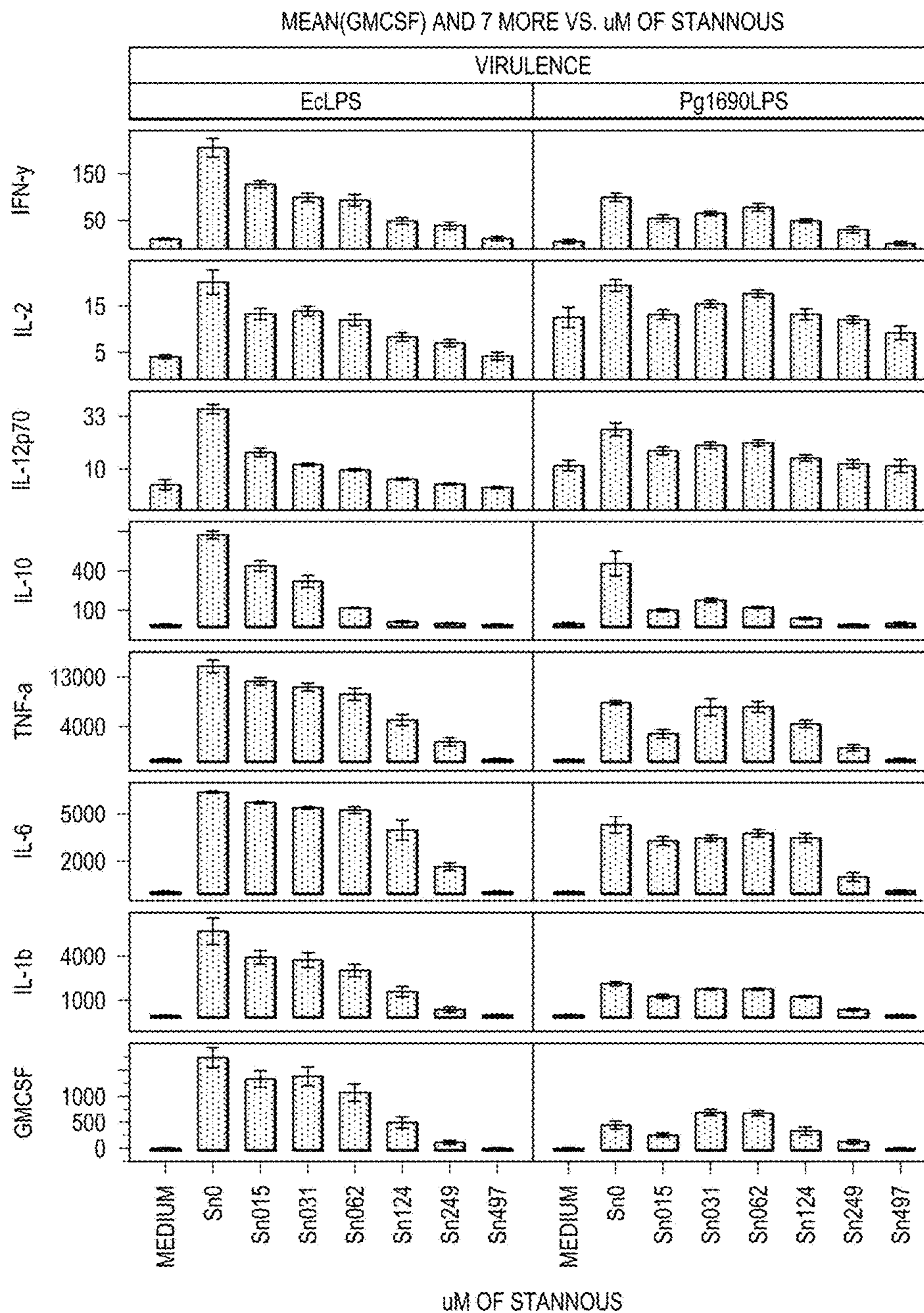


FIG. 33

FIG. 34



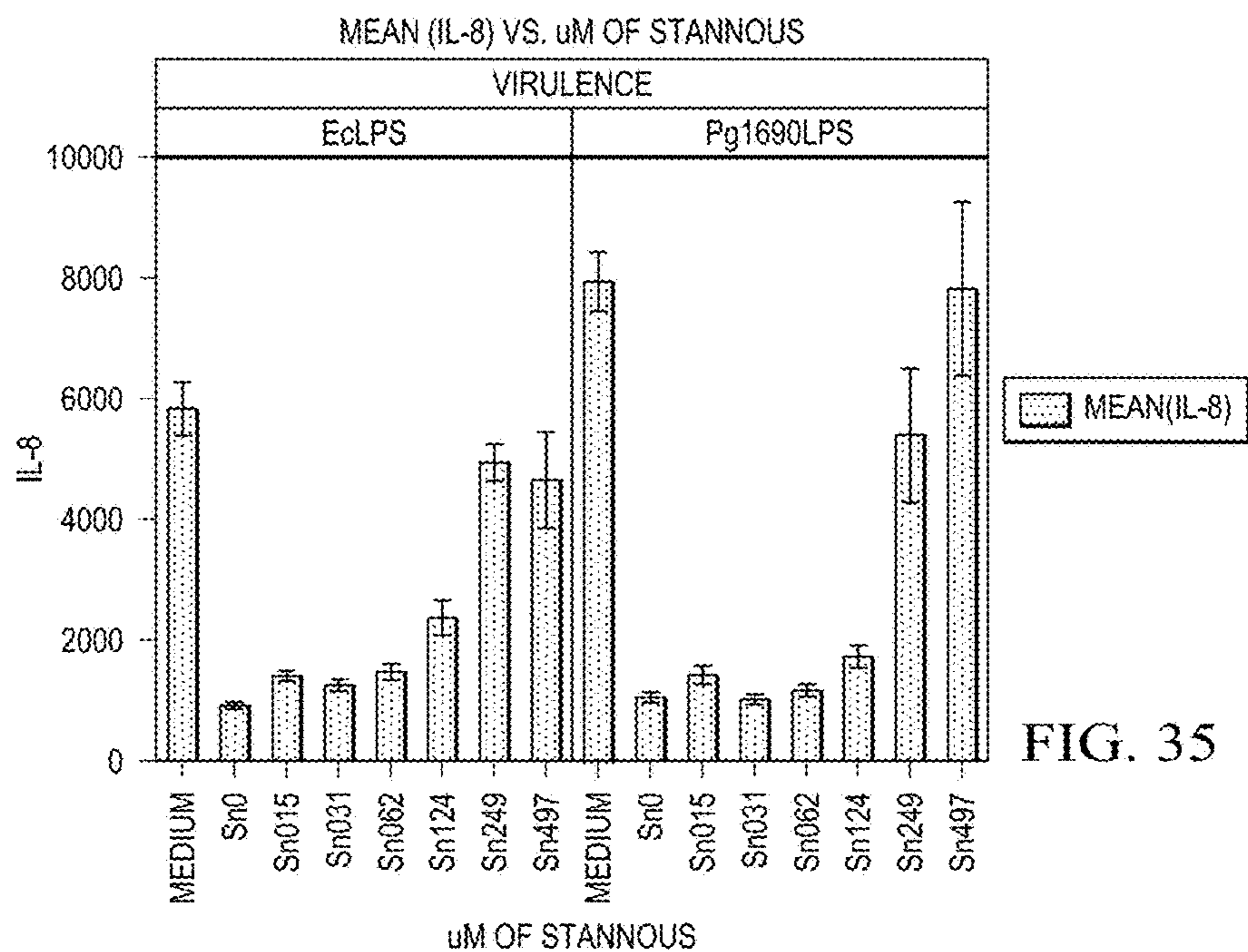


FIG. 35

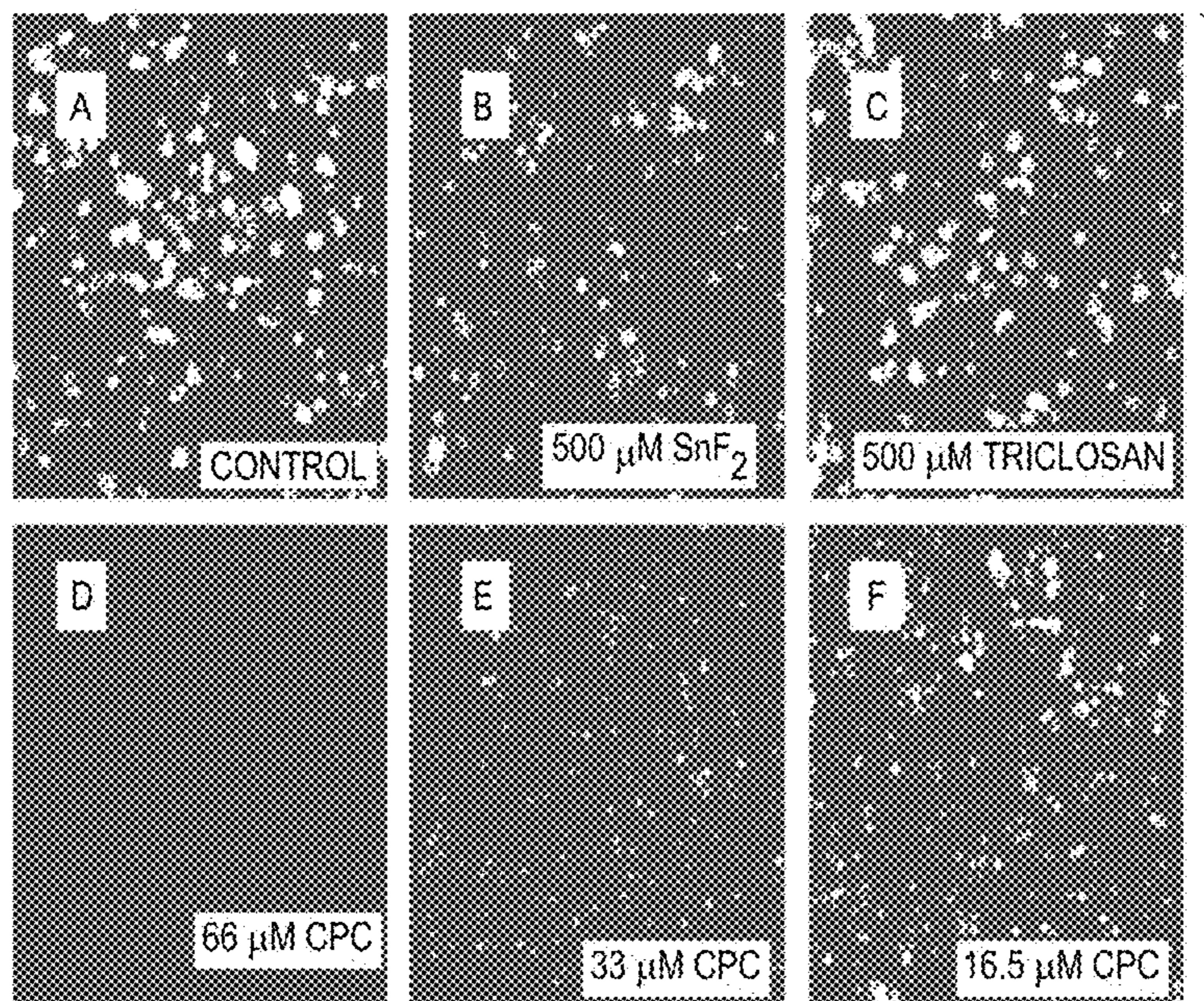
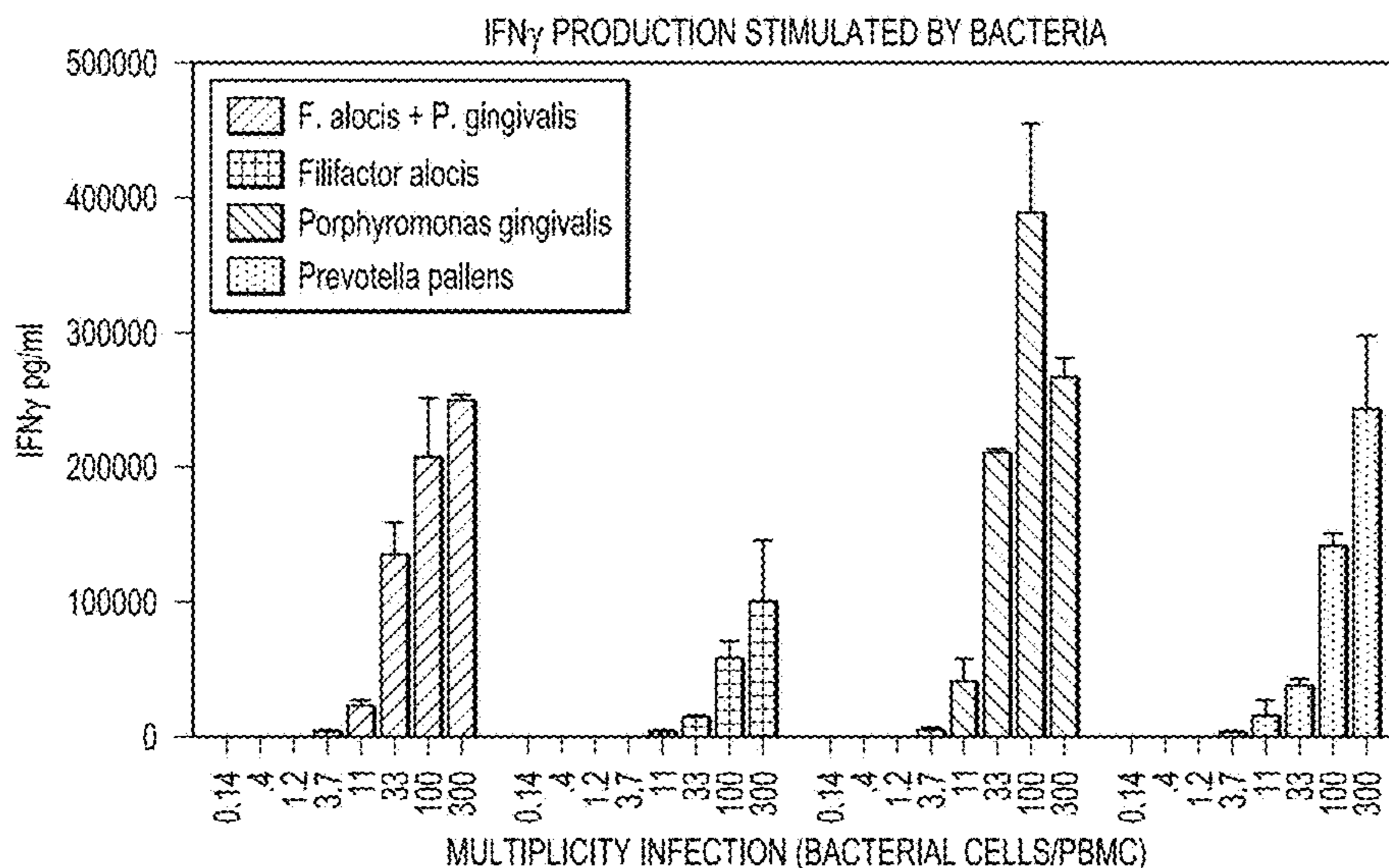
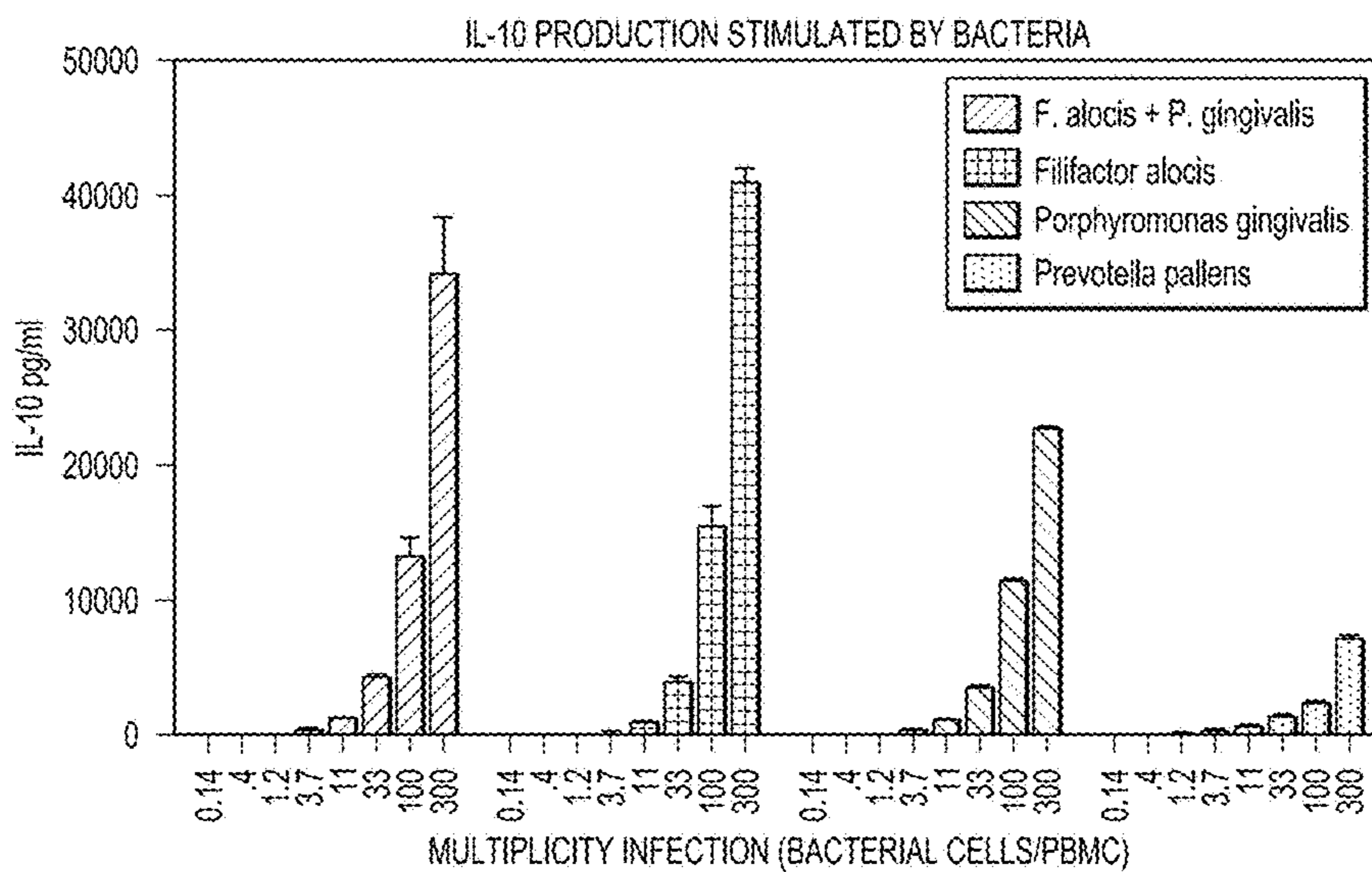


FIG. 36

FIG. 37A

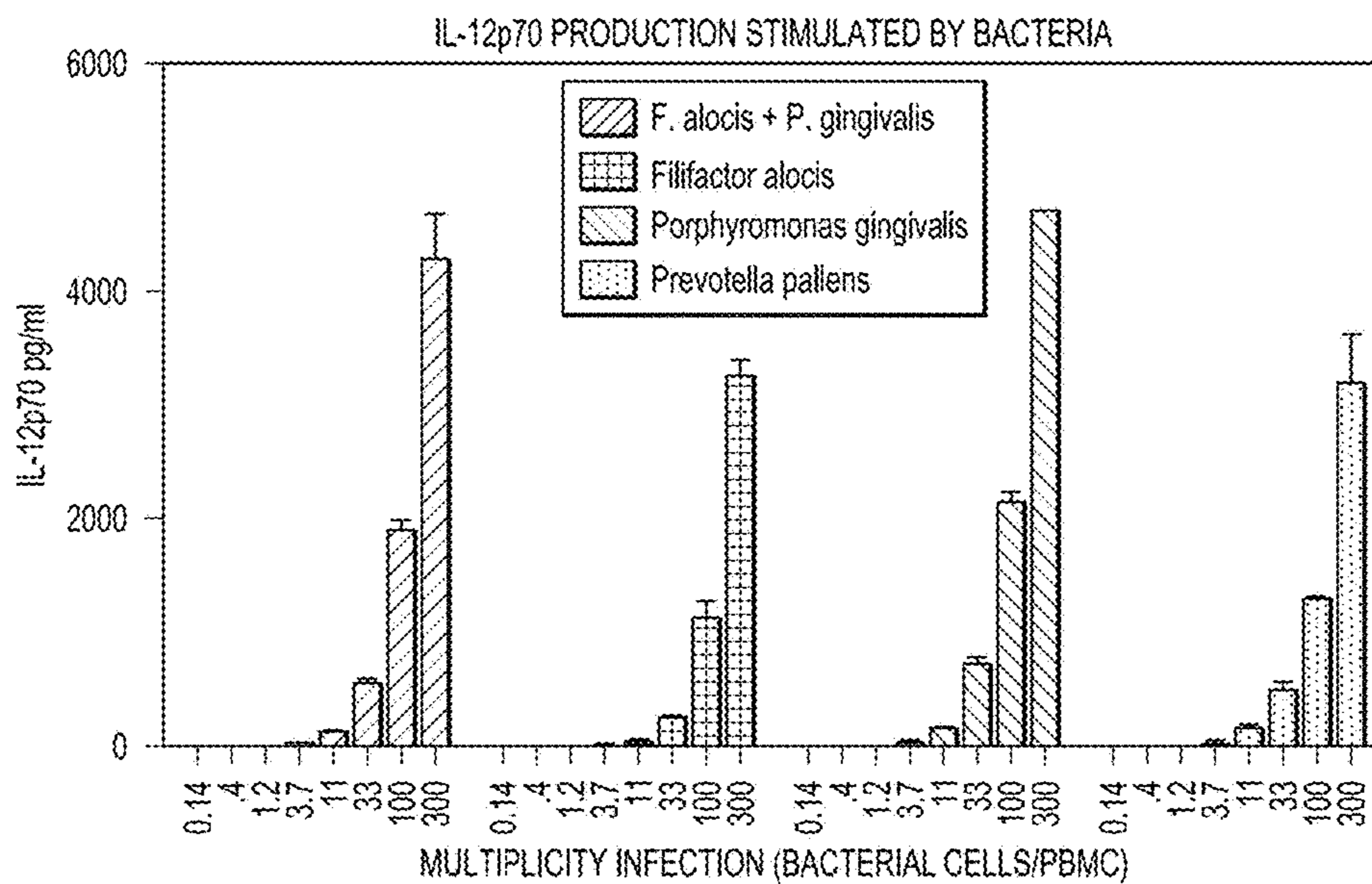


	F. alocis + P. gingivalis	Filifactor alocis	Porphyromonas gingivalis	Prevotella pallens
EC50	31.91	98.34	26.56	102.2

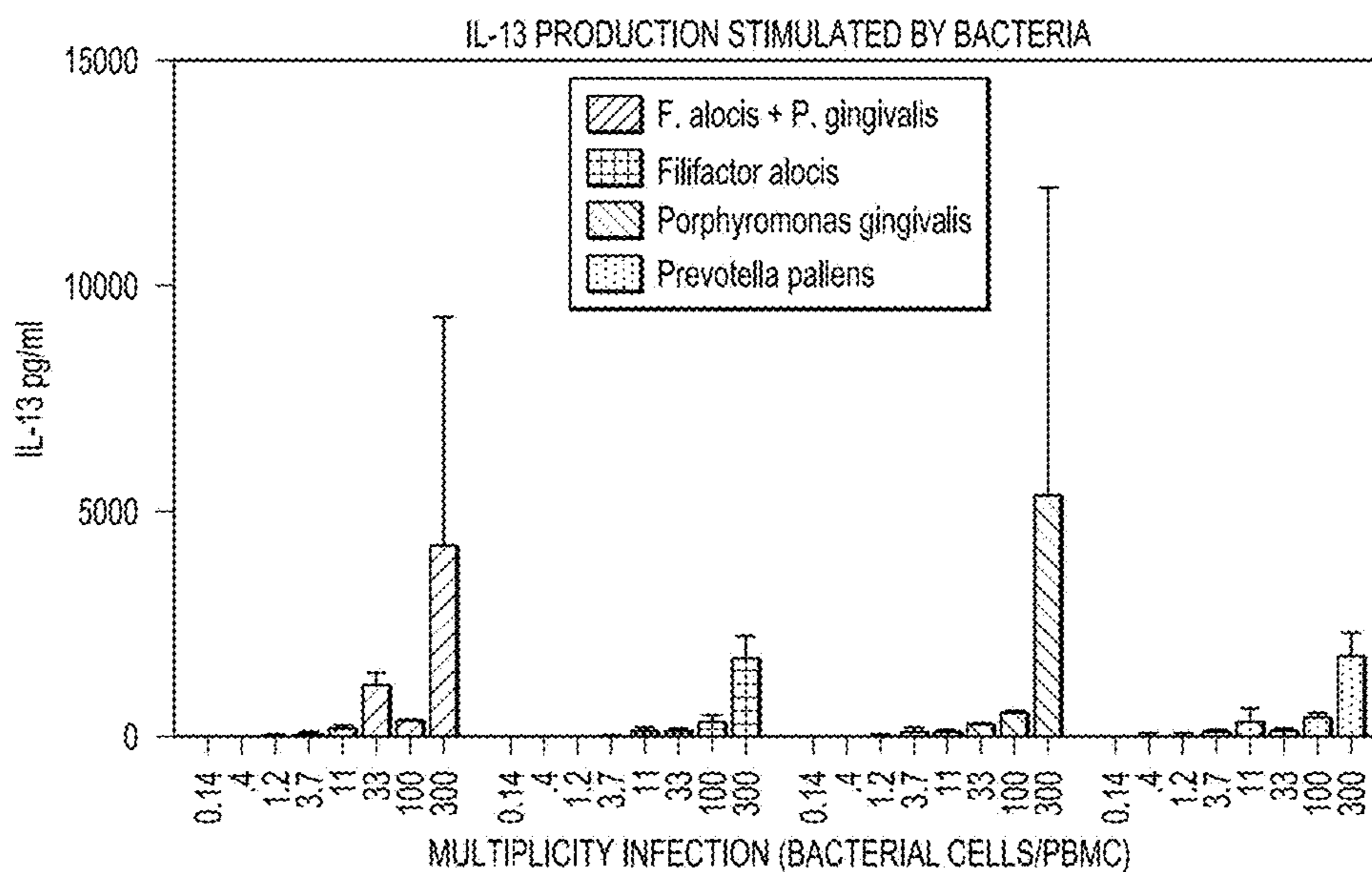


	F. alocis + P. gingivalis	Filifactor alocis	Porphyromonas gingivalis	Prevotella pallens
EC50	484.0	256.0	156.8	-1.846e+006

FIG. 37B

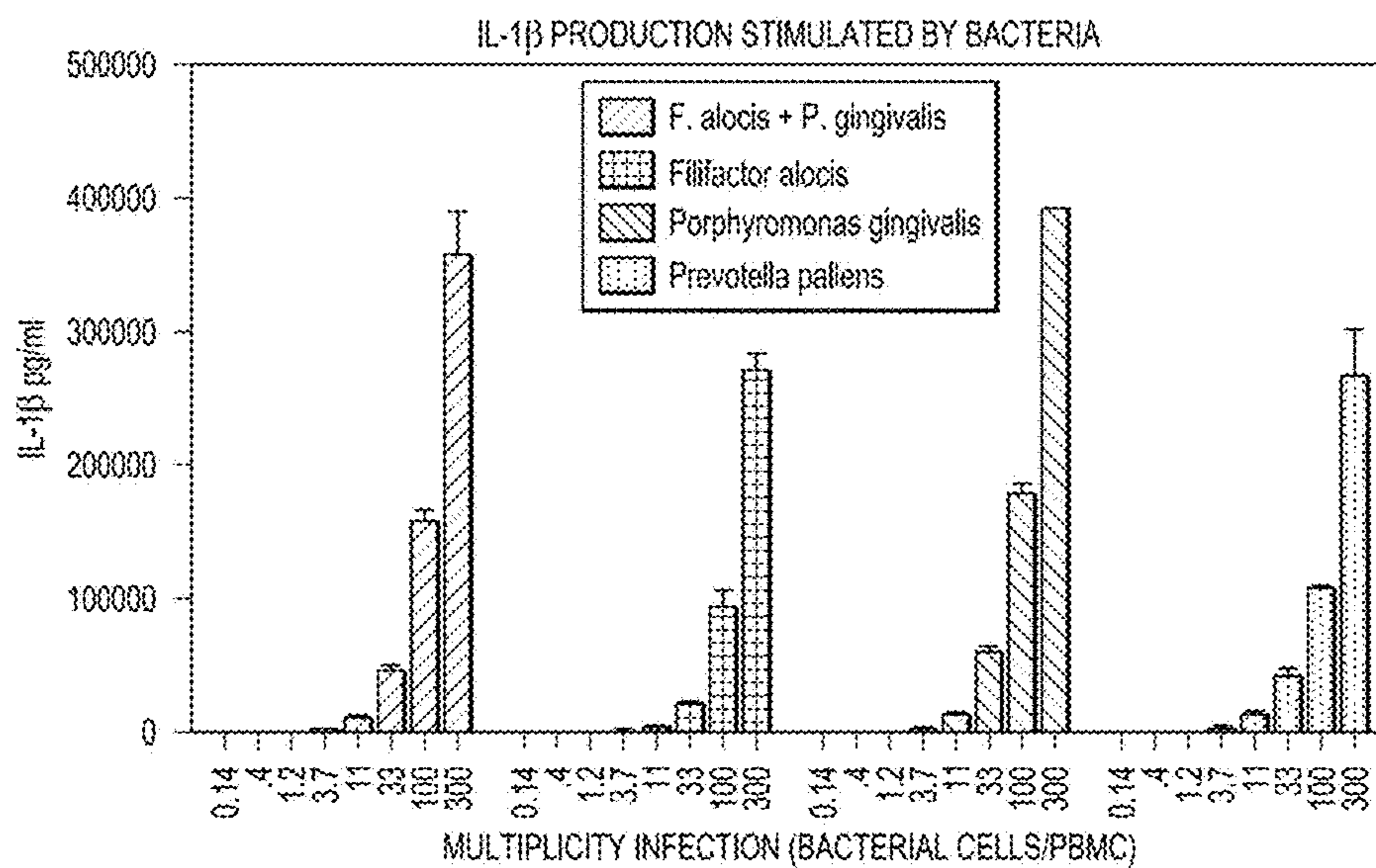


	F. alocis + P. gingivalis	Filifactor alocis	Porphyromonas gingivalis	Prevotella pallens
EC50	-76013	64.81	24.80	113.5

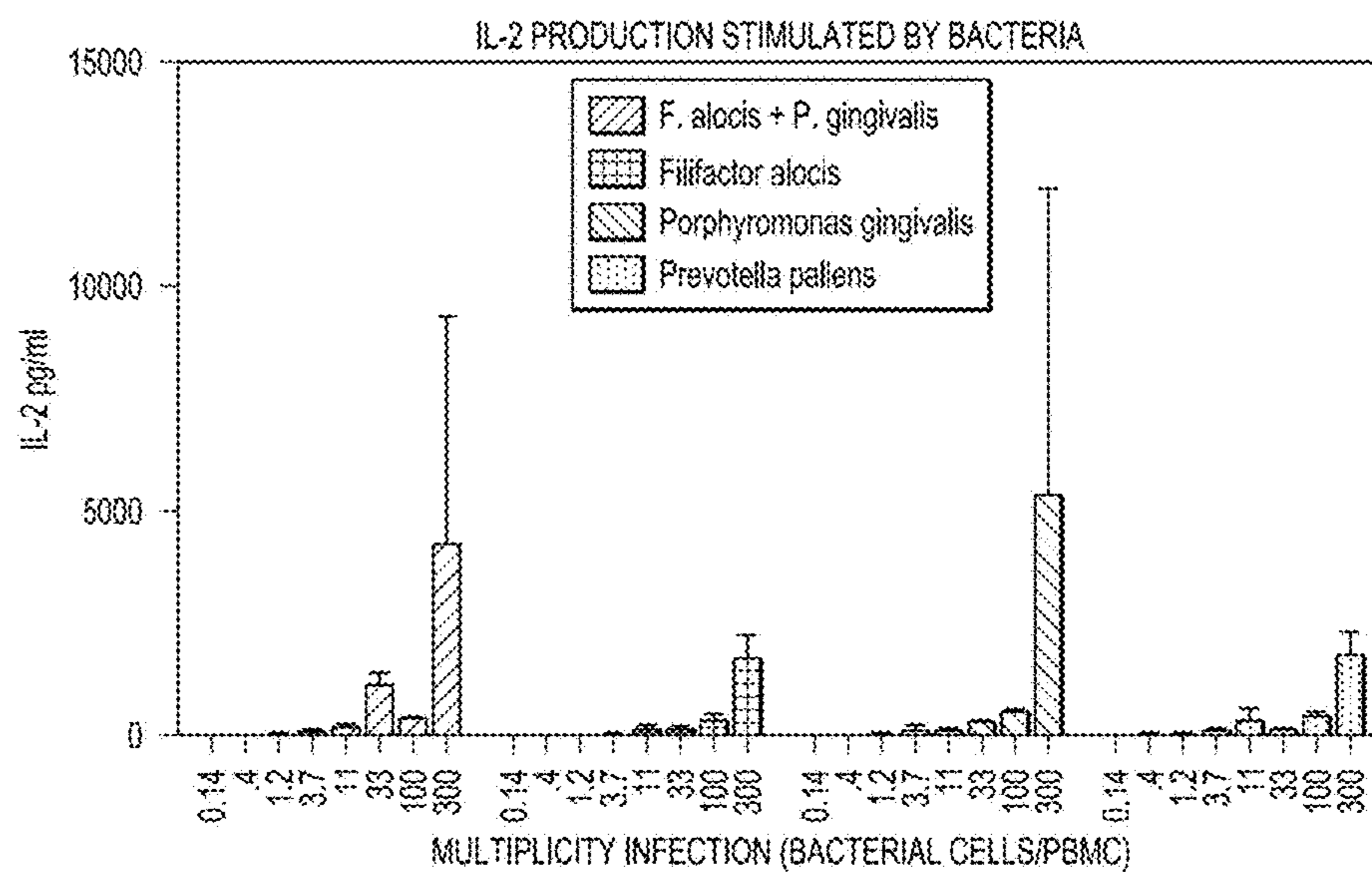


	F. alocis + P. gingivalis	Filifactor alocis	Porphyromonas gingivalis	Prevotella pallens
EC50	-1.041e+006	81.19	-185415	-938807

FIG. 37C

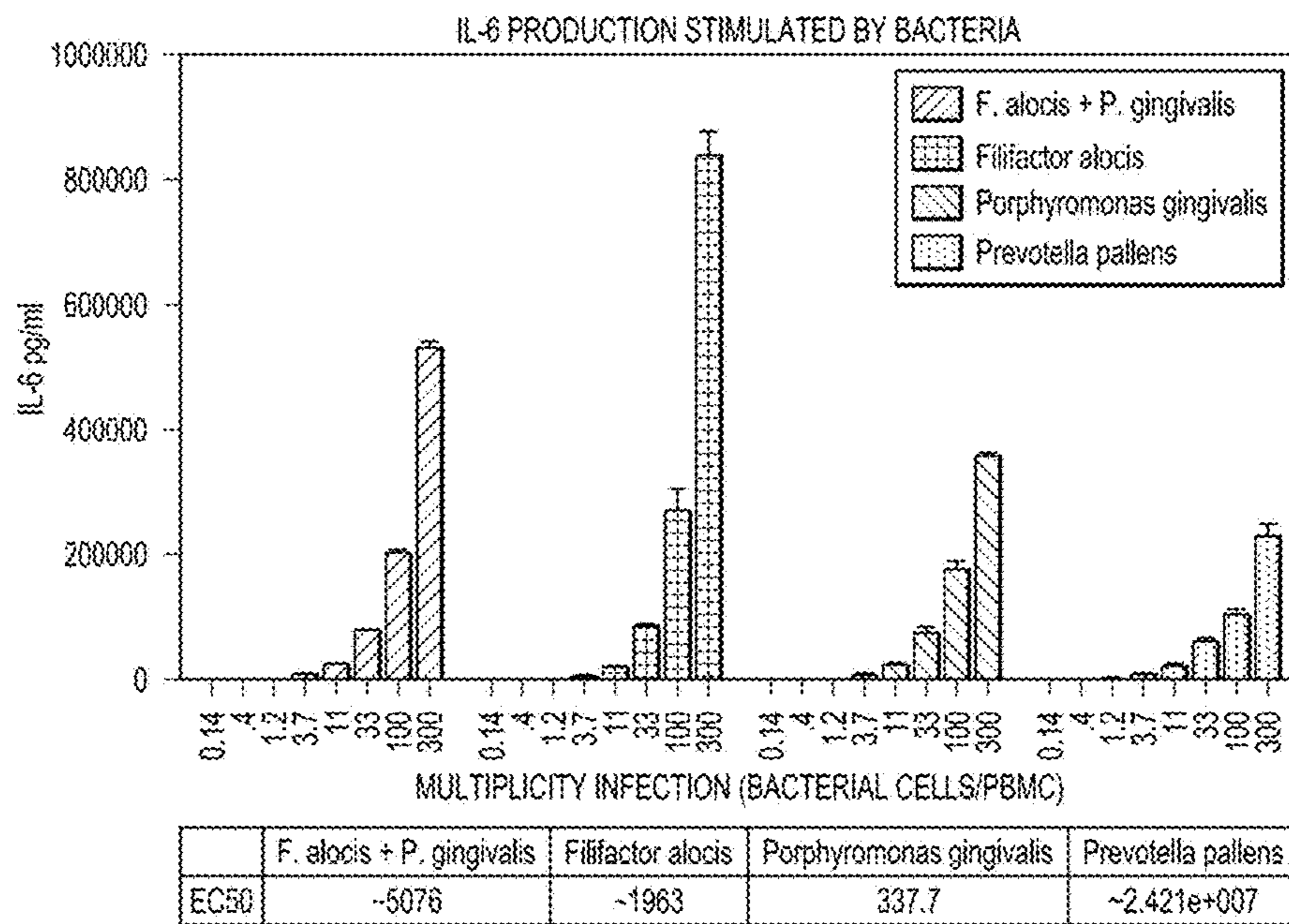
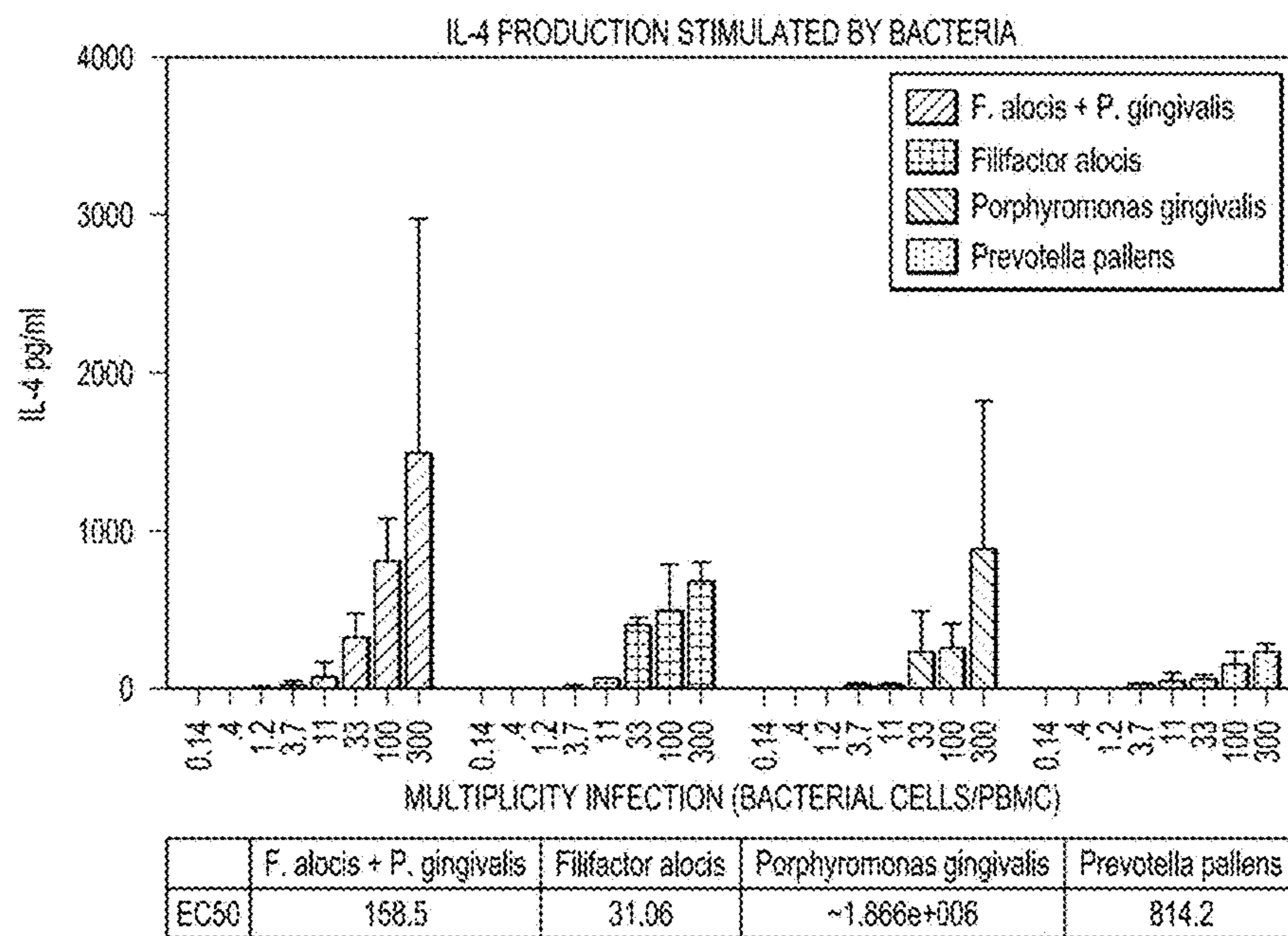


	F. alocis + P. gingivalis	Filifactor alocis	Porphyromonas gingivalis	Prevotella pallens
EC50	211.6	262.6	236.1	~1837

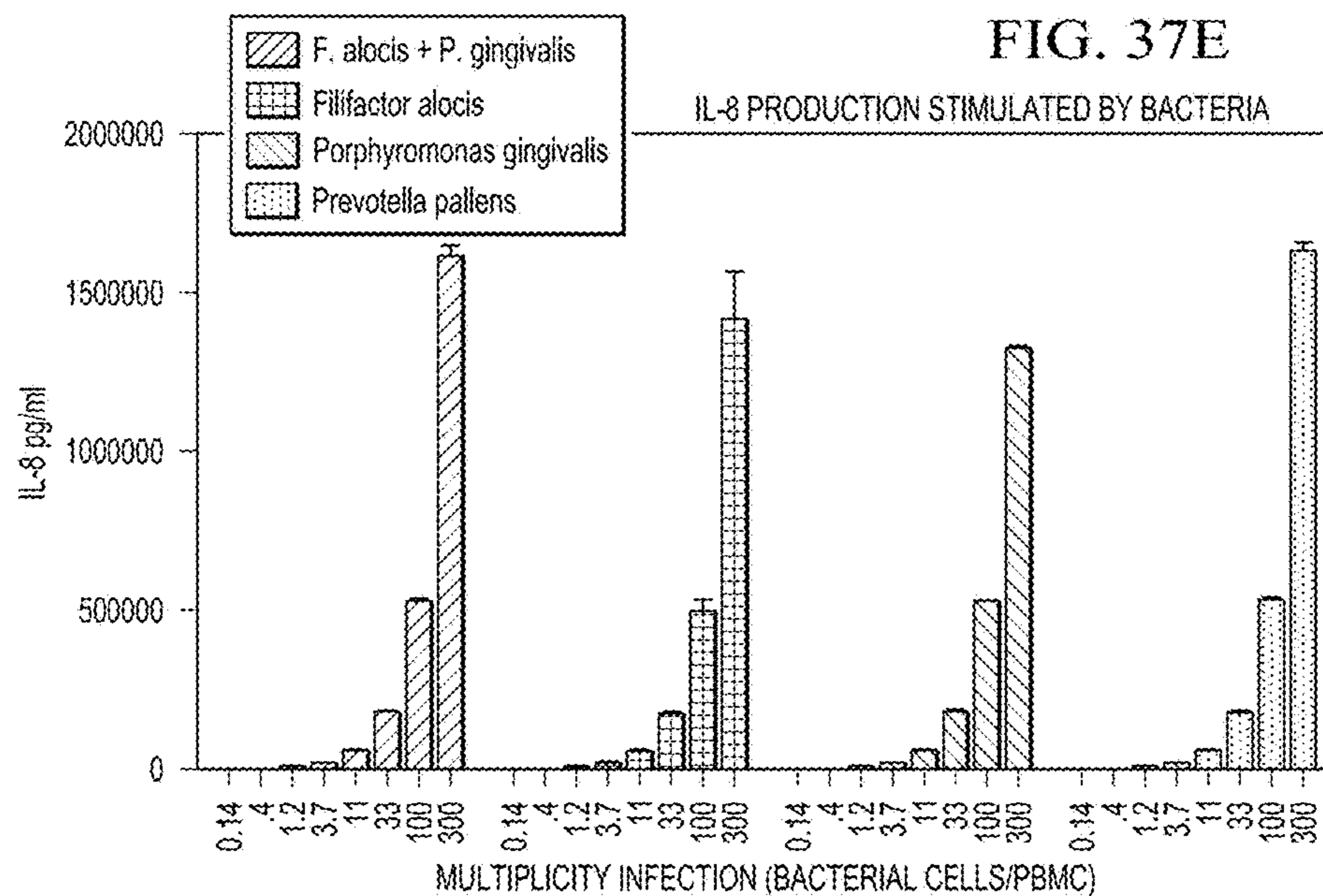


	F. alocis + P. gingivalis	Filifactor alocis	Porphyromonas gingivalis	Prevotella pallens
EC50	~2540	~37557	~5151	~45485

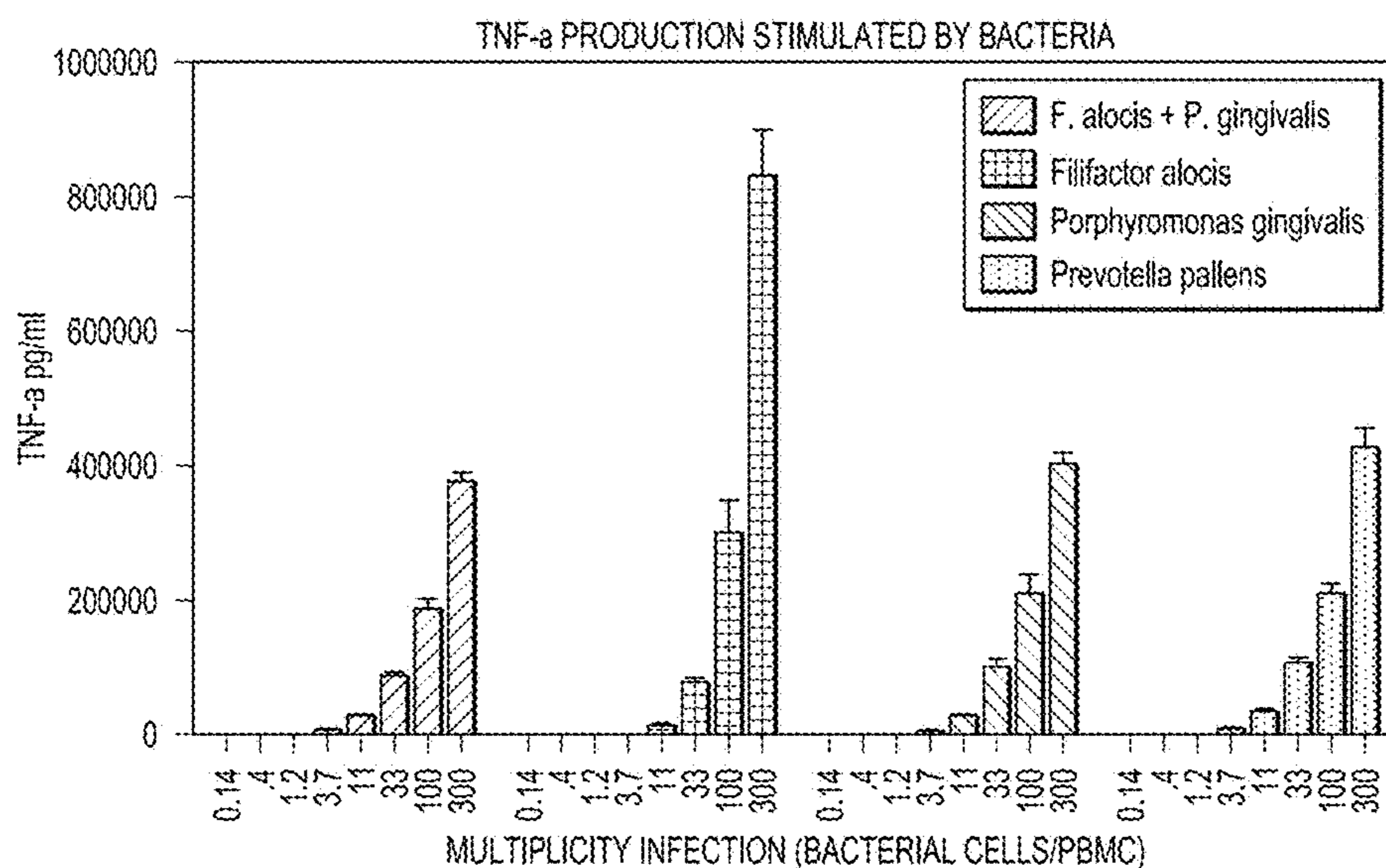
FIG. 37D



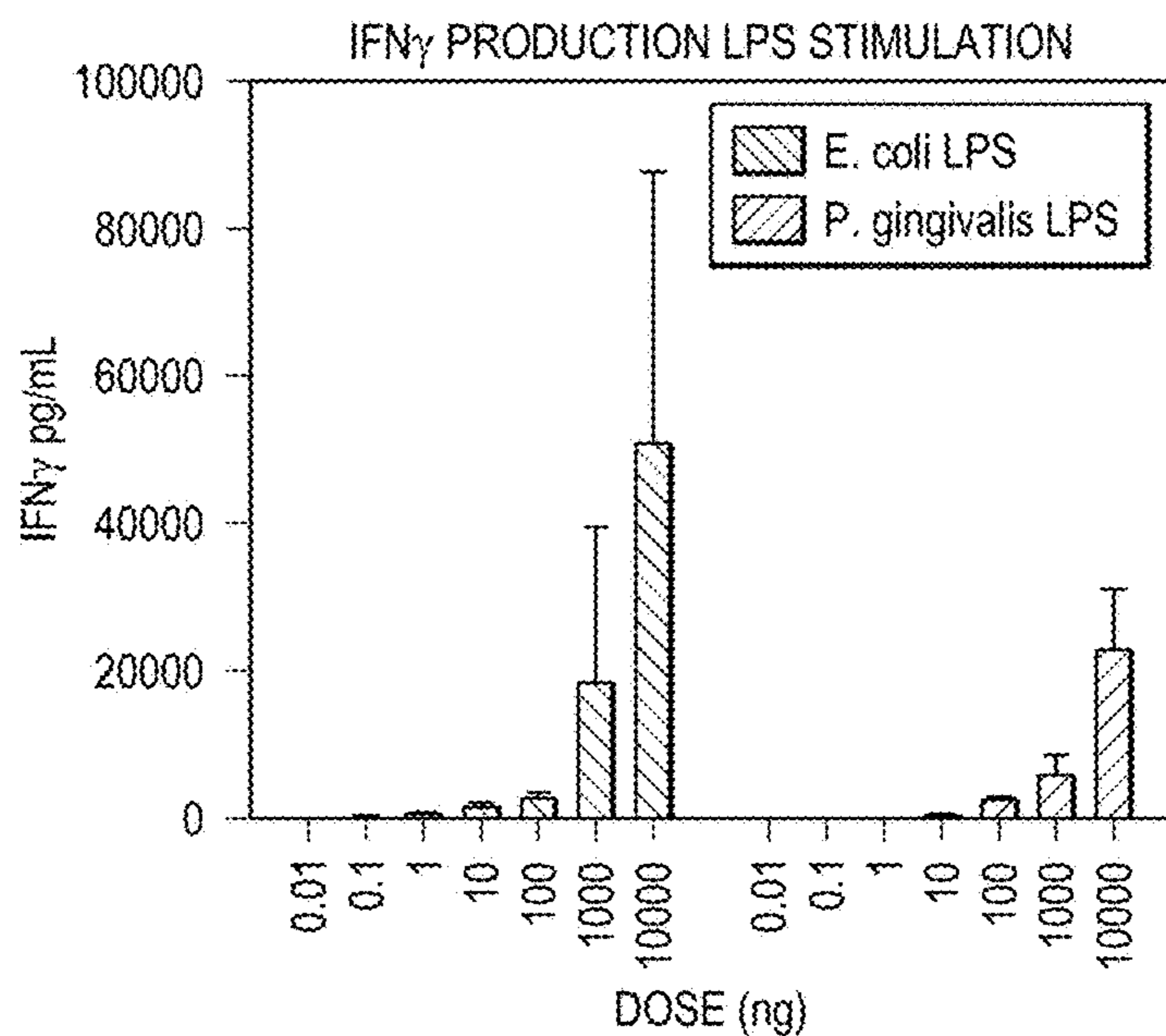




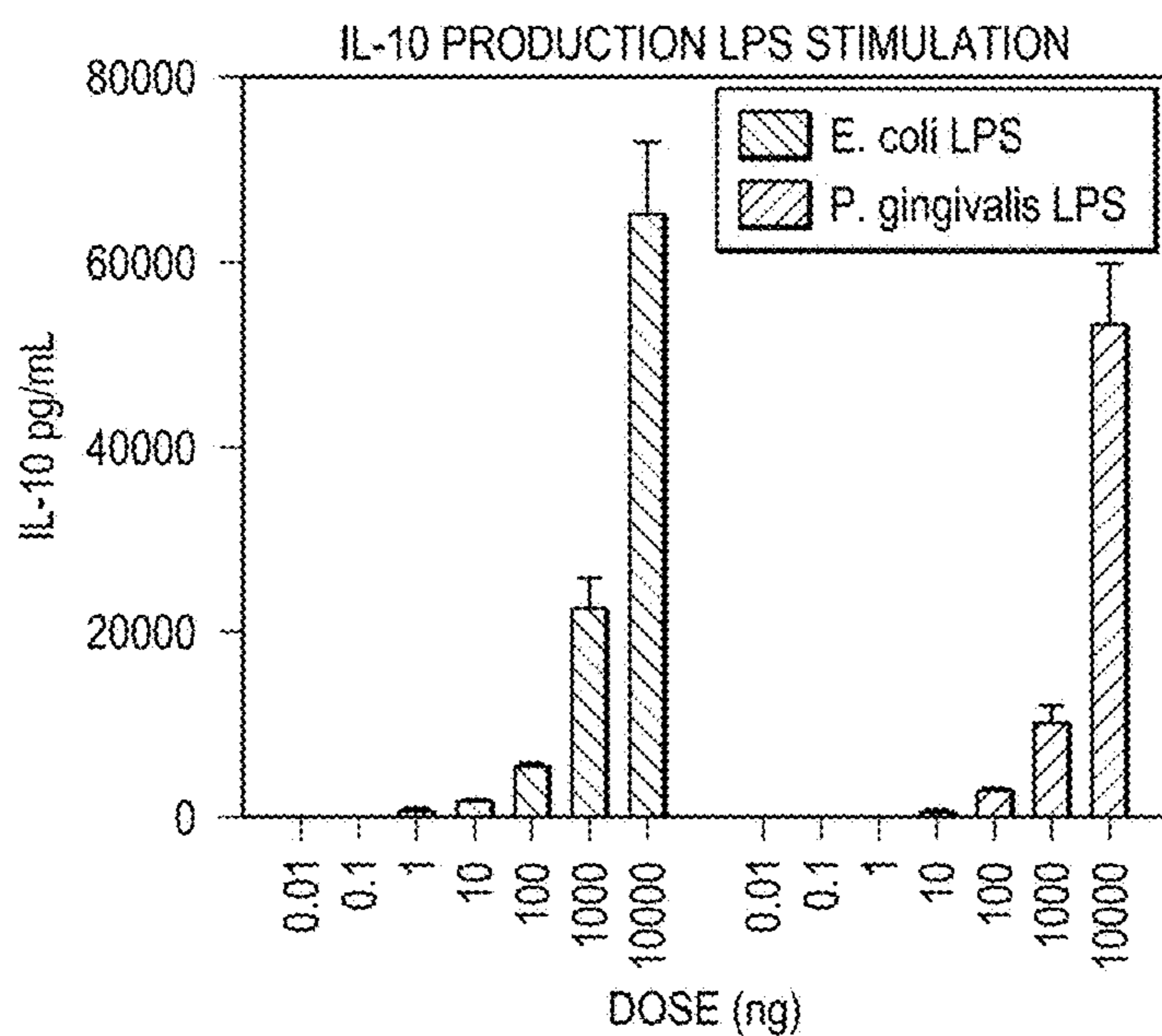
	F. alocis + P. gingivalis	Filifactor alocis	Porphyromonas gingivalis	Prevotella pallens
EC50	385909	~36413	691.6	321206



	F. alocis + P. gingivalis	Filifactor alocis	Porphyromonas gingivalis	Prevotella pallens
EC50	465.3	305.7	305.2	1168

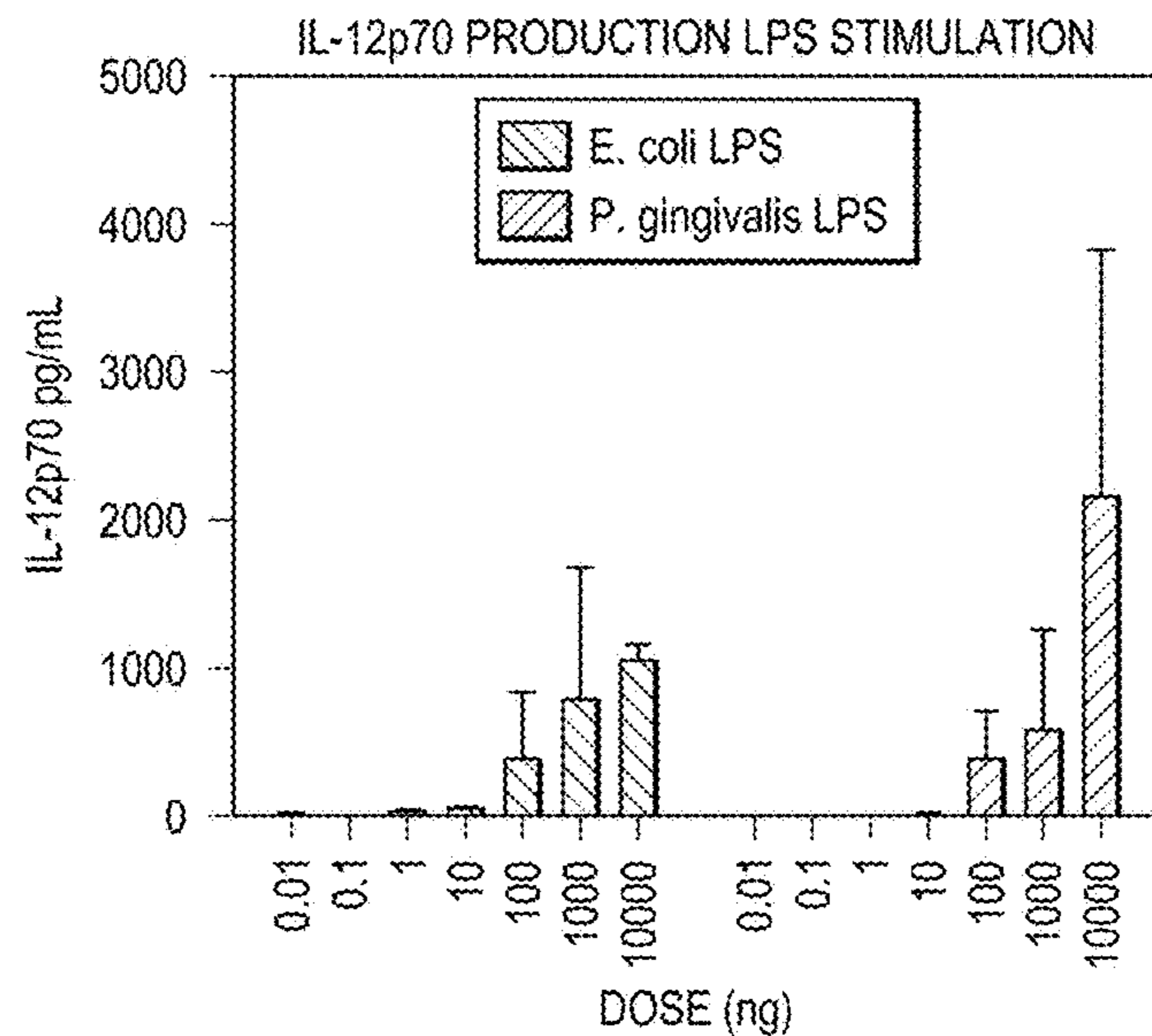


	E. coli LPS	P. gingivalis LPS
EC50	2541	$\sim 1.101e+010$

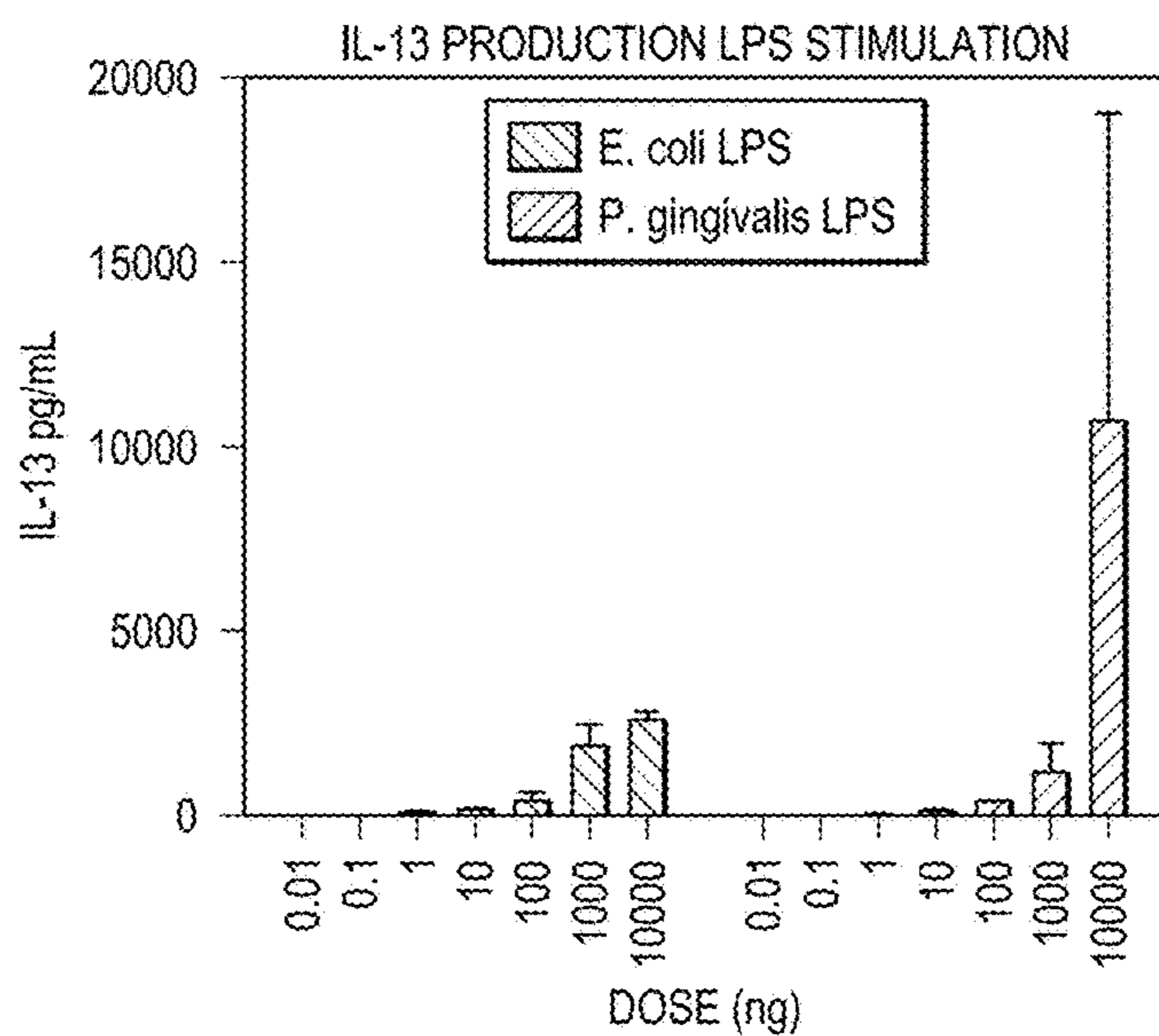


	E. coli LPS	P. gingivalis LPS
EC50	8355	$2.865e+008$

FIG. 38A

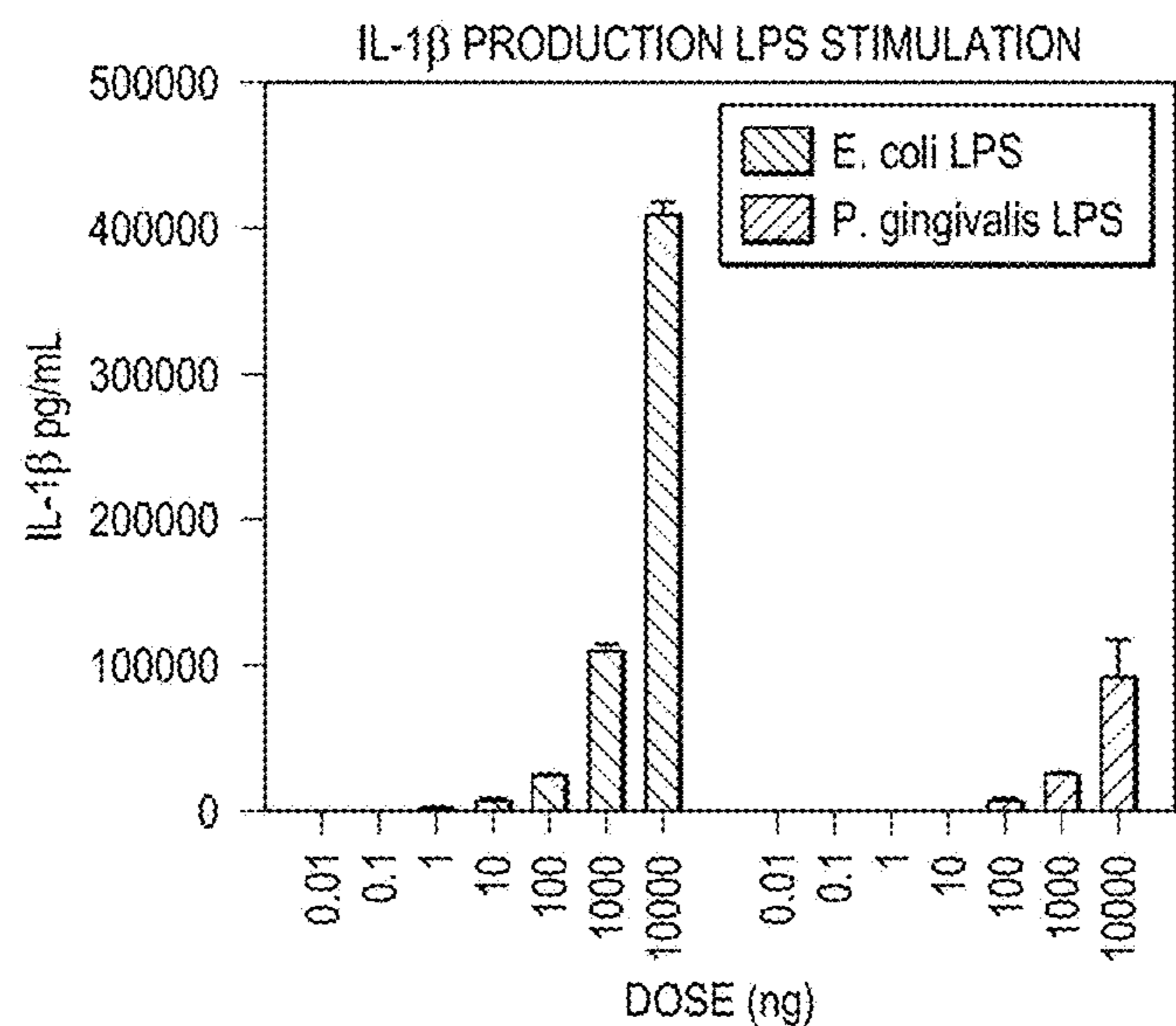


	E. coli LPS	P. gingivalis LPS
EC50	282.1	-7.326e+010

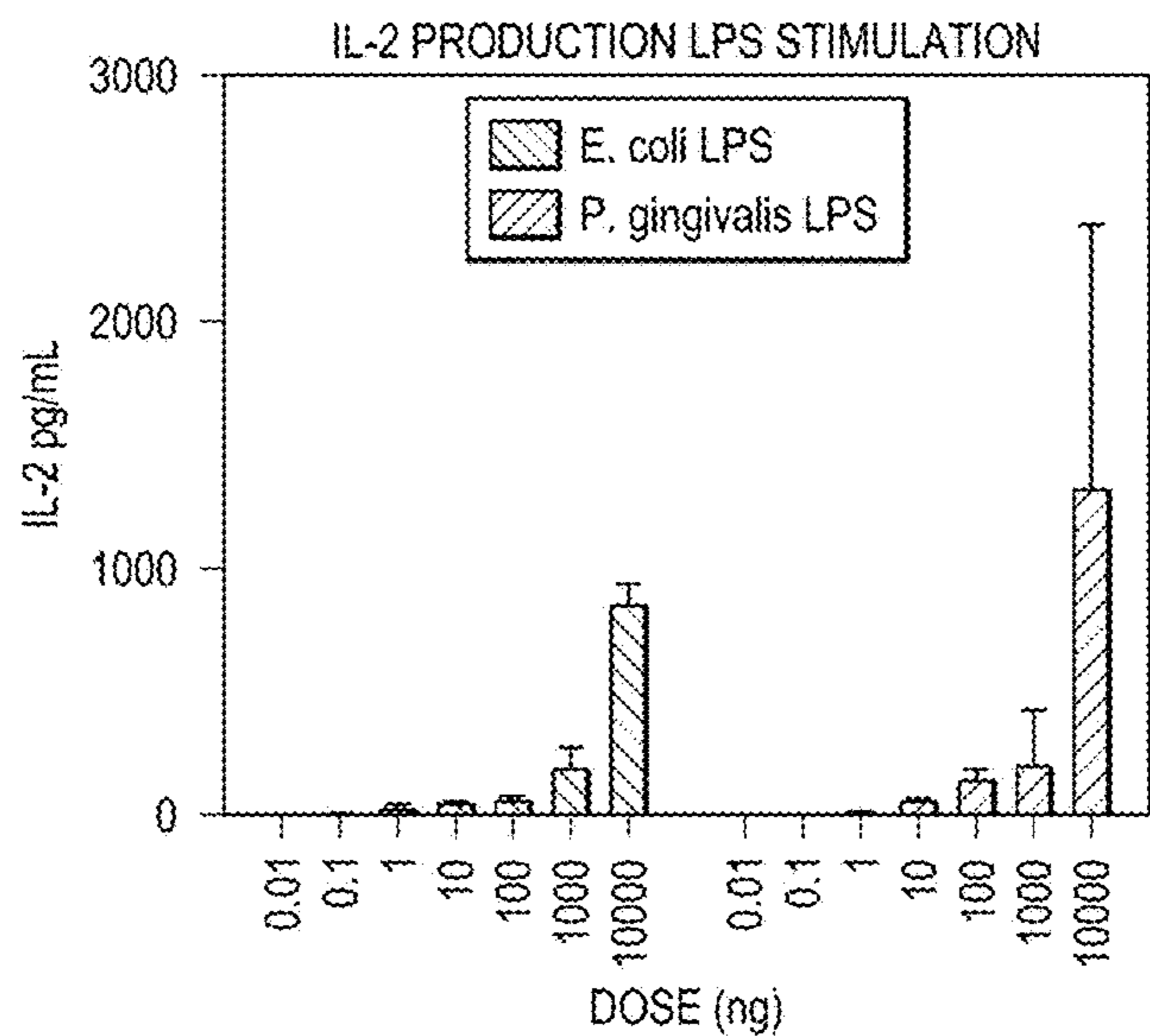


	E. coli LPS	P. gingivalis LPS
EC50	494.6	9.848e+006

FIG. 38B

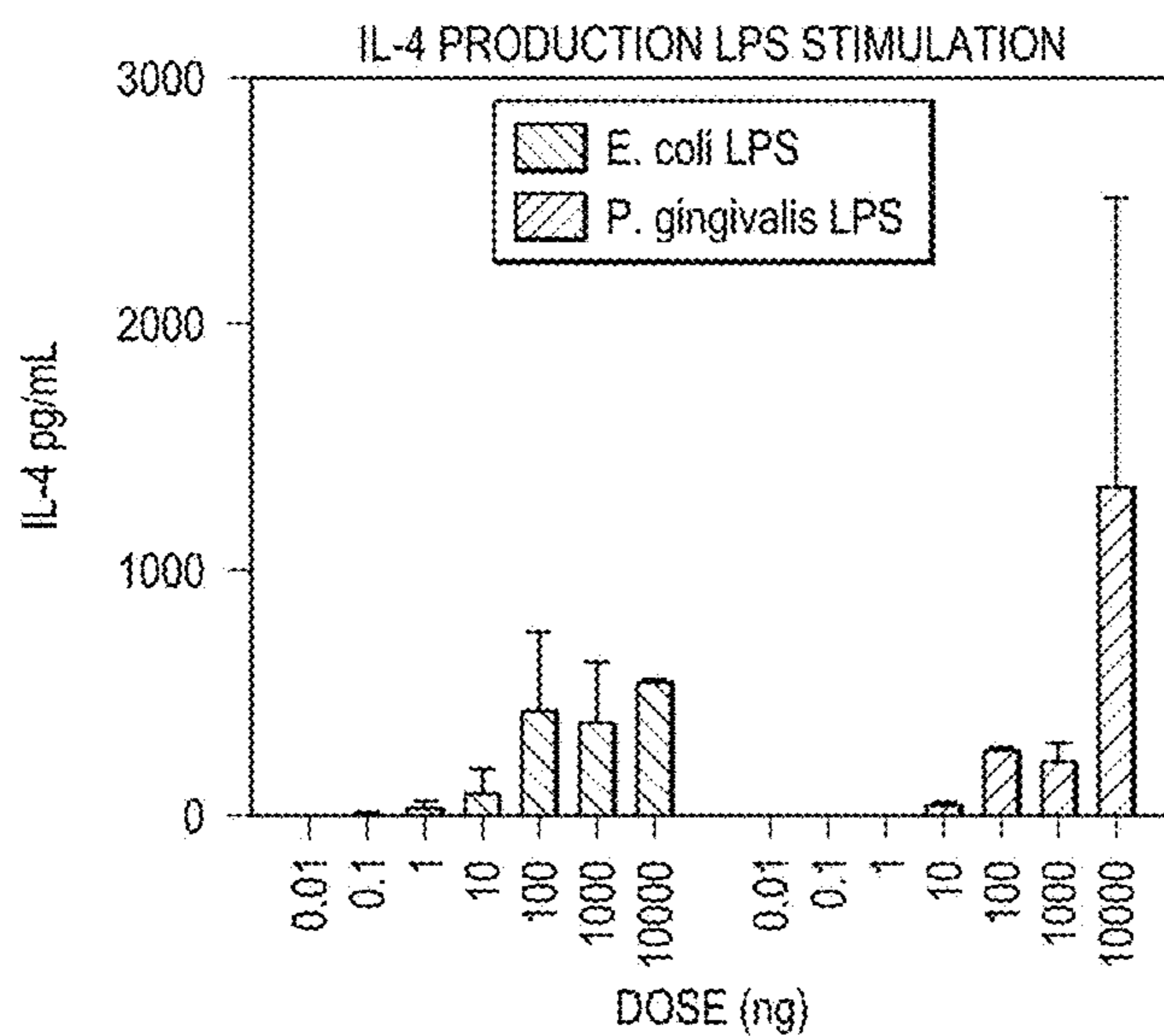


	E. coli LPS	P. gingivalis LPS
EC50	~57882	-7.853e+006

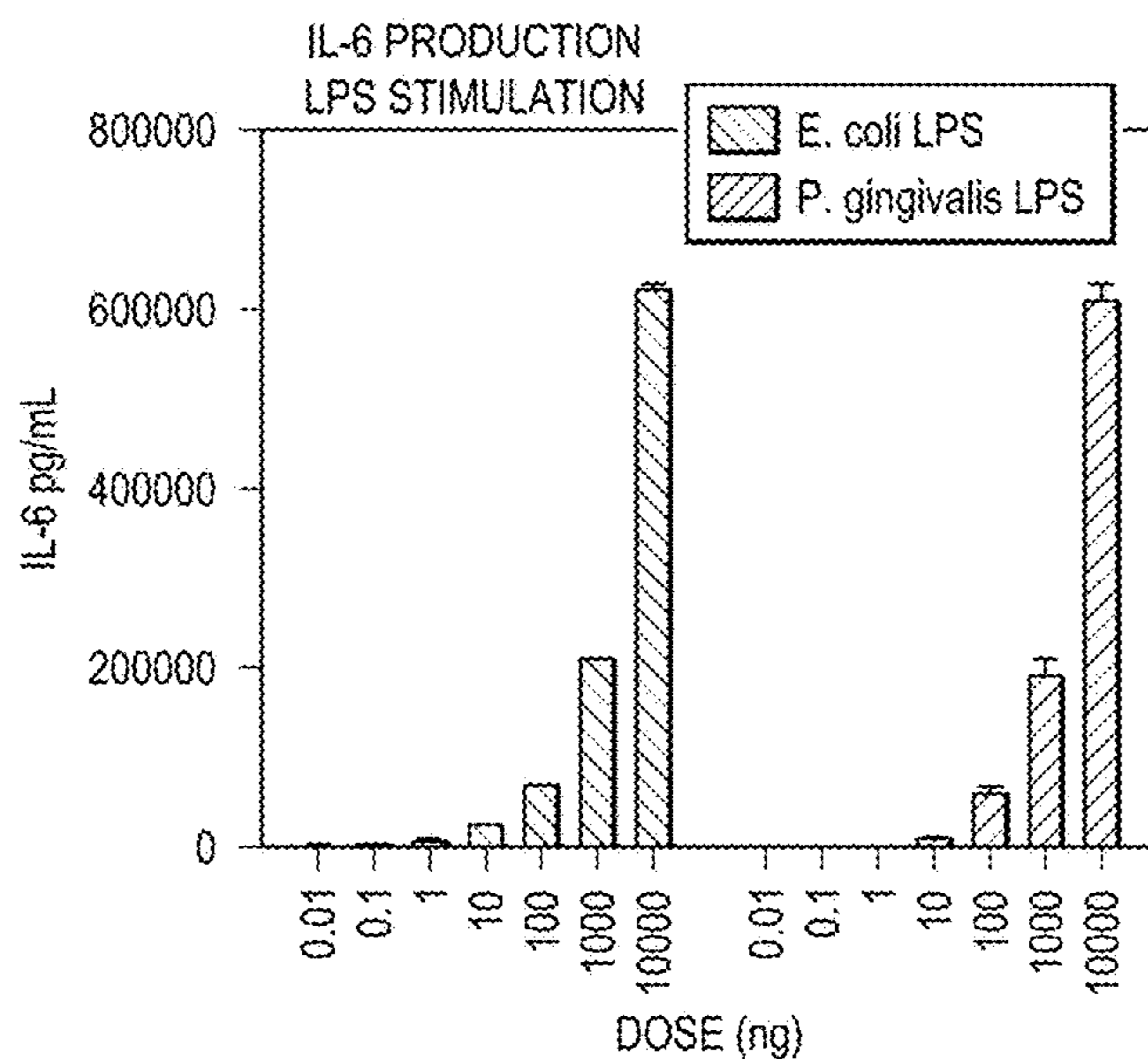


	E. coli LPS	P. gingivalis LPS
EC50	~4.806e+008	~7.437e+007

FIG. 38C

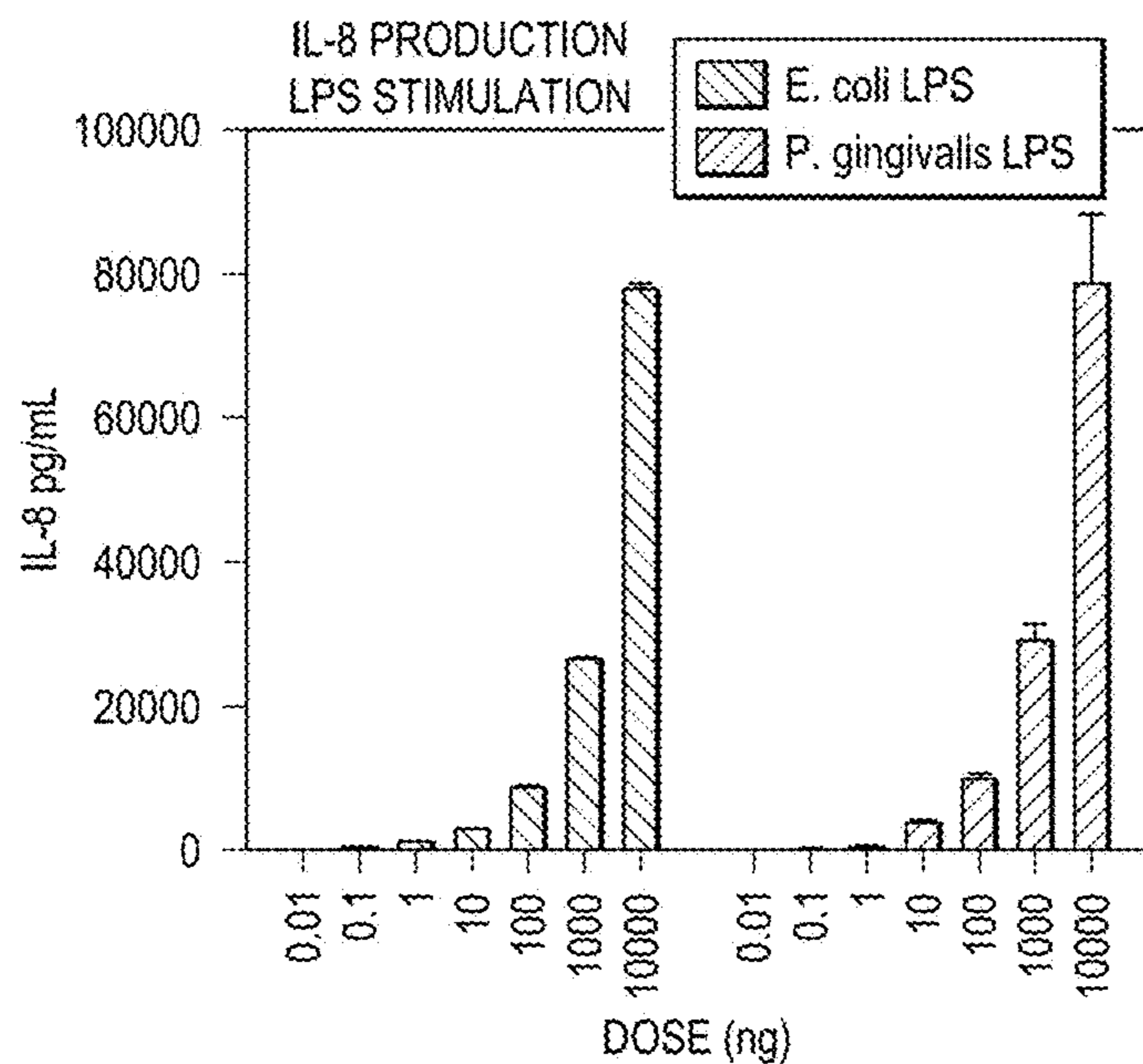


	E. coli LPS	P. gingivalis LPS
EC50	282.1	-7.326e+010

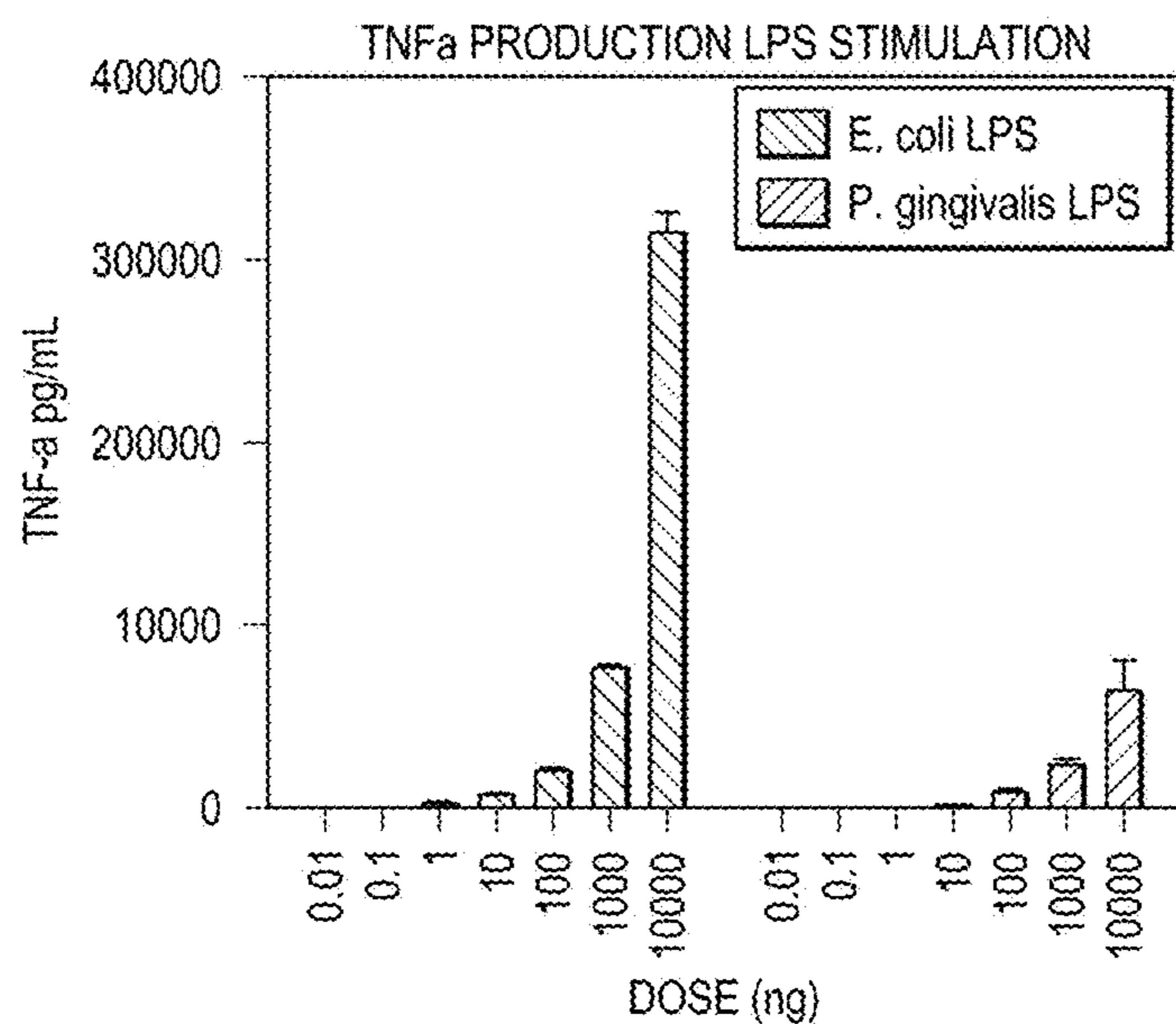


	E. coli LPS	P. gingivalis LPS
EC50	~2.229e+006	~859657

FIG. 38D



	E. coli LPS	P. gingivalis LPS
EC50	~3.300e+006	~516645



	E. coli LPS	P. gingivalis LPS
EC50	8.922e+008	~181521

FIG. 38E

## DETOXIFICATION OF MICROBIAL VIRULENCE FACTORS IN ORAL CAVITY

### FIELD OF THE INVENTION

The invention relates to a method for identifying agents that detoxify 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 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 to obtain a sufficient quantity of LTA or LPS for detection. Thus, their invention lacks the ability to detect the non-culturable species present, and would not allow for measuring the 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 were unable to quantify the level of virulence of the endo-

toxin. Further, their invention limited them to the Gram-negative endotoxin, as the LTA is not detectable via the LAL kit.

TLR signal transduction involves association of the chaperonin 10 with a Toll-like receptor in an activation cluster. In US Pub. No. 2009/0047240, the chaperonin 10 (Cpn10) antibodies were used to modulate the clustering of Cpn10 in a cell line, murine RAW264, thus changing the signal transduction of TLR. 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 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 cytokine. The methods outlined in '544 were predicated on the workings of Dongari-Bagtzoglou and Ebersole in *J. Perio. Res.* 31: 90-8 1996, where Dongari showed that human gingival fibroblast cells could be used to screen bacteria for an inflammatory response by measuring the production of either IL-1 beta, IL-6, or IL-8 cytokines. The '544 application was analogous to the work of Madiano et al., in *J. Clinical Perio.* 32: 57-71 2005, where Madiano reviewed the literature around bacterial virulence factors and disclosed the concept of gingival cells as methods to determine the anti-inflammatory properties of multiple bacterial components from several species of bacteria. Their system proposes the use of indicator materials (cytokines) generated from the host immune response by the activation of any Toll-like receptor. Though their proposed system may give rise to a cytokine, as was demonstrated in the literature (Shapira et al., *J. Dent. Res.* 76: 1381-6 1997), their proposed invention would not allow for quantification of the specified cytokine, nor would it allow for determination of the virulence potential of the bacterial components. Thus, what is needed is an invention that can quantify virulence and determine level of potency.

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 for identifying agents for detoxifying oral virulence factors is provided that comprises providing a reporter cell that expresses at least one of TLR2 or TLR4; providing an oral virulence factor; providing a potential detoxifying agent; combining oral virulent factor and potential detoxifying agent with reporter cell; determining EC50 of virulence factor.

A method to identify detoxifying agents that bind to at least one of lipopolysaccharide or lipoteichoic acid is provided that comprises providing a reporter cell that expresses at least one of TLR2 or TLR4; providing at least one of lipopolysaccharide or lipoteichoic acid; combining at least one of lipopolysaccharide or lipoteichoic acid and potential detoxifying agent with reporter cell; measuring binding of potential detoxifying agents using at least one of BODIPY method or LAL method of low EC50 lipopolysaccharide or lipoteichoic acid; and determining EC50 of at least one of lipopolysaccharide or lipoteichoic acid.

An oral care composition is provided that comprises a detoxifying agent that is at least one of myristyl-gamma-picolinium chloride; potassium linoleate; Sodium hexadecyl sulfate; fenugreek; steapyrium chloride; alpha.-glyceryl monooloeate; stearyldiethanolamine; Dimethylaminopropylaurylamide; Stearylamidopropyl dimethylamine; linoleamide; N-Lauryldiethanolamine; Steartrimonium chloride; hexaethylene glycol, monodecyl ether; Laureth-6; Laureth-9; Quaternium-17; Bromoenol lactone; Oleyl amine; cetrimonium chloride; or palmitamide monoethanolamine.

#### BRIEF DESCRIPTION OF THE DRAWINGS

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

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

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

FIG. 1D has a graph showing secretion of LPS during bacterial growth in culture at 24 hours.

FIG. 1E has a graph showing the amount of LPS per bacterium during bacterial growth in culture at 24 hours.

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

FIG. 2B has a graph showing bacteria activation of the TLR4 reporter gene in culture.

FIG. 2C has a graph showing the supernatant of bacterial growth activates TLR4 reporter gene in culture.

FIG. 3 has a graph showing stannous fluoride inhibited TLR4 reporter gene expression stimulated by *E. coli* LPS.

FIG. 4 has a graph showing stannous fluoride prevented *P. gingivalis* LPS from activating TLR2 reporter gene expression.

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

FIG. 6 has graphs showing EC50 determination of subgingival plaques that activate TLR4-SEAP signal transduction.

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

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

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

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

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

FIG. 9 has graphs showing EC50 determination of subgingival plaques that activate TLR2-SEAP signal transduction.

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

FIG. 11 has graphs showing cell culture and virulence determination of flagellins from different bacteria.

FIG. 12 has graphs showing LPS Stimulated TLR Activity in Human Monocytes THP-1. THP-1 cells produce several functional TLR receptors.

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

FIG. 14 has graphs showing clinical measurements.

FIG. 15 has a graph showing changes in the abundance of the total amount of DNA in the marginal gingival plaques.

FIG. 16A has graphs showing decreases in bacterial abundance in the supragingival plaques during the six week of regimen treatment.

FIG. 16B has graphs showing decreases in relative abundance of bacteria in the supragingival plaques during the six week of regimen treatment.

FIG. 17 has a graph showing citrulline concentrations decreased in Buccal-brush samples over the period of six-week regimen treatment.

FIG. 18 has a graph showing protein bound ornithine in Buccal brush samples decreased during treatment.

FIG. 19 has a diagram showing enzymes in the ornithine, citrulline and arginine cycle.

FIG. 20 has graphs showing expression of ornithine, citrulline and arginine genes was changed during a 6-week treatment.

FIG. 21 has a graph showing citrulline concentrations increased in Buccal-brush samples in experimental gingivitis.

FIG. 22 has graphs showing protein bound citrulline in Buccal brush samples decreased in experimentally induced gingivitis.

FIG. 23 has graphs showing concentrations of protein bound ornithine increased in Buccal-brush samples in experimentally induced gingivitis.

FIG. 24 has graphs showing concentrations of total ornithine increased in Buccal-brush samples in experimentally induced gingivitis.

FIG. 25 has graphs showing concentrations of protein bound arginine decreased in Buccal-brush samples in experimentally induced gingivitis.

FIG. 26 has graphs showing concentrations of total arginine decreased in Buccal-brush samples in experimentally induced gingivitis.

FIG. 27 has graphs showing citrulline inhibited cytokine production stimulated by *P. gingivalis* LPS 1690 in THP-1 cells.

FIG. 28 has a graph showing bacterial LTA reduces fluorescent intensity of BC fluorescent dye in a dose dependent manner.

FIG. 29 has a graph showing LPS reduces the fluorescent intensity of BC fluorescent dye in a dose-dependent manner.



FIG. 30 has a graph showing the supernatant of bacterial culture reduces the fluorescent intensity of BC fluorescent dye in a dose-dependent manner.

FIG. 31 has a graph showing bacteria reduce the fluorescent intensity of BC fluorescent dye in a dose-dependent manner.

FIG. 32 has a graph showing stannous fluoride binds to *E. coli* LPS. Binding of stannous fluoride to LPS was examined using MALDI-TOF mass spectrometry.

FIG. 33 has graphs showing stannous fluoride prevents LPS from activation of TLR-reporter gene in THP-1 cells.

FIG. 34 has graphs showing stannous fluoride inhibits LPS from stimulating production of cytokines in human primary monocytes.

FIG. 35 has a graph showing stannous fluoride removes suppression of LPS on production of cytokines in human primary monocytes.

FIG. 36 has pictures showing *E. coli* LPS binding to TLR4 receptors.

FIG. 37A has graphs showing bacteria stimulated production of proinflammatory cytokines in human primary blood mononuclear cells.

FIG. 37B has graphs showing bacteria stimulated production of proinflammatory cytokines in human primary blood mononuclear cells.

FIG. 37C has graphs showing bacteria stimulated production of proinflammatory cytokines in human primary blood mononuclear cells.

FIG. 37D has graphs showing bacteria stimulated production of proinflammatory cytokines in human primary blood mononuclear cells.

FIG. 37E has graphs showing bacteria stimulated production of proinflammatory cytokines in human primary blood mononuclear cells.

FIG. 38A has graphs showing LPS stimulated production of proinflammatory cytokines in human primary blood mononuclear cells.

FIG. 38B has graphs showing LPS stimulated production of proinflammatory cytokines in human primary blood mononuclear cells.

FIG. 38C has graphs showing LPS stimulated production of proinflammatory cytokines in human primary blood mononuclear cells.

FIG. 38D has graphs showing LPS stimulated production of proinflammatory cytokines in human primary blood mononuclear cells.

FIG. 38E has graphs showing LPS stimulated production of proinflammatory cytokines in human primary blood mononuclear cells.

#### DETAILED DESCRIPTION OF THE INVENTION

As disclosed herein, it was surprisingly discovered that the invention 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 present invention can use non-gingival engineered cells to determine the oral cavity state of health by detecting the level of bacterial toxins, such as endotoxins. Additionally, the way a host responds to the virulence factors can be determined by utilizing the host's metabolic pathways, such as quantifying products of the urea cycle.

Virulence factors are molecules produced by a microbe, such as a bacteria, fungi, or protozoa, or is a microbial agent, such as a virus, both of which contribute to the pathogenicity

of the microbe or microbial agent with regard to a host inflammatory response. Non-limiting examples of virulence factors include one or more of the following: lipopolysaccharide from gram negative bacteria; lipoteichoic acid from gram positive bacteria; nucleic acids from bacteria, such as DNA and/or RNA; proteins and/or enzymes from microbes, such as a protease, lipase, or coagulase; guanosine triphosphate hydrolase enzyme hijacking agents, such as *Salmonella* protein SopE, which may accelerate host cell colonization; fimbriae; flagellum; adhesions; exopolysaccharide; exotoxins, such as tetanus toxin; siderophores; cytotoxin; superantigens; and pili.

Detoxification, detoxify, and/or detox as used herein refer to the inactivation of virulence factors. Non-limiting examples of virulence factors are lipopolysaccharide or endotoxin from gram negative bacteria; or lipoteichoic acid from gram positive bacteria. The inactivation of a virulence factor is defined as the virulence factor losing its ability to stimulate a host immune response. A non-limiting example of the stimulation of the host immune response would be the activation of one or more of the Toll-like Receptors, such as TLR-4, where lipopolysaccharide may have a level of activation on this receptor. A reduction of the activation of this receptor by 30% or more would fall under the definition of detoxification. Further, detoxification may be the removal of the virulence factor from the system.

Toll-like receptors (TLR) are a class of proteins in eukaryotic cells that play a role in the immune response. The class currently has 13 proteins identified and may grow as research expands in this area. They are typically abbreviated TLR-#, where the # is 1 to 13. Some of these are membrane-spanning proteins that reside on the surface of the eukaryotic cell and allow a response to the environment around the cell. Specific responses include the detection of pathogenic bacterial components, such as lipopolysaccharide from gram negative bacteria. Additionally, some of the TLR proteins have been referred to as pattern recognition receptors or pathogen-associated molecular patterns (PAMPs).

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 it 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 is needed to educate consumers on the efficacy of their oral hygiene, which is lacking in the art.

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. The methodologies allow understanding of what virulence factor types are present in the dental plaques, and how the host responds. Importantly, those measurements provide a detailed assessment on the

severity of gingivitis in terms of virulence factors of the microbes in dental plaques and healthy status in the host. In addition, these methods help evaluate the effectiveness of a technology in preventing and treating gingivitis.

The methodology of the present invention includes: (1) using an 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 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) determining citrulline activities in inhibiting LPS-induced production of proinflammatory cytokines, (6) measuring binding of  $\text{SnF}_2$  to LPS, and (7) determining the effective dose 50 of bacteria and LPS on stimulating cytokine production in human peripheral blood mononuclear cells.

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 type of execution of the described methods would allow for consumers to ascertain the state of their dental health at home or allow professionals, such as dentists, to rapidly measure the state of a patient's oral health.

In practicing the present invention, a strip of material may be applied to the desired oral surface by the wearer. The side of the material facing the oral surface is at least the side wherein the composition herein is applied. This oral care composition provides a vehicle for the active as well as tackiness between the oral surfaces and the strip of material, holding the strip of material in place for extended periods of time. The period over which the strip of material is used may be, for example, from about one to about thirty minutes.

A strip could comprise one or more layers where the first layer may contain an antibacterial agent and biofilm/plaque dissolving agent, such as an essential oil. The first layer would dissolve leaving a second layer containing the detoxifying agent. The third layer would comprise a backing agent. Alternatively, the first two layers may be entirely dissolving without the need for a backing agent. In addition in certain embodiments, the strip may be a single dissolvable layer containing a detoxifying agent.

The strip of material readily conforms to the oral care surface by lightly pressing it there against. The strip of material is easily removed by the wearer by peeling it off using a finger or fingernail. In certain embodiments each successive treatment uses a fresh strip of material.

In the situation where the oral care surface is the surface of teeth, it is not necessary to prepare the teeth surface before applying the delivery system of the present invention. For example, the wearer may or may not choose to brush his teeth or rinse his mouth before applying the delivery system. The surfaces of the teeth are not required to be dried or to be excessively wet with saliva or water before the strip of material is applied.

The delivery system herein may optionally be applied with two fingers to the oral surface in need of treatment and allowed to remain in place for at least five minutes. The

delivery system may be applied to cover at least a portion of a tooth and at least a portion of the adjoining soft tissue (gum) area.

The methods described above can be used to determine the cellular impact of organic and inorganic molecules, for example if there is an interaction between the molecule and the targeted receptor. Further, the methods of the present invention could be used to ascertain if a molecule would cause an irritation or inflammatory response, if the receptor for that response is known. The receptor in question could 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 could be determined on the receptor associated with the biological response, thus reducing or eliminating the need to do animal testing.

Metabolic changes occur in gingival tissue during gingivitis, including the degradation and synthesis of carbohydrates, lipids, proteins and nucleic acids and their metabolic products. Those changes will alter the levels of RNA, nucleic acids, carbohydrates, lipids, fatty acids, proteins, peptides, amino acids and their metabolic products in the gingival tissues, saliva, gingival crevicular fluid and dental plaques. Such biological active molecules are very potent on stimulating inflammatory responses or promoting tissue repairs. Consequently, metabolic changes in gingivitis either facilitate resolution of gingivitis or exacerbate inflammatory or injurious damages to the periodontal tissue.

Citrulline and ornithine are two amino acids in the urea cycle. Citrulline was first isolated from watermelon in 1914 by Koga and Odake. It is a key intermediate in the urea cycle pathway. Citrulline is also produced as a byproduct of an enzymatic reaction catalyzed by nitric oxide synthases. The latter oxidize arginine into nitric oxide and citrulline. Nitric oxide is an important vasodilator and also contributes to host defence and seems to have cytostatic and cytotoxic effects against certain pathogens. In addition, some Gram-positive bacteria, such as *Staphylococcus aureus*, can also convert arginine to nitric oxide and citrulline. Ornithine is one of the products of arginases, which hydrolyze arginine into ornithine and urea.

Both citrulline and ornithine are not coded for by any gene codons, that is, not in any newly synthesized proteins. However, both host and bacterial enzymes can modify arginine residues in the proteins into either citrulline or ornithine residues. As a result, the modified proteins either lose their original functions or gain new activities. Importantly, bacteria produce virulence factors, which possess enzymes to convert arginine in proteins to ornithine or citrulline, thus, modifying the function of the proteins to cause pathogenic conditions or help tissue heal. Metabolic changes also alter the production and expression of cytokines, chemokines, extra cellular matrix proteins and other cellular proteins. Similarly, metabolic changes shift different population of immune cells. All those changes, including small molecules related to carbohydrates, lipids, proteins and nucleic acids in the gingival tissue, will reflect the healthy status of gingival tissues, and can function as biomarkers individually. As a whole, those changes will give a detailed assessment of the potency and levels of virulence factors in the oral cavity.

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.

The present invention may include 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 µl 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 O1, 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 T H1, 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 “13838M\_AA\_Sequence\_Listing\_ST25.” The ASCII text file was created on 6 Apr. 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.

The receptor genes are attached to the regulatory sequence of another gene (reporter gene), such as a fluorescent or luminescent gene, which may encode green fluorescent protein, luciferase, alkaline phosphatase, and/or red fluorescent protein. An example of a reporter gene is the SEAP reporter gene. The SEAP reporter gene encodes a secreted enzyme, called embryonic alkaline phosphatase or SEAP. For instance, in certain embodiments, the SEAP reporter gene is placed under the control of an interferon- $\beta$  minimal promoter fused to five NFkB and AP-1-binding sites. A recombinant Toll-like receptor binds its ligand, and initiates a chain of responses, leading to recruitment of NFkB and API transcription factors to the reporter gene promoter, which induce expression of SEAP. As compared to measuring an immune response the reporter genes of the present invention allow rapid, specific and reproducible measurements of virulence factors. Other receptors can be used in combination with Toll-like receptors, for example,

nucleotide-binding oligomerization domain (NOD)1 and NOD2 recognize the peptidoglycan derivatives, meso-di-aminopimelic acid (meso-DAP) and muramyl dipeptide (MDP), and trigger host innate immune responses.

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. 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.

In certain embodiments a determination of virulence is assigned based on the activation of one or more of the Toll-like receptors. 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; bacterial metabolites; fimbriae; outer membrane vesicles; and bacterial proteins and/or 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” refers to the neutralization, reduction, and/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.

The oral care compositions of the present invention may include one or more of the following components, which can include metal salts, sweeteners, carrier materials, antimicrobial agents, bad breath reduction agents, bleaching agents (hydrogen peroxide), surfactants, flavors (menthol), anti-tartar agents, colorants, sensates, abrasive polishing materials, thickening materials, humectants, and other additives.

- A. A method for identifying agents for detoxifying oral virulence factors comprising: providing a reporter cell that expresses at least one of TLR2 or TLR4; providing an oral virulence factor; providing a potential detoxifying agent; combining oral virulent factor and potential detoxifying agent with reporter cell; determining EC50 of virulence factor.
- B. The method of paragraph A, wherein the virulence factor is lipopolysaccharide, preferably wherein the lipopolysaccharide is from a Gram negative bacterium.
- C. The method of paragraph A, wherein the virulence factor is lipoteichoic acid, preferably wherein the lipoteichoic acid is from a Gram positive bacterium.
- D. The method of according to any of paragraphs A to C, wherein the potential detoxifying agent is at least one of myristyl-gamma-picolinium chloride; potassium linoleate; Sodium hexadecyl sulfate; fenugreek; steapyrium chloride; alpha.-glyceryl monooloeate; stearyldiethanolamine; Dimethylaminopropyl laurylamide; Stearylamidopropyl dimethylamine; linoleamide; N-Lauryldiethanolamine; Steartrimonium chloride; hexaethylene glycol, monodecyl ether; Laureth-6; Laureth-9; Quaternium-17; Bromoenol lactone; Oleyl amine; cetrimonium chloride; or palmitamide monoethanolamine.
- E. A method to identify detoxifying agents that bind to at least one of lipopolysaccharide or lipoteichoic acid comprising:
- a) providing a reporter cell that expresses at least one of TLR2 or TLR4;
  - b) providing at least one of lipopolysaccharide or lipoteichoic acid;
  - c) combining at least one of lipopolysaccharide or lipoteichoic acid and potential detoxifying agent with reporter cell;
  - d) measuring binding of potential detoxifying agents using at least one of BODIPY method or LAL method of low EC50 lipopolysaccharide or lipoteichoic acid; and
  - e) determining EC50 of at least one of lipopolysaccharide or lipoteichoic acid.
- F. An oral care composition comprising:
- a detoxifying agent that is at least one of myristyl-gamma-picolinium chloride; potassium linoleate; Sodium hexadecyl sulfate; fenugreek; steapyrium chloride; alpha.-glyceryl monooloeate; stearyldiethanolamine; Dimethylaminopropyl laurylamide; Stearylamidopropyl dimethylamine; linoleamide; N-Lauryldiethanolamine; Steartrimonium chloride; hexaethylene glycol, monodecyl ether; Laureth-6; Laureth-9; Quaternium-17; Bromoenol lactone; Oleyl amine; cetrimonium chloride; or palmitamide monoethanolamine.

G. The oral care composition of paragraph F, wherein the detoxifying agent is present in an amount of from about 0.001% to about 2.0%, by weight of the oral care composition.

- 5 H. The oral care composition paragraph F or G, wherein the oral care composition is a strip.

## EXAMPLES

### Example 1

#### Determination of Secreted Lipopolysaccharide by Bacteria

15 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 (OD<sub>600</sub>) 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, thereafter referred to as “supernatant of bacterial culture”, was subsequently analyzed for LPS content using the procedure as described below.

20 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 N<sub>2</sub>:CO<sub>2</sub>:H<sub>2</sub> 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 (OD<sub>600</sub>) 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.

25 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 OD<sub>600</sub> (OD<sub>600</sub> of 1.0=8×10<sup>8</sup> cells/ml).

30 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 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 the plate covered for 5 min at 37° C. Then 50 μl LAL was added to each well.

35 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 platereader (Molecular Device, Sunnyvale, Calif.).

## 15

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 is 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. 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 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.

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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	1644
Unhealthy Marginal Plaque	80277

It was expected that the marginal plaques in unhealthy sites had more endotoxins than those in the healthy sites (TABLE) 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 SnF<sub>2</sub> 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.).

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As shown in TABLE 2, SnF<sub>2</sub> and some other compounds inhibited LPS activities in LAL assays

TABLE 2

Inhibition of LPS activities on LAL Assays				
Samples	Inhibition of LAL activity %			
	<i>P. gingivalis</i> LPS 1690 1 ng/ml		<i>E. coli</i> 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 monohydrate	103	77	103	46
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 zinc salt	0	0	2	6
sodium fluoride	0	0	4	5
Carboxymethyl cellulose sodium	0	0	2	0

## 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. 5 *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 SNF<sub>2</sub> and cetylpyridinium chloride in 30 µl of 50 mM Tris buffer at pH 7.4 for 10 min, 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.

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TABLE 3

Displacement of LPS from binding to BODIPY TR cadaverine		
Concentrations mM	Displacement %	
	Stannous fluoride	Cetylpyridinium chloride
0.16	2.45	9.98
0.31	2.79	31.11
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 an IL-12 p40 minimal promoter fused to five NF-κB 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 API 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% CO<sub>2</sub>, 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% CO<sub>2</sub>, 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 proinflam-

matory 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 NFκB-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 μM to 1,000 μM on 100 ng/ml *E. coli* LPS, as reported by activation of TLR-4. At stannous concentrations of 500 μM 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 μM to 1,000 μM on *P. gingivalis* LPS, as reported by activation of TLR-2. At stannous concentrations of 1000 μM, 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 <sup>13</sup>/<sub>14</sub>, <sup>15</sup>/<sub>16</sub>, <sup>11</sup>/<sub>12</sub>, <sup>7</sup>/<sub>8</sub>, <sup>1</sup>/<sub>2</sub>.) Each Gracey curette is designed to adapt to a specific area or tooth surface. For example, Gracey <sup>13</sup>/<sub>14</sub> 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 μl 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.



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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. 7 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. 7, 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.

## 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 NFκB 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 NFκB 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 NFκB and API 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% CO<sub>2</sub>, and 95% humidity. For treatment with LTA, wells of a

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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% CO<sub>2</sub>, and 95% humidity until day 4. On day 4, medium (100 μL) 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. 7 of EXAMPLE 7 were examined using the TLR2-SEAP reporter gene assay. The results are shown in FIG. 10. 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.

## 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- $\beta$  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 API 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% CO<sub>2</sub>, and 95% humidity. For treatment with flagellin, wells of a 96-well plate were seeded with 10,000 cells/well in 100  $\mu$ L of growth medium. The cells were incubated for 72 hours at 37° C., 5% CO<sub>2</sub>, 95% humidity until day 4 after cells were seeded onto wells of a 96-well plate. On day 4, medium (100  $\mu$ L) 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  $\mu$ g/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 EC<sub>50</sub> of flagellin on TLR5 was 0.02 ng/ml at 5 hours and 0.014 ng/ml at 23 hours. The EC<sub>50</sub> 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<sup>TM</sup> 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- $\beta$  minimal promoter fused to five copies of the NF-kB 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% CO<sub>2</sub>. 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  $\mu$ l of growth medium. 10  $\mu$ l 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% CO<sub>2</sub>, 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. 12. 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. 12, *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.

#### Example 12

##### 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 stimulating 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,

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  $\mu\text{g/ml}$ . The cultures were harvested at 24 and 48 hours after treatment and six proinflammatory cytokines (interferon- $\gamma$ , IL-1 $\beta$ , IL-2, IL-10, IL-12p70 and TNF- $\alpha$ ) were analyzed using ELISA kits from Meso Scale Discovery. As shown in TABLE 4, expression of interferon- $\gamma$ , IL-1 $\beta$ , IL-2, IL-10, IL-12p70 and TNF- $\alpha$  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.

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> genomic DNA				<i>P. gingivalis</i> genomic DNA		
	DNA	24 hours	48 hours		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	1	IL-12p70	PG DNA	0	0.25
	0 ug				0 ug		
	PG DNA	0.67	0.33		PG DNA	0.67	0
	0.3 ug				0.3 ug		
	PG DNA	0	0		PG DNA	0	0.67
	1 ug				1 ug		
IL-2	PG DNA	0.33	0.33	TNF-alpha	PG DNA	0	0.33
	2 ug				2 ug		
	PG DNA	0.5	1		PG DNA	1	1.5
	0 ug				0 ug		
	PG DNA	0	0.67		PG DNA	1.67	1.33
	0.3 ug				0.3 ug		
	PG DNA	0.33	1		PG DNA	0.67	1
	1 ug				1 ug		
	PG DNA	0.33	0.67		PG DNA	1.33	1.33
	2 ug				2 ug		

Switzerland) in T75 flasks at 37° C. under a 5% CO<sub>2</sub> 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  $\mu\text{l}$  of CELLnTEC growth medium at 37° C. under a 5% CO<sub>2</sub> 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  $\mu\text{l}$  of CellnTec medium was added without supplements in each well. If the assay medium was the growth medium, 100  $\mu\text{l}$  of growth medium was added to each well. The *P. gingivalis* LPS and bacterial DNA were added to the cells. At 24 h, 48

Human primary gingival epithelial cells were treated with *P. gingivalis* LPS at 0, 0.3, 1 and 2  $\mu\text{g/ml}$  in the procedures described above. As shown in TABLE 5, again, expression of interferon- $\gamma$ , IL-1 $\beta$ , IL-2, IL-10, IL-12p70 and TNF- $\alpha$  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
IL-10	PG LPS 2 ug	0.00	0.00	IL-12p70	PG LPS 2 ug	0.00	1.00
	PG IPS 0 ug	0.50	1.00		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
IL-2	PG LPS 1 ug	0.00	0.67	TNF-alpha	PG LPS 1 ug	0.00	0.33
	PG LPS 2 ug	0.33	0.67		PG LPS 2 ug	0.67	0.00
	PG IPS 0 ug	0.50	1.00		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  $-70^{\circ}$  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).

The DNA samples were isolated from margin gingival plaques which contain both human host and bacterial cells. After meta-sequencing, bacterial DNA and human DNA sequences were identified and quantitated. As shown in FIG. 15, human DNA contribution to the plaque samples was about 5-10 ng/ $\mu$ l from baseline to week 6 of the treatment. However, the microbial DNA was reduced dramatically from baseline to weeks 1, 3 and 6 of the treatment.

The relative abundance of bacterial genera underwent significant changes during treatment (TABLE 6A). *Capnocytophaga* and *Campylobacter* were increased during the treatment. *Prevotella* is a genus of Gram-negative bacteria, existing in microbiota of oral cavities, vagina and respiratory tract. This genus of bacteria can cause infections including aspiration pneumonia, lung abscess, pulmonary empyema, and chronic otitis media and sinusitis. *Prevotella* bacteria were abundant at baseline. Its number was reduced in weeks 1, 3 and 6.

TABLE 6A

Changes in relative abundance of bacterial genera in the supragingival plaques from baseline to week 6 of regimen treatment.				
Genera	Relative Abundance %			
	Baseline	Week 1	Week 3	Week 6
<i>Streptococcus</i>	26.01	18.13	6.94	27.59
<i>Prevotella</i>	16.02	8.87	7.06	8.76
<i>Rothia</i>	10.04	12.84	14.75	15.57
<i>Veillonella</i>	9.93	5.85	7.64	7.43
<i>Actinomyces</i>	9.45	17.83	22.13	8.10
<i>Porphyromonas</i>	4.75	1.69	1.13	2.50
<i>Haemophilus</i>	3.97	4.49	7.24	7.03
<i>Gemella</i>	3.48	3.67	1.61	3.00
<i>Neisseria</i>	1.99	1.81	5.30	2.56
<i>Granulicatella</i>	1.87	2.34	2.23	2.22
<i>Atopobium</i>	1.26	1.47	1.18	0.62
<i>Megasphaera</i>	1.11	0.36	0.02	0.30
<i>Eubacterium</i>	1.09	1.14	0.93	0.99
<i>Peptostreptococcus</i>	1.00	0.42	0.23	0.55
<i>Fretibacterium</i>	0.88	1.46	1.20	0.62
<i>Solobacterium</i>	0.70	0.34	0.28	0.37
<i>Capnocytophaga</i>	0.55	2.13	3.27	1.50
<i>Alloprevotella</i>	0.51	0.45	1.03	0.38
<i>Stomatobaculum</i>	0.48	0.32	0.06	0.20
<i>Parvimonas</i>	0.47	0.62	0.25	0.53
<i>Oribacterium</i>	0.42	0.32	0.26	0.33

TABLE 6A-continued

Changes in relative abundance of bacterial genera in the supragingival plaques from baseline to week 6 of regimen treatment.				
Genera	Relative Abundance %			
	Baseline	Week 1	Week 3	Week 6
<i>Bifidobacterium</i>	0.42	0.10	0.04	0.18
<i>Legionella</i>	0.37	0.79	0.43	0.41
<i>Corynebacterium</i>	0.35	3.32	2.74	0.31
<i>Mogibacterium</i>	0.30	0.25	0.11	0.30
<i>Lachnoanaerobaculum</i>	0.26	0.24	0.10	0.14
<i>Abiotrophia</i>	0.22	0.31	0.85	0.57
<i>Scardovia</i>	0.20	2.01	1.67	0.10
<i>Campylobacter</i>	0.17	0.52	0.69	0.24
<i>Filifactor</i>	0.15	0.18	0.08	0.18
<i>Dialister</i>	0.13	0.82	1.23	0.26
<i>Tannerella</i>	0.11	0.14	0.06	0.12
<i>Bulleidia</i>	0.11	0.10	0.05	0.09
<i>Treponema</i>	0.11	0.50	0.51	0.12
<i>Slackia</i>	0.08	0.09	0.03	0.17
<i>Lautropia</i>	0.07	0.21	1.92	2.31
<i>Kingella</i>	0.07	0.14	0.41	0.05
<i>Alloscardovia</i>	0.06	0.00	0.00	0.04
<i>Bacteroidetes</i>	0.06	0.18	0.05	0.10
<i>Aggregatibacter</i>	0.06	0.14	0.22	0.08
<i>Selenomonas</i>	0.06	0.34	0.24	0.20
<i>Eikenella</i>	0.06	0.21	0.35	0.05
<i>Lactobacillus</i>	0.06	0.09	0.09	1.54
<i>Actinobacillus_unclassified</i>	0.06	0.18	0.15	0.04
<i>candidate_division_TM7</i>	0.05	0.53	0.34	0.05
<i>Olsenella</i>	0.05	0.06	0.03	0.05
<i>Eggerthia_catenaformis</i>	0.04	0.04	0.00	0.02
<i>Shuttleworthia</i>	0.03	0.05	0.11	0.03
<i>Cardiobacterium</i>	0.03	0.11	0.24	0.04
<i>Catonella</i>	0.02	0.06	0.08	0.02
<i>Actinobaculum</i>	0.02	0.54	1.60	0.07
<i>Simonsiella</i>	0.02	0.01	0.00	0.00
<i>Anaerococcus</i>	0.01	0.08	0.11	0.00
<i>Propionibacterium</i>	0.01	0.12	0.15	0.20
<i>Mitsuokella</i>	0.01	0.01	0.00	0.00
<i>Riemerella_unclassified</i>	0.01	0.01	0.02	0.01
<i>Peptoniphilus</i>	0.01	0.00	0.00	0.00
<i>Pseudoramibacter</i>	0.00	0.00	0.00	0.01
<i>Desulfobulbus</i>	0.00	0.02	0.02	0.00
<i>Staphylococcus</i>	0.00	0.03	0.00	0.00
<i>Eubacteriaceae</i>	0.00	0.01	0.00	0.01
<i>Gardnerella</i>	0.00	0.01	0.00	0.00
<i>Centipeda</i>	0.00	0.00	0.00	0.00
<i>Parascardovia</i>	0.00	0.52	0.12	0.02
<i>Mycoplasma</i>	0.00	0.00	0.00	0.00
<i>Pseudomonas</i>	0.00	0.00	0.00	0.46
<i>Ruminococcus_torques</i>	0.00	0.00	0.00	0.00
<i>Xanthomonas</i>	0.00	0.00	0.00	0.00

TABLE 6A-continued

Changes in relative abundance of bacterial genera in the supragingival plaques from baseline to week 6 of regimen treatment.				
Genera	Relative Abundance %			
	Baseline	Week 1	Week 3	Week 6
<i>Desulfovibrio</i>	0.00	0.00	0.00	0.00
<i>Johnsonella</i>	0.00	0.03	0.00	0.00

The abundance of certain bacteria in the supragingival plaques changed in the six week treatments as shown in Table 6B and FIG. 16A. Certain bacteria were decreased either in weeks 1, 3 or 6. Those microbes comprise *Actinomyces\_graevenitzii*, *Actinomyces\_massiliensis*, *Actinomyces\_sp\_ICM47*, *Atopobium\_parvulum*, *Campylobacter\_conciscus*, *Catonella\_morbi*, *Eubacterium\_brachy*, *Eubacterium\_infirmum*, *Fusobacterium\_nucleatum*, *Fusobacterium\_periodonticum*, *Lachnoanaerobaculum\_saburreum*, *Lachnospiraceae\_bacterium\_oral\_taxon\_082*, *Megasphaera\_micronuciformis*, *Mitsuokella\_unclassified*, *Neisseria\_subflava*, *Oribacterium\_sinus*, *Parvimonas\_micra*, *Parvimonas\_unclassified*, *Peptostreptococcus\_stomatis*, *Peptostreptococcus\_unclassified*, *Pestivirus\_Giraffe\_1*, *Porphyromonas\_endodontalis*, *Porphyromonas\_sp\_oral\_taxon\_279*, *Prevotella\_histicola*, *Prevotella\_maculosa*, *Prevotella\_melaninogenica*, *Prevotella\_nanceiensis*, *Prevotella\_oris*, *Prevotella\_pallens*, *Prevotella\_salivae*, *Prevotella\_sp\_C561*, *Prevotella\_veroralis*, *Rothia\_dentocariosa*, *Rothia\_mucilaginoso*, *Selenomonas\_flueggei*, *Selenomonas\_sputigena*, *Solobacterium\_moorei*, *Stomatobaculum\_longum*, *Streptococcus\_anginosus*, *Streptococcus\_australis*, *Streptococcus\_constellatus*, *Streptococcus\_cristatus*, *Streptococcus\_oligofermentans*, *Streptococcus\_parasanguinis*, *Streptococcus\_sanguinis*, *Veillonella\_atypica*, *Veillonella\_dispar*, *Veillonella\_parvula*, *Veillonella\_unclassified*. 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. 16A. The relative amount of each bacterial species was plotted over the three time periods of the treatment (FIG. 16B). The relative amount of certain bacteria, such as *Actinomyces\_massiliensis*, *actinomyces\_naslundii*, *capnocytophaga\_gingivalis*, and *campylobacter\_gracilis*, was increased, while the abundance of *Prevotella\_pallens*, *Prevotella\_melaninogenica*, and *Porphyromonas\_sp\_oral\_taxon\_279* was reduced during the three weeks of treatment.

TABLE 6B

Decrease of gingivitis-associated bacteria in supragingival plaques during the period of 6-week regimen treatment.							
Species	Relative Abundance Baseline	Relative Abundance Week 1	Relative Abundance Week 3	Relative Abundance Week 6	p. value	p. value	p. value
					Week 1 v. Baseline - Relative Abundance	Week 3 v. Baseline - Relative Abundance	Week 6 v. Baseline - Relative Abundance
<i>Actinomyces_graevenitzii</i>	3.61	1.2	0.0016	2.18	0.125	0.0198	0.302
<i>Actinomyces_massiliensis</i>	0.0147	0.203	0.176	0.0365	0.026	0.00739	0.0885
<i>Actinomyces_sp_ICM47</i>	2.69	1.36	0	1.82	0.289	0.00982	0.547
<i>Atopobium_parvulum</i>	1.02	0.738	0.309	0.379	0.43	0.0199	0.0375
<i>Campylobacter_conciscus</i>	0.0966	0.0544	0.0092	0.0664	0.19	0.0015	0.404
<i>Catonella_morbi</i>	0.0241	0.0592	0.0755	0.0219	0.108	0.187	0.972
<i>Eubacterium_brachy</i>	0.619	0.792	0.592	0.587	0.604	0.935	0.904
<i>Eubacterium_infirmum</i>	0.298	0.141	0.267	0.127	0.122	0.816	0.112
<i>Fusobacterium_nucleatum</i>	0.282	1.21	1.07	0.31	0.0361	0.0688	0.785
<i>Fusobacterium_periodonticum</i>	0.567	0.186	0.0856	0.244	0.0268	0.00707	0.101
<i>Lachnoanaerobaculum_saburreum</i>	0.0116	0.12	0.0635	0.0106	0.115	0.118	0.632

TABLE 6B-continued

Decrease of gingivitis-associated bacteria in supragingival plaques during the period of 6-week regimen treatment.							
Species	Bacterial Abundance (ng/uL) - Baseline	Bacterial Abundance (ng/uL) - Week 1	Bacterial Abundance (ng/uL) - Week 3	Bacterial Abundance (ng/uL) - Week 6	p. value Week 1 v. Baseline - Bacterial Abundance	p. value Week 3 v. Baseline - Bacterial Abundance	p. value Week 6 v. Baseline - Bacterial Abundance
Lachnospiraceae_bacterium_oral_taxon_082	0.0974	0.0605	0.00232	0.0427	0.296	0.00055	0.0883
Megasphaera_micronuciformis	1.1	0.347	0.0134	0.295	0.0703	0.0108	0.0565
Mitsuokella_unclassified	0.00705	0.0071	0.00377	0.00461	0.988	0.27	0.382
Neisseria_subflava	0.374	0.0431	0.018	0.178	0.0615	0.0446	0.331
Oribacterium_sinus	0.29	0.101	0.00147	0.176	0.0371	0.00144	0.284
Parvimonas_micra	0.0686	0.107	0.0485	0.075	0.412	0.481	0.69
Parvimonas_unclassified	0.393	0.507	0.2	0.443	0.638	0.163	0.65
Peptostreptococcus_stomatis	0.54	0.38	0.231	0.43	0.39	0.101	0.779
Peptostreptococcus_unclassified	0.449	0.0396	0.00028	0.109	0.00428	0.00217	0.0146
Pestivirus_Giraffe_1	0.00083	0.00146	0.00066	0.00221	0.478	0.814	0.0952
Porphyromonas_endodontalis	0.264	0.197	0.301	0.479	0.425	0.808	0.0902
Porphyromonas_sp_oral_taxon_279	4.39	1.15	0.284	1.86	0.0276	0.00497	0.0977
Prevotella_histicola	2.77	0.851	0.00025	0.721	0.109	0.0119	0.0666
Prevotella_maculosa	0.0148	0.133	0.15	0.0105	0.0413	0.0912	0.705
Prevotella_melaninogenica	4.9	1.24	0.0236	4.19	0.00246	0.00017	0.895
Prevotella_nanceiensis	1.61	0.646	0.0228	0.835	0.0818	0.00129	0.144
Prevotella_oris	0.319	1.07	1.46	0.211	0.0358	0.0307	0.434
Prevotella_pallens	2.53	0.553	0.00042	0.712	0.00322	0.0003	0.00705
Prevotella_salivae	0.501	0.179	0.0147	0.312	0.0528	0.00298	0.332
Prevotella_sp_C561	1.47	0.295	0	0.232	0.0792	0.0263	0.0655
Prevotella_veroralis	0.257	0.282	0.541	0.181	0.871	0.363	0.694
Rothia_dentocariosa	1.18	3.53	7.2	1.87	0.02	0.022	0.183
Rothia_mucilaginosa	8.35	7.28	1.24	12.7	0.731	3.8E-05	0.0299
Selenomonas_flueggei	0.0179	0.0264	0.0361	0.0107	0.342	0.362	0.4
Selenomonas_sputigena	0.0121	0.0314	0.0271	0.00853	0.116	0.145	0.558
Solobacterium_moorei	0.693	0.341	0.279	0.357	0.0141	0.01	0.0303
Stomatobaculum_longum	0.476	0.318	0.056	0.197	0.335	0.00305	0.0518
Streptococcus_anginosus	0.226	0.819	0.44	0.211	0.143	0.414	0.947
Streptococcus_australis	1.02	0.288	0.0591	0.784	0.031	0.00575	0.684
Streptococcus_constellatus	0.31	0.38	0.00031	0.0929	0.825	0.116	0.302
Streptococcus_cristatus	0.0873	0.127	0.122	0.0705	0.311	0.567	0.496
Streptococcus_oligofermentans	0.0518	0.0389	0.0273	0.018	0.518	0.194	0.0562
Streptococcus_parasanguinis	7.99	4.46	0.285	7.68	0.132	0.00117	0.961
Streptococcus_sanguinis	0.602	1.22	2.82	1.48	0.085	0.0675	0.0218
Veillonella_atypica	1.52	0.413	0.00603	0.696	0.0118	0.0009	0.0988
Veillonella_dispar	0.448	0.164	0.0159	0.418	0.0356	0.002	0.811
Veillonella_parvula	0.772	1.88	2.7	0.517	0.0741	0.0102	0.161
Veillonella_unclassified	6.08	2.69	4.14	4.76	0.00212	0.181	0.146
Actinomyces_graevenitzi	270	9.95	0.0425	45.3	0.00266	0.00201	0.00735
Actinomyces_massiliensis	1.21	3.28	2.51	0.906	0.378	0.185	0.595
Actinomyces_sp_ICM47	166	17	0	35.1	0.00276	0.00106	0.00669
Atopobium_parvulum	88.9	6.85	5.47	8.28	0.0261	0.0241	0.0284
Campylobacter_conciscus	8.42	0.661	0.208	1.38	0.00326	0.00212	0.0066
Catonella_morbi	1.7	0.977	1.76	0.511	0.329	0.962	0.0475
Eubacterium_brachy	35.9	10.8	10.2	11.6	0.0397	0.0421	0.048
Eubacterium_infirmum	22	1.61	3.13	2.88	0.00434	0.00733	0.00671
Fusobacterium_nucleatum	21	20.9	17.8	6.96	0.993	0.698	0.0129
Fusobacterium_periodonticum	44.2	1.86	1.63	5.61	0.00702	0.00684	0.0129
Lachnoanaerobaculum_saburreum	0.952	2.89	0.966	0.285	0.422	0.984	0.0317
Lachnospiraceae_bacterium_oral_taxon_082	7.05	0.394	0.0477	1.07	0.00804	0.00582	0.0163
Megasphaera_micronuciformis	86.2	2.98	0.305	6.39	0.00923	0.0076	0.0118
Mitsuokella_unclassified	0.621	0.0852	0.0631	0.118	0.032	0.0257	0.0434
Neisseria_subflava	23.3	0.57	0.327	3.09	0.0161	0.0152	0.0299
Oribacterium_sinus	21.7	1.19	0.0294	3.74	0.00289	0.00191	0.0074
Parvimonas_micra	4.27	1.25	0.776	1.55	0.00609	0.00139	0.0277
Parvimonas_unclassified	26.6	5.36	4.69	8.46	0.00696	0.00751	0.0233
Peptostreptococcus_stomatis	39.1	5.42	6.17	7.11	0.01	0.0131	0.0136
Peptostreptococcus_unclassified	37.4	0.358	0.00485	2.38	0.00571	0.00537	0.00817
Pestivirus_Giraffe_1	0.0653	0.0158	0.00479	0.041	0.192	0.104	0.544
Porphyromonas_endodontalis	19.7	2.33	6.48	7.69	0.00853	0.0745	0.0748
Porphyromonas_sp_oral_taxon_279	402	13.3	5.5	32.8	0.0497	0.0458	0.0608
Prevotella_histicola	312	7.36	0.00819	15.5	0.047	0.0425	0.0524
Prevotella_maculosa	1.3	2.11	1.82	0.231	0.525	0.517	0.0289
Prevotella_melaninogenica	447	10.3	0.488	76.7	0.00692	0.00601	0.019
Prevotella_nanceiensis	126	7.55	0.532	14.2	0.00985	0.00678	0.0136
Prevotella_oris	25.1	16.8	18.4	4.41	0.508	0.496	0.0219
Prevotella_pallens	196	3.94	0.00783	16.3	0.00169	0.00145	0.0028
Prevotella_salivae	52.7	1.45	0.117	6.26	0.0288	0.0254	0.0449
Prevotella_sp_C561	144	1.17	0	6.8	0.0959	0.0935	0.109
Prevotella_veroralis	18.6	3.14	7.56	3.27	0.0333	0.133	0.0348

TABLE 6B-continued

Decrease of gingivitis-associated bacteria in supragingival plaques during the period of 6-week regimen treatment.							
Rothia_dentocariosa	92.5	29.6	88.3	33.9	0.0162	0.903	0.0324
Rothia_mucilaginosa	651	53.9	16.5	219	0.0009	0.00054	0.0103
Selenomonas_flueggei	1.63	0.306	0.293	0.31	0.0469	0.0451	0.0495
Selenomonas_sputigena	0.848	0.586	0.437	0.227	0.571	0.15	0.022
Solobacterium_moorei	50.7	4.23	3.55	6.75	0.00028	0.00025	0.00046
Stomatobaculum_longum	33.2	3.03	1.41	4.29	0.00231	0.00156	0.00318
Streptococcus_anginosus	14.3	9.14	9.94	3.15	0.379	0.62	0.00894
Streptococcus_australis	84.8	2.79	0.983	14.2	0.0234	0.021	0.0468
Streptococcus_constellatus	16.6	4.39	0.0059	1.13	0.138	0.0327	0.0447
Streptococcus_cristatus	5.84	1.46	2.3	1.67	0.00594	0.0585	0.00838
Streptococcus_oligofermentans	3.59	0.514	0.665	0.506	0.0212	0.029	0.0211
Streptococcus_parasanguinis	748	41.2	3.81	129	0.00773	0.00554	0.0168
Streptococcus_sanguinis	43	15.5	49.5	37.1	0.0373	0.76	0.709
Veillonella_atypica	157	2.96	0.135	15.4	0.0184	0.0167	0.0283
Veillonella_dispar	37.3	1.75	0.254	9.91	0.00689	0.00526	0.0317
Veillonella_parvula	71.1	20.8	38.9	12.9	0.0306	0.153	0.0118
Veillonella_unclassified	548	23.4	56.6	107	0.00213	0.00345	0.00711

## 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 the freezer, thawed and extracted by placing the samples on a tube

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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 µl) was analyzed for protein concentrations using the Bio-Rad protein assay (BioRad, Hercules, Calif.).

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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.

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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 6C). Those include FN-γ, IL-1β, 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-1α, VEGF-A, IL-17A, IL-15, TNF-β, IL-8 (HA), MCP-1, MCP-4, Eotaxin, IP-10, MDC, Eotaxin-3, TARC, MIP-1α, MIP-1β, VEGF-C, VEGF-D, Tie-2, Flt-1/VEGFR1, P1GF, FGF (basic), SAA, CRP, VCAM-1, and ICAM-1.

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TABLE 6C

	Changes in abundance of proteins in the gingival-brush samples.							
	Mean				α = 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-1α	3.554	2.331	2.181	1.891	A	A, B	B, C	C
IL-1β	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
IFNγ	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
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

TABLE 6C-continued

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
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
Fk-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 $\alpha$	0.091	0.091	0.084	0.080	A	A	A	A
MIP-1 $\beta$	0.091	0.100	0.110	0.094	A	A	A	A
TNF $\alpha$	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 measurement. 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
deoxycarnitine	3.2381	0.6088	0.19	0.0003	0.0168	146.1
proline	1.405	0.99	0.7	0.0033	0.0546	116.1
2-methylbutyrylcarnitine (C5)	1.7769	0.6997	0.39	0.0034	0.0546	246.1
lysylleucine	1.2009	0.5275	0.44	0.0036	0.0546	260.2
threonylleucine	1.1513	0.4931	0.43	0.0044	0.0594	231.2
trehalose	2.3563	0.9084	0.39	0.0054	0.0647	361.2
arginylleucine	1.4447	0.6819	0.47	0.0084	0.0777	288.3
lysylphenylalanine	1.1682	0.4563	0.39	0.0095	0.0777	294.3
citrulline	1.0147	0.71	0.7	0.0104	0.0777	176.1
arginylphenylalanine	0.9616	0.3335	0.35	0.0119	0.0777	322.2
pyroglutamine*	1.3424	0.7873	0.59	0.0136	0.0777	129.2
EDTA	1.5985	0.8384	0.52	0.0138	0.0777	291.1
tyrosine	1.383	1.0299	0.74	0.0161	0.0787	182.1
propionylcarnitine	1.2565	0.7688	0.61	0.0201	0.0877	218.2
phosphate	1.4008	0.8376	0.6	0.0208	0.0877	298.9
maltose	0.8727	1.4481	1.66	0.022	0.0877	204.1
threonylphenylalanine	1.7693	0.918	0.52	0.0233	0.0877	267.2
leucylleucine	0.9505	0.4393	0.46	0.0251	0.0877	245.1
adenosine 5'-monophosphate (AMP)	1.4092	0.8451	0.6	0.0295	0.0956	348.1
valine	1.1598	0.9271	0.8	0.0304	0.0956	118.1
alanine	0.8721	1.102	1.26	0.0318	0.0973	115.9
ornithine	1.2916	0.7069	0.55	0.0367	0.1035	141.9
glutamine	1.2459	0.8366	0.67	0.0374	0.1035	147.2
1-arachidonoylglycerophosphoethanolamine	1.2294	0.8274	0.67	0.038	0.1035	500.3
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
mannitol	1.3004	0.7982	0.61	0.042	0.107	319.1
tryptophan	1.1518	0.9089	0.79	0.0487	0.1185	205.1
valylvaline	0.9347	0.8231	0.88	0.0508	0.1207	215.2
erythritol	1.625	0.8085	0.5	0.0582	0.1325	217



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
13-HODE + 9-HODE	1.0877	0.7088	0.65	0.0601	0.1338	295.2
leucine	1.2158	0.9359	0.77	0.0613	0.1338	132.2
spermidine	1.1613	0.8678	0.75	0.0687	0.1465	146.2
asparagylleucine	0.9295	0.6122	0.66	0.0698	0.1465	246.2
succinate	1.2929	0.8113	0.63	0.0754	0.1548	247
1-oleoylglycerophosphoethanolamine	0.7378	1.0747	1.46	0.0767	0.1548	478.3
palatinitol	1.4395	0.8272	0.57	0.0782	0.1549	204
serylisoleucine*	1.1753	0.7169	0.61	0.0814	0.1583	219.2
fructose	1.9933	1.1106	0.56	0.0847	0.1605	217
maltotriose	1.0456	1.8347	1.75	0.0858	0.1605	204
glutathione, oxidized (GSSG)	1.0161	1.4669	1.44	0.087	0.1605	613.1

## Example 17

## Citruilline was Reduced in Regimen Treatment Over 6 Weeks

Quantitation of citruilline 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 citruilline and ornithine analysis, the extracts of the Gingival-brush samples were analyzed either directly (50  $\mu$ l undiluted sample solution) in 50/50 acetonitrile/ultra-pure water with 0.754% formic acid or diluted fivefold. For total citruilline and ornithine analysis, the extracts of the Gingival-brush samples were first hydrolyzed using 6 N HCl (50  $\mu$ L of extract with 450  $\mu$ 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 Lifetechnology, 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 citruilline or ornithine was calculated as the result of subtracting the concentration of free citruilline or ornithine from the concentration of total citruilline or ornithine, respectively. The result was reported as the concentration of citruilline or ornithine or the result was standardized by dividing by the amount of citruilline 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
Citruilline	176 $\rightarrow$ 159	d <sub>7</sub> -Citruilline	181 $\rightarrow$ 164
Ornithine	133 $\rightarrow$ 70	d <sub>6</sub> -Ornithine	139 $\rightarrow$ 76

All samples from the 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. 17, citruilline 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. 18. Protein bound ornithine was reduced gradually as gingivitis was decreased in severity.

## Example 19

## Expression of Enzymes in the Ornithine-citruilline-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 RNAlater solution (1 ml in in a 1.5 ml Eppendorf tube) for keeping RNA from degrading during transport and storage (Qiagen, Valencia, Calif.). The microcentrifuge 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 were 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. 19). 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. 20). 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 (liver) and II increased (FIG. 20).

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. 20).

#### 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 ul DPBS (Dulbecco's phosphate-buffered saline) (Lifetechnologies, Grand Island, N.Y.) with protease inhibitors, including AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) 2 mM, aprotinin 0.3 μM, Bestatin 130 μM, EDTA (Ethylenediaminetetraacetic acid) 1 mM, E-64 1 μM, and leupeptin 1 μM. 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. 21. 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. 22 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. 23) 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. 24) in both groups.

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## 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. 25) 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. 26).

## 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 treatment, 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. 27. Citrulline inhibited *P. gingivalis* LPS 1690 effects on proinflammatory cytokine production in a dose-dependent manner. Those cytokines include IL-6, TNF- $\alpha$ , IL-12p70, IL-10, IL-2, IFN-r and IL-1 $\beta$ .

## 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-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 room temperature. *E. coli* LPS (15  $\mu$ g/ml) was mixed in a well of a 96-well solid black flat bottom plate (Corning Incorporated, Corning, N.Y.) with SnF<sub>2</sub> and cetylpyridinium chloride in 30  $\mu$ l of 50 mM Tris buffer at pH 7.4 for 10 min, and then 20  $\mu$ l of 60  $\mu$ 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 concen-

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tration (IC<sub>50</sub>) 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. 28 and 29, 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. 30 and 31).

## Example 26

Chemistry Targeting Both LPS and LTA: Binding  
of Stannous Fluoride to *E. coli* LPS Using  
MALDI-TOF Mass Spectrometry

Stannous fluoride is a leading anti-gingivitis technology in P&G toothpaste products. Tests were conducted to understand whether stannous fluoride could bind LPS thus neutralizing LPS proinflammatory activities.

Analysis of *E. coli* LPS and Stannous Fluoride LPS Binding Using MALDI-TOF Mass Spectrometry: Matrix assisted laser desorption ionization (MALDI) is a soft ionization technique used in mass spectrometry. MALDI mass spectrometry can be used for the analysis of biomolecules such as peptides, proteins, polysaccharides and large organic molecules, such as polymers. In MALDI, the analyte is first co-crystallized with a UV absorbing matrix, such as alpha-cyano-4-hydroxycinnamic acid (CHCA), then subjected to pulse YAG laser radiation. This causes the desorption of the analyte/matrix crystals and produces ions which are transmitted into a mass analyzer for detection. In MALDI-TOF, a time-of-flight mass analyzer is used. MALDI-TOF data can be acquired in MS mode to generate molecular weight information and in MS/MS mode for structure information. Typical MALDI mass spectrum acquisition takes less than a minute so it can be used for fast screening of molecular species in samples of interest. In this study, *E. coli* LPS molecular weight profiles and its interaction with SnF<sub>2</sub> were investigated. An equal volume of the *E. coli* LPS solutions with or without SnF<sub>2</sub> was mixed with the 10  $\mu$ l MALDI matrix CHCA (10 mg/ml in 80% acetonitrile/20% water). 0.7  $\mu$ l of the sample solution was spotted on a MALDI plate, air-dried, and analyzed in negative ion mode using a MALDI-TOF/TOF 4800 plus system (AB-Sciex, Framingham, Mass., USA). Typical data acquisition uses mass scan range 500-3000 Da and a laser power of 4500. Data was collected in an automated fashion using random sampling over the sample spot with 250 shots per subspectrum and a total of 2500 shots per spectrum.

The data shown in FIG. 32 showed *E. coli* LPS is heterogeneous with atomic masses range from 1130 to 1220. This observation was consistent with literature reports. Some of the LPS did bind to one molecule of stannum, as marked with an arrow. Similarly, stannum is the element with the largest number of stable isotopes having atomic masses ranging from 112 to 124. The most abundant ones are 120Sn, 118Sn, and 116Sn, while the least abundant one is 115Sn. Three different isotopes were identified in the sample, with 116Sn most abundant.

## Example 27

Targeting Both LPS and LTA: Identification of  
Materials that Displace Binding of LPS from  
Fluorescent Dye BODIPY-TR-Cadaverine

Virulence factors are toxic to host cells and tissue, causing damages, injuries, death or serious debilitation. A detoxify-

ing agent is able to target, bind to and neutralize bacterial virulence factors, such as LPS and LTA. In certain embodiments a detoxifying agent may be present in an amount of from about 0.001% to about 2.0%, by weight of the oral care composition. To develop such LPS and LTA sequestration technologies, or detoxifying agent, a high throughput screening, as described briefly in EXAMPLE 4, 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.) *E. coli* LPS (15 µg/ml) was mixed with test materials believed to be safe to humans cosmetically in 30 µl of 50 mM Tris buffer at pH 7.4 for 10 min, and then 20 µl of 60 µM BC fluorescent dye was added right before fluorescence measurement at 21° C. Fluorescence measurements were performed on automated 96 well plate reader SpectraMax M3 (Molecular Device, Sunnyvale, Calif.). The excitation wavelength was 580 nM and the fluorescence emission was measured at 620 nM.

To identify technologies that sequester LPS from binding to BODIPY TR cadaverine, internal chemical libraries were screened. Compounds that displaced LPS binding by 15% were considered as hits; with the initial hits tested again.

As shown in TABLE 9, certain materials showed strong displacement activities.

TABLE 9

Compounds replacing LPS from binding to BODIPY TR cadaverine.				
Chemicals	LPS displacement %			
	Reading 1	Reading 2	Reading 3	Mean
Steapyrium chloride	~100	100	~100	~100
N-Laurylpyridinium chloride	66	56	59	60
N,N-Dimethyldodecylamine	36	18	13	22
Myristyl- $\gamma$ -picolinium chloride	~100	~100	~100	~100
Diethylaminoethyl stearamide	77	62	58	66
Laurylisoquinolinium bromide	~100	39	66	69
N-Myristoylglycine	~100	39	74	76
Stearylamidopropyl dimethylamine	85	71	74	77
Diallyldimethylammonium chloride	40	38	28	35
3-Dodecanamidopropyl dimethylamine	87	62	87	79
dl-lactic acid hemizinc salt	15		24	19
1,3-Diphenylacetone (1,3 diphenyl-2-propanone)	16		5	11
alpha-Amylcinnamaldehyde	17		13	15
cis-Oleic acid	54		9	31
Isopentyl benzoate	22		13	18
Undecylenic aldehyde	29		15	22
iron(iii) chloride hexahydrate	~100	100	~100	~100
3-(4-Isopropylphenyl)-2-methylpropionaldehyde	21	29	65	39
Copper(I) iodide	23	12	20	18
Methyl linoleate	~100	53	~100	88

### Chemistry Targeting Both LPS and LTA: Identification of LPS Binding Materials Using Nearest Neighbor Similarity Searching

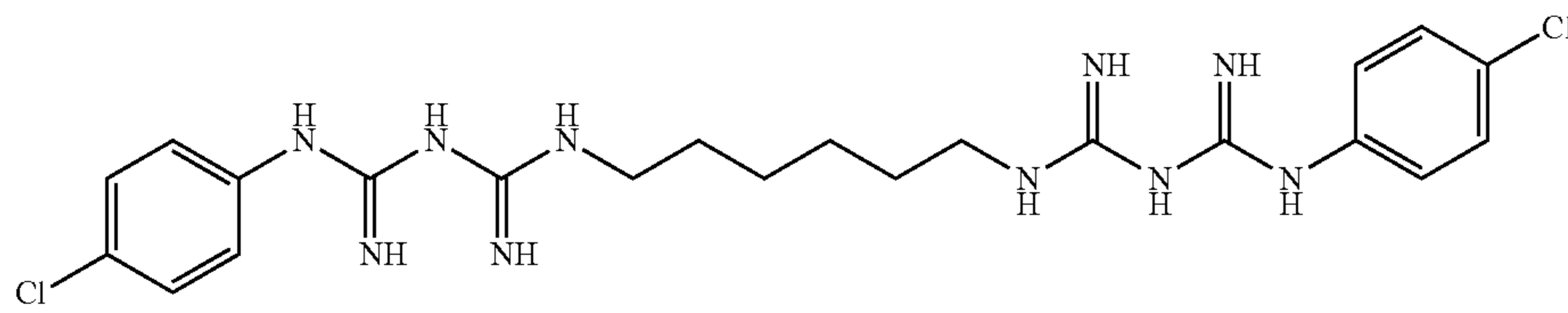
Compounds listed in TABLE 9 were used as starting materials for nearest neighbor similarity searching versus with test materials believed to be safe to humans cosmetically. The software used for nearest neighbor similarity searching was Pipeline Pilot software (version 8.5.0.2 from BIOVIA, San Diego, Calif.), which included cheminformatics components.

Nearest neighbor similarity searching involves 4 basic steps—Structure generation; Fingerprint generation from the structure; Calculation of the distance between two fingerprints; and Application of a cutoff to filter results.

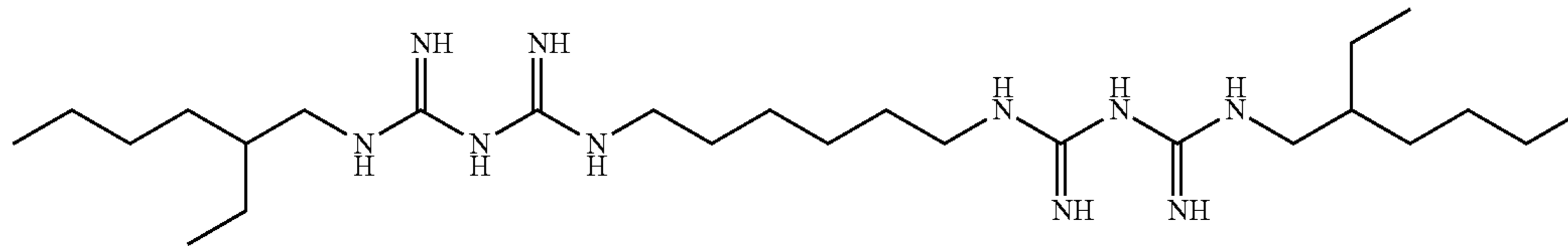
- Structure generation—structures for the 6 lead compounds, as shown below (Chlorhexidine, Alexidine, Yan-673, DS-176, Cetylpyridinium chloride, DS-347), were drawn manually using ChemBioDraw Ultra v.14 and converted into SMILES strings (1D representation of structure). The target ListNet database already contained structures as SMILES strings. All lead and target structures were imported into Pipeline Pilot and the “Molecule from SMILES” component was used to convert all SMILES to 2D molecular structures.

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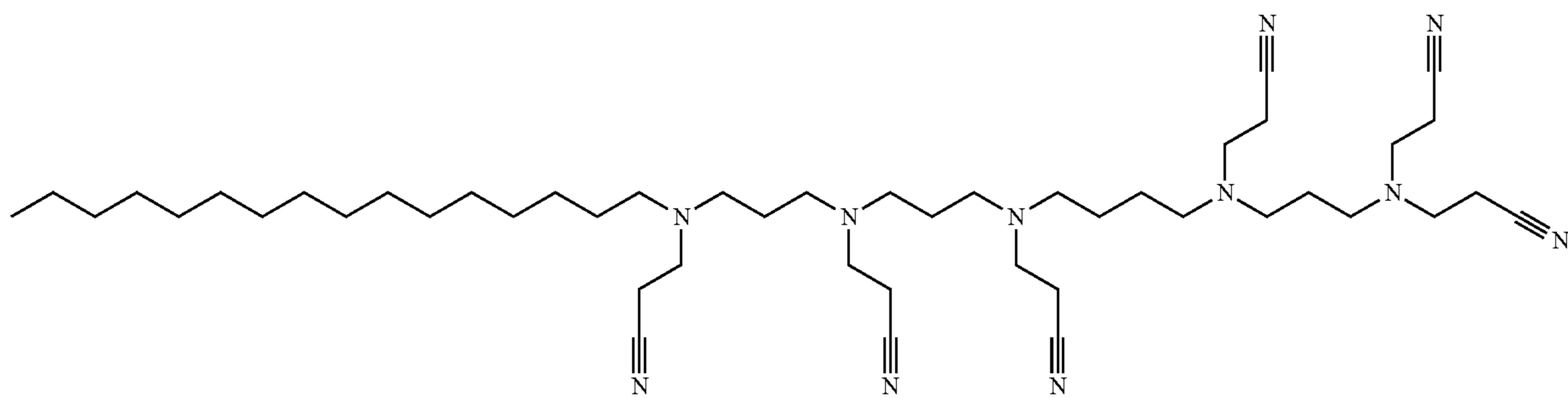
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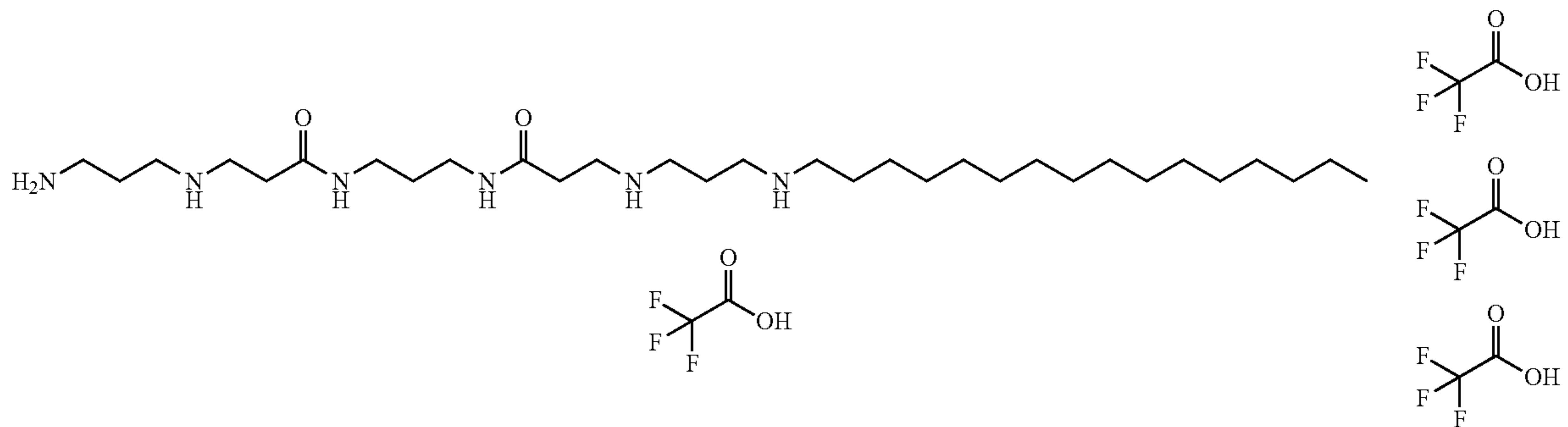
Chlorhexidine



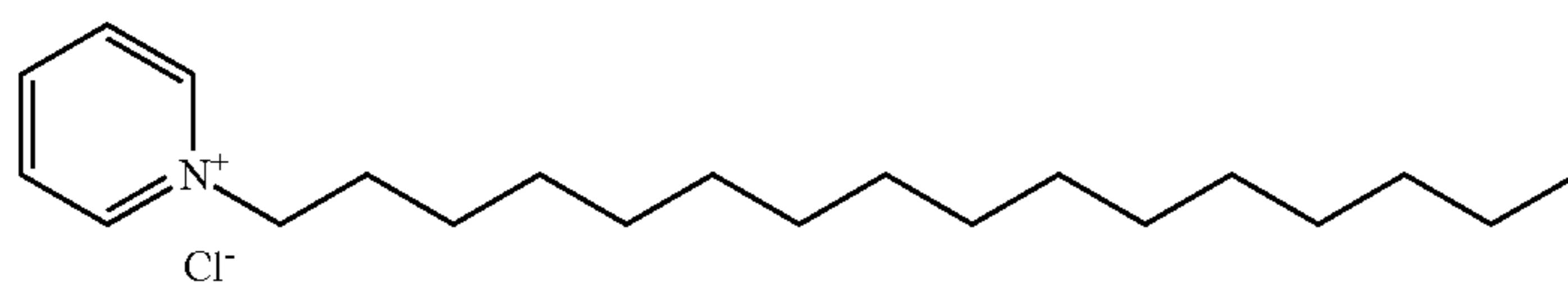
Alexidine



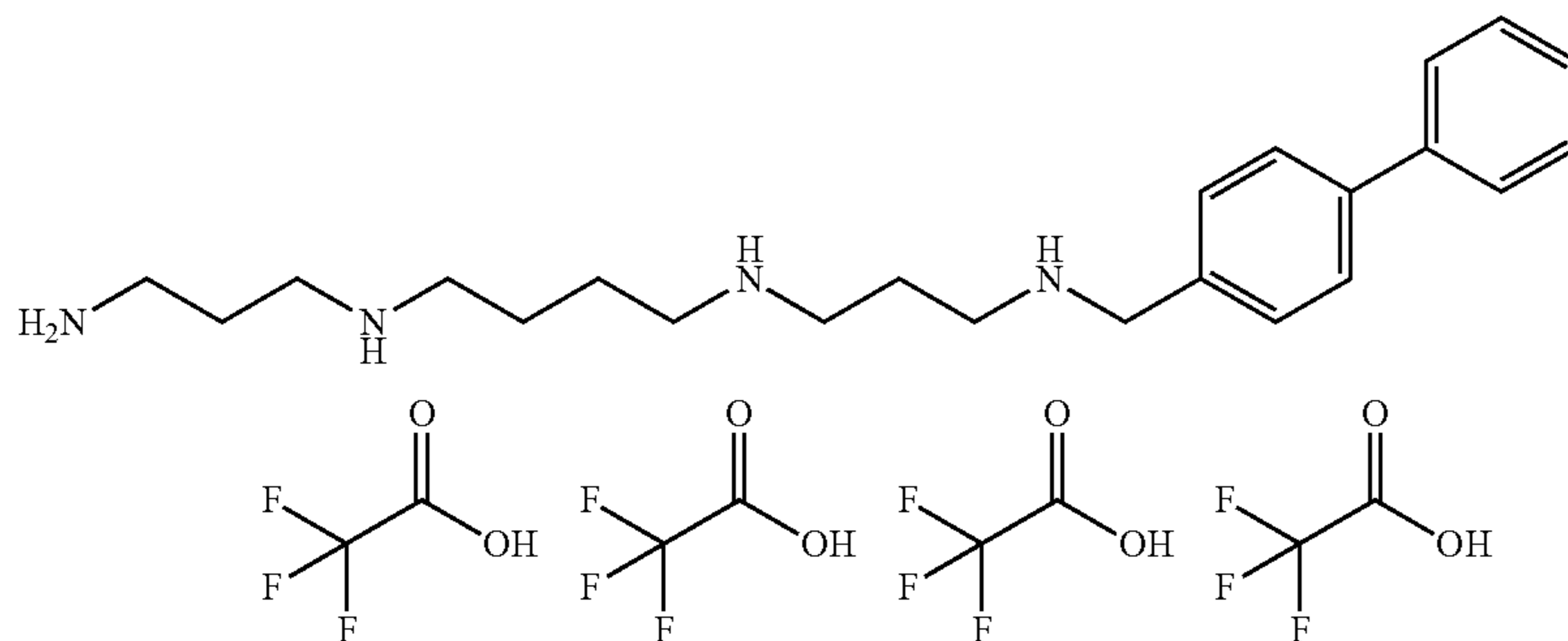
Yan-673



DS-176



Cetylpyridinium chloride



DS-347

2. Fingerprint generation—The FCFP\_6 fingerprint method was used to generate all molecular fingerprints.

Functional Class Extended-Connectivity FingerPrint is a method for generating atom feature codes for all heavy (non-hydrogen) atoms in a molecule. It applies the following rapid functional-role codes to each atom: Hydrogen-bond acceptor, Hydrogen-bond donor, Positively ionized or positively ionizable, Negatively ionized or negatively ionizable, Aromatic and Halogen. The number after the underscore indicates the maximum distance for extending the connectivity. In this case, FCFP\_6 indicates that the connectivity is extended up to 6 bonds in diameter.

FCFPs were developed by Accelrys Inc. and incorporated in their Pipeline Pilot software (“Extended-Connectivity Fingerprints”, D. Rogers and M. Hahn, *J. Chem. Inf Model.*, 2010, 50 (5), pp 742-754). The protocols were run as described in the Accelrys’ Pipeline Pilot user guide (Accelrys Software Inc., *Chemistry Collection: Basic Chemistry User Guide, Pipeline Pilot*, San Diego: Accelrys Software Inc., 2011).

3. Calculation of distance—The Tanimoto similarity coefficient was used to calculate similarity values between each database molecule and the lead molecules encoded by the FCFP\_6 molecular fingerprints described above using Pipeline Pilot’s “Find similar molecules fingerprint” component.

The Tanimoto similarity coefficient is defined by the expression:

$$\text{Tanimoto} = \frac{SA}{SA + SB + SC}$$

where:

SA=Number of bits defined in both the target and the reference

SB=Number of bits defined in the target but not the reference

SC=Number of bits defined in the reference but not the target

The Tanimoto similarity ranges from zero (there are no common ‘bits’ between the reference and the target molecules) to one (the reference and the target molecules have exactly the same ‘bits’)

4. Cutoff and filtering—A cutoff distance of 0.3 was applied to filter the results. This resulted in 76 materials being selected out of 4456. The 76 compounds were manually/visually analyzed to remove self-matches, multiple salt forms and duplicates and narrowed to 29 top candidates which were recommended for lab testing. 10 of the 29 materials were confirmed hits in an LPS displacement assay.

Using nearest neighbor similarity searching, we found a list of compounds (TABLE 10) that share structural similarities to LPS binders listed in TABLE 9.

TABLE 10

Materials of sharing structural similarities to LPS binders.

CAS	Smiles	Common Name
14246-55-0	CCCCCCCCCCCC(=O)NCC(=O)O	N-Myristoyl-glycine
14246-53-8	CCCCCCCC(=O)NCC(=O)O	Caprylylglycine
112-63-0	CCCCC/C=C/C=C\CCCCCCCC(=O)OC	Methyl Linoleate
112-62-9	CCCCCCCC/C=C\CCCCCCCC(=O)OC	Methyl Oleate
1120-34-9	CCCCCCCC/C=C\CCCCCCCCCCCC(=O)OC	Methyl cis-13-Docosenoate
301-00-8	CC/C=C/C=C\C/C=C\C\CCCCCCCC(=O)OC	Methyl linolenate
22882-95-7	CCCCC/C=C/C=C\C\CCCCCCCC(=O)OC	isopropyl linoleate
544-35-4	CCCCC/C=C/C=C\C\CCCCCCCC(=O)OC	Ethyl Linoleate
17673-59-5	CCCCCCCC/C=C\CCCCCCCC(=O)CCCCCCCC/C=C\C\CCCC	oleyl linoleate
106-73-0	CCCCCCC(=O)OC	methyl heptanoate
7132-64-1	CCCCCCCCCCCCCCCC(=O)OC	Methyl pentadecanoate
1731-88-0	CCCCCCCCCCCCCCCC(=O)OC	Methyl tridecanoate
124-10-7	CCCCCCCCCCCCCCCC(=O)OC	Methyl Myristate
1731-84-6	CCCCCCCC(=O)OC	methyl nonanoate
1808-26-0	CCCCC/C=C/C=C\C/C=C\C\CCCC(=O)OC	Arachidonic Acid ethyl ester
111-82-0	CCCCCCCCCCCC(=O)OC	methyl laurate
111-11-5	CCCCCCCC(=O)OC	methyl octanoate
112-39-0	CCCCCCCCCCCCCCCC(=O)OC	Palmitic Acid methyl ester
110-42-9	CCCCCCCC(=O)OC	Methyl Decanoate
112-11-8	CCCCCCCC/C=C\CCCCCCCC(=O)OC(C)C	isopropyl oleate
111-62-6	CCCCCCCC/C=C\CCCCCCCC(=O)OC	Oleic Acid ethyl ester
56219-10-4	CCCCC/C=C\CCCCCCCC(=O)OC	Palmitoleic Acid ethyl ester
1191-41-9	CC/C=C/C=C\C/C=C\C\CCCCCCCC(=O)OC	ethyl linolenate
141-24-2	CCCCC[C@H](C/C=C\C\CCCCCCCC(=O)OC)O	Methyl Ricinoleate
106-70-7	CCCCC(=O)OC	methyl hexanoate
2432-77-1	CCCCC(=O)SC	S-Methyl thiohexanoate
140-03-4	CCCCC[C@H](C/C=C\C\CCCCCCCC(=O)OC)OC(=O)C	Methyl O-Acetylricinoleate
537-40-6	CCCCC/C=C/C=C\C\CCCCCCCC(=O)OC(=O)C(=O)C=C\C\CCCC	Glyceryl trilinoleate
111-57-9	CCCCCCCCCCCCCCCC(=O)NCCO	Stearoyl Ethanolamide
142-78-9	CCCCCCCCCCCC(=O)NCCO	Lauramide MEA
13481-87-3	CCCCC/C=C/C=C\C\CCCCCCCC(=O)NCCO	methyl 3-nonenoate
544-31-0	CCCCCCCCCCCCCCCC(=O)NCCO	Palmitoyl Ethanolamide
2277-28-3	CCCCC/C=C/C=C\C\CCCCCCCC(=O)NCCO	(S)-glyceryl linoleate
142-77-8	CCCCCCCC/C=C\CCCCCCCC(=O)OC(=O)O	Butyl oleate
111-81-9	CCC(=O)CCCCCCCC=C	methyl 10-undecenoate
3687-46-5	CCCCCCCCCCCC(=O)CCCCCCCC/C=C\C\CCCCCCCC	Decyl Oleate
22393-86-8	CCCCCCCCCCCCCCCC(=O)CCCCCCCC/C=C\C\CCCCCCCC	Palmityl Oleate
36078-10-1	CCCCCCCCCCCCCCCC(=O)CCCCCCCC/C=C\C\CCCCCCCC	Dodecyl Oleate
22393-93-7	CCCCCCCCCCCCCCCC(=O)CCCCCCCCCCCC/C=C\C\CCCCCCCC	Oleyl Myristate
2441-41-0	CCCCCCCCCCCCCCCC(=O)NCC(=O)[O-]	N-Palmitoyl Glycine
56255-31-3	CCCCCCCCCCCCCCCC(=O)N[C@H](C)(=O)O	N-Hexadecanoyl-alanine
21394-64-9	CCCCCCCCCCCCCCCC(=O)NC(CO)C(=O)O	N-Palmitoyl Serine
4500-01-0	CCCCCCCC/C=C\CCCCCCCC(=O)OCCO	Glycol Oleate
52315-75-0	CCCCCCCCCCCC(=O)NCCCC[C@H](C(=O)O)N	Lauroyl Lysine
111-79-5	CCCCC/C=C\C\CCCCCCCC(=O)OC	Methyl trans-2-nonenoate
30606-27-0	CCCCC/C=C\C\CCCCCCCC(=O)OC(=O)C(=O)C=C\C\CCCC	Glyceryl Dilinoleate
106-79-6	CCC(=O)CCCCCCCC(=O)OC	Dimethyl Sebacate





The outlined methodology of using: (1) the LAL kit to quantify total LPS present and to detect technologies that inhibit the LAL assay (EXAMPLES 1-3); (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 (EXAMPLES 4-12); and (3) the high throughput screening and nearest neighbor similarity searching to identify new LPS and LTA mitigation technology (EXAMPLES 25-30); surprisingly lead to molecules that reduced the virulence of subgingival plaque, which led to a reduction in toxicity of the plaque. This reduction in toxicity was a result of the identified chemistry detoxifying the subgingival environment, which surprisingly led to better oral health as was manifested in a reduction in gingivitis.

TABLE 11

LPS Inhibition Via Limulus Amebocyte Lysate Assay.	
STRUCTURE_NAME	LAL % Inhibition
Hexaethylene glycol, monodecyl ether	100.50
Laureth-6	100.44
Steapyrium chloride	100.13
N-Lauryldiethanolamine	100.05
Quaternium-17	100.01
Laureth-9	99.92
Bromoenoil lactone	99.69
N-Lauryldiethanolamine	99.64
Oleyl amine	99.60
Cetrimonium chloride	99.51
Cetyltrimethylammonium bromide	99.45
Dimethylaminopropyl laurylamide	99.40
Cetylpyridinium chloride	99.13
(Laurylamidopropyl)dimethyl amine	99.09
potassium linoleate	98.92
Dimethyldodecylbenzylammonium chloride	98.25
Dimethyldodecylbenzylammonium bromide	98.09
.alpha.-linoleic acid	97.88
Benzalkonium chloride	97.50
Didecyldimethylammonium chloride	97.49
Diethylaminoethyl stearamide	97.42
Laurylisoquinolinium bromide	97.41
(R)-Bromoenoil lactone	97.27
Laurylisoquinolinium bromide	96.94
C-8 Ceramine	96.71
N-Laurylpyridinium chloride	96.53
Stearalkonium chloride	96.47
Myristyl-.gamma.-picolinium chloride	96.23
TRIDECYL SULFONIC ACID SODIUM SALT	95.58
Dimethylbenzylcetylammmonium chloride	95.55
Domiphen bromide	95.54
(S)-Bromoenoil lactone	95.15
Benzethonium chloride	94.57
Tetradecylbenzyltrimethylammonium chloride	93.78
Diocetyltrimethylammonium chloride	93.30
Abacross Flaxseed Oil	93.24
Tea Tree Oil Fractionated (Melafresh T96)	93.16
Methylbenzethonium chloride	92.98
N-Myristoylglycine	91.40
DODECYLTRIPHENYLPHOSPHONIUM BROMIDE, 98%	91.14
fenugreek	90.00
Sodium hexadecyl sulfate	89.44
STEARYLDIETHANOLAMINE	88.09
Sandalwood Oil (West Australian)	87.78
Asian ginseng	87.07
Tin tetrachloride	86.74
Marjoram Sweet Oil	86.64
Sodium pentadecyl sulfate	86.61
Propylpyrazole Triol	86.58
Sandalwood Oil (Australian)	86.06
Sandalwood Oil	85.98
N-Palmitoyl Glycine	85.78
N-Docosanoyl Taurine	85.15
Stearylamidopropyl dimethylamine	83.77
Eicosapentaenoyl PAF C-16	82.66

TABLE 11-continued

LPS Inhibition Via Limulus Amebocyte Lysate Assay.	
STRUCTURE_NAME	LAL % Inhibition
5.alpha.-hydroxy-6-keto Cholesterol	81.72
N-Nervonoyl Taurine	79.82
FTY720	79.08
linoleamide	75.25
10 Abacross Pepper Black Oil	73.67
PAz-PC	72.82
Steartrimonium chloride	71.27
1,2-Didecanoyl PC	67.90
diglycol monooleate	67.01
Frankincense Oil	66.66
C-8 Ceramide	65.48
15 D-erythro-Sphingosine C-18	63.76
Oleic Acid	61.04
Methylcarbanyl PAF C-16	59.72
Lavender Oil	58.69
Ferric chloride hexahydrate	58.13
N,N-Dimethylsphingosine	58.11
20 Oleyl Trifluoromethyl Ketone	57.56
<i>Arisaema Amurense</i> Rhizome	56.66
Curcumin	55.88
1-Stearoyl-2-Arachidonoyl PC	54.96
.delta.-Tocotrienol	52.44
Arachidonoyl Trifluoromethyl Ketone	52.11
25 1,2-Dioctanoyl-sn-glycerol	51.18
N-Arachidonoyl Dopamine	49.13
.alpha.-glyceryl monooleate	48.98
<i>aloe Vera</i>	48.93
POV-PC	48.72
C-6 Ceramide	48.05
30 AM404	47.57
1,2-Didecanoyl-sn-glycerol	47.40
MK 886 (sodium salt)	47.19
9-octadecenoic acid (z)-,2-hydroxyethyl ester	46.04
Dodecanamide MEA	44.32
Lavender Eucalyptus blend	44.25
35 Abacross Mandarin Red Oil	43.62
C-8 Ceramide-1-phosphate	42.66
Hexadecyl Acetyl Glycerol	41.98
1-Arachidonoyl Glycerol	41.75
Hexadecyl Methyl Glycerol	39.97
Montelukast (sodium salt)	38.55
.gamma.-Tocotrienol	38.53
40 methyl linoleate	36.90
1-Oleoyl-2-acetyl-sn-glycerol	36.17
Celastrol	35.93
2-Arachidonoyl Glycerol ether	34.98
(z,z,z)-9,12,15-octadecatrienoic acid ethyl ester	34.94
Ebselen	34.70
45 Oleoylethanolamide	34.14
Palmityl Trifluoromethyl Ketone	34.08
Oxirane, polymer with formaldehyde and 4-(1,1,3,3-tetramethylbutyl)phenol	33.80
Hexadecanamide MEA	32.47
ethyl cis-9-hexadecenoate	31.75
50 Anandamide (N-arachidonylethanol amide)	31.31
Olive Oil	30.72
alpha.-Tocotrienol	29.93
ethyl arachidonate	28.72
Aluminum phenol sulfonate	27.39
BENZALKONIUM BROMIDE	26.00
55 .alpha.-linolenic acid methyl ester	24.93
Orange Flower Extract OS	22.78
palmitamide mea	13.20
Octoxynol-9	9.83

60 The data in TABLE 11 showed the rank order of Lipopolysaccharide inhibition as measured by the LAL method. Molecules with the lipopolysaccharide inhibition of >50% would provide enough lipopolysaccharide inhibition to have an effect on reducing the lipopolysaccharide in vivo treatment applications, such as when delivered from a dentifrice or toothpaste. However, some of the tested molecules may interact with the enzymes in the LAL method to give false

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positive readings, the BODIPY method was used to further confirm the activity of the detoxifying agents in binding to virulence factors, such as LPS and LTA.

TABLE 12

LPS Inhibition Via BODIPY Method	
STRUCTURE_NAME	% LPS Displacement
palmitamide mea	295.10
Myristyl-.gamma.-picolinium chloride	262.64
potassium linoleate	153.94
Sodium hexadecyl sulfate	124.89
Aluminum phenol sulfonate	109.40
fenugreek	107.75
Steapyrium chloride	102.04
.alpha.-glyceryl monooleate	92.32
Tin tetrachloride	89.26
Ferric chloride hexahydrate	82.16
STEARYLDIETHANOLAMINE	78.64
Dimethylaminopropyl laurylamide	74.49
Stearylamidopropyl dimethylamine	72.47
Lavender Eucalyptus blend	70.84
Laureth-9	70.45
linoleamide	66.62
Lavender Oil	64.00
diglycol monooleate	63.16
Diethylaminoethyl stearamide	60.23
Laureth-6	59.27
N-Laurylpyridinium chloride	57.72
N-Myristoylglycine	56.24
N-Lauryldiethanolamine	55.07
MANGANESE ACETATE	52.70
Laurylisoquinolinium bromide	52.58
Oxirane, polymer with formaldehyde and 4-(1,1,3,3-tetramethylbutyl)phenol ethyl arachidonate	48.38
Methylbenzethonium chloride	47.52
Dimethyldodecylbenzylammonium chloride	47.47
<i>Arisaema Amurensis</i> Rhizome	45.85
BENZALKONIUM BROMIDE	45.31
Diocetyl dimethylammonium chloride	44.16
Domiphen bromide	43.50
Octoxynol-9	42.56
Myristyl-.gamma.-picolinium chloride	41.80
Steartrimonium chloride	40.81
ethyl cis-9-hexadecenoate	39.25
Quaternium-17	38.09
Dimethyldodecylbenzylammonium bromide	37.86
DODECYLTRIPHENYLPHOSPHONIUM BROMIDE, 98%	37.20
Abacross Pepper Black Oil	35.88
Tetradecylbenzyl dimethylammonium chloride	35.48
(z,z,z)-9,12,15-octadecatrienoic acid ethyl ester	34.15
Cetrimonium chloride	34.00
Didecyl dimethylammonium chloride	32.86
Sodium pentadecyl sulfate	32.82
Oleyl amine	30.12
Dimethylbenzylcetyl ammonium chloride	29.94
Cetyltrimethylammonium bromide	29.07
Hexaethylene glycol, monodecyl ether	28.94
.alpha.-linolenic acid methyl ester	27.98
Benzalkonium chloride	25.75
Stearalkonium chloride	23.93
Cetylpyridinium chloride	23.42
Benzethonium chloride	22.75
.alpha.-linoleic acid	22.30
N-Lauryldiethanolamine	20.21
9-Aminoacridine hydrochloride monohydrate	19.18
TRIDECYL SULFONIC ACID SODIUM SALT	18.48
methyl linoleate	17.85
Laurylisoquinolinium bromide	16.86

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TABLE 12-continued

LPS Inhibition Via BODIPY Method	
STRUCTURE_NAME	% LPS Displacement
.beta.-Cyclodextrin,2-hydroxypropyl ethers	16.01

TABLE 12 showed molecules which inhibited LPS by using the BODIPY method. Some molecules, such as palmitamide monoethanolamine, potassium linoleate, and myristyl gamma picolinium chloride caused a greater than 100% displacement. This is likely due to them enhancing the fluorescence of the BODIPY dye. The confirmatory test is to show displacement using both BODIPY and LAL, which is shown below in TABLE 13.

TABLE 13

LPS Inhibition Via BODIPY and LAL Cross Comparison		
STRUCTURE_NAME	LAL % Inhibition	BODIPY % LPS Displacement
Myristyl-.gamma.-picolinium chloride	95.96	262.64
potassium linoleate	98.92	153.94
Sodium hexadecyl sulfate	89.44	124.89
fenugreek	90.00	107.75
Steapyrium chloride	100.13	102.04
.alpha.-glyceryl monooleate	48.98	92.32
Tin tetrachloride	86.74	89.26
Ferric chloride hexahydrate	58.13	82.16
STEARYLDIETHANOLAMINE	88.09	78.64
Dimethylaminopropyl laurylamide	99.40	74.49
Stearylamidopropyl dimethylamine	83.77	72.47
Lavender Eucalyptus blend	44.25	70.84
Laureth-9	99.92	70.45
linoleamide	75.25	66.62
Lavender Oil	58.69	64.00
diglycol monooleate	67.01	63.16
Diethylaminoethyl stearamide	97.42	60.23
Laureth-6	100.44	59.27
N-Laurylpyridinium chloride	96.53	57.72
N-Myristoylglycine	91.40	56.24
N-Lauryldiethanolamine	100.05	55.07
Laurylisoquinolinium bromide	97.41	52.58
Methylbenzethonium chloride	92.98	47.52
Dimethyldodecylbenzylammonium chloride	98.25	47.47
<i>Arisaema Amurensis</i> Rhizome	56.66	45.85
Diocetyl dimethylammonium chloride	93.30	44.16
Domiphen bromide	95.54	43.50
Myristyl-.gamma.-picolinium chloride	96.23	41.80
Steartrimonium chloride	71.27	40.81

The compounds shown in TABLE 13 have inhibition using the LAL method and the BODIPY method. These molecules would be ideal candidates for inhibiting LPS in a biological system, such as in a gingival pocket to prevent or reduce gingivitis or periodontitis.

## Example 29

## 60 Stannous Fluoride Binds to Bacterial Cell Wall Components

Bacteria are coated with a well-developed cell wall which is responsible for many of their unique biological structures. The primary function of the cell wall is to protect the cell from internal turgor pressure caused by the much higher concentrations of proteins and other molecules inside the

cell compared to its external environment. The bacterial cell wall is composed of peptidoglycan which is located immediately outside of the cytoplasmic membrane, lipoteichoic acids (LTA), lipopolysaccharides (LPS) and others.

Stannous fluoride (SnF<sub>2</sub>) was shown to bind to *E. coli* LPS as shown in FIG. 33. Experiments were carried out, in a procedure as described in EXAMPLE 11, to determine whether SnF<sub>2</sub> inhibited expression of the NFκB-SEAP and IRF-Luciferase reporter genes in THP-1 dual cells that were stimulated by LPS and LTA from different bacteria. In the experiments, various concentrations of SnF<sub>2</sub> were applied to the THP-1 dual reporter cells. As shown in FIG. 33, SnF<sub>2</sub> inhibited expression of the NFκB-SEAP reporter gene by LPS and LTA in a dose dependent manner. Only *E. coli* LPS promoted strong expression of the IRF-luciferase reporter gene in the THP-1 dual cells. As shown in FIG. 33, SnF<sub>2</sub> inhibited luciferase production in the THP-1 dual cells stimulated by *E. coli* LPS.

To determine whether SnF<sub>2</sub> can inhibit LPS-stimulated production of proinflammatory cytokines in human primary monocytes, various concentrations of SnF<sub>2</sub> were applied to monocyte culture in the presence of 100 ng of *E. coli* LPS or *P. gingivalis* LPS 1690 for 24 hours. The primary human monocytes were purchased from Astarte Biologics (Bothell, Wash.). 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 LPS and various concentrations of SnF<sub>2</sub>. The medium was harvested at 24 hours after adding LPS and SnF<sub>2</sub>, and analyzed for proinflammatory cytokines in a kit following manufacturer's instruction (Meso Scale Diagnostics, Rockville, Md.). As shown in FIG. 34, SnF<sub>2</sub> inhibited cytokine production, stimulated by LPS, in a dose-dependent manner. Surprisingly, neither *E. coli* LPS nor *P. gingivalis* LPS 1690 stimulated production of IL-8 in primary human monocytes at 100 ng/ml, as shown in FIG. 35. Instead, they suppressed production of IL-8. SnF<sub>2</sub> reversed the suppression of LPS on IL-8 production in a dose-dependent manner, as shown in FIG. 35.

### Example 30

#### Stannous Fluoride Blocks Fluorescence-labeled *E. coli* LPS from Binding to TLR4 Receptors

Procedures: Green-fluorescent Alexa Fluor 488 conjugated LPS was purchased from Thermo Fisher, Waltham, Mass. HEK-Blue-hTLR4 cell line was purchased from InvivoGen as described in EXAMPLE 5. Stannous fluoride, Cetylpyridium chloride (CPC) and Triclosan were purchased from Sigma-Aldrich, St. Louis, Mo. The cells were grown and maintained in the 75 cm<sup>2</sup> flasks with 15 ml growth medium at 37° C. incubator, 95% humidity and 5% CO<sub>2</sub>. The growth medium: DMEM, with 4.5 g/l glucose, supplemented 10% fetal bovine serum, 50 U/ml penicillin and streptomycin, 100 μg/ml Normocin, and 2 mM L-glutamine, purchased from Thermo Fisher, Waltham, Mass. LPS-TLR4 Binding:

- A. Make solution A (3× concentration of the test compound solution in the cell growth medium).
- B. Make solution B (3×(3 ug/ml) LPS+0.15 ug/ml LPS binding protein in the cell growth medium).
- C. Take one flask of HEK-blue-TRL4, the cells were detached directly from in growth medium by hand shaking, the suspend cells were transferred into a 50 μl

tube, after 3 minutes centrifugation at low speed (800 rpm), the cells were re-suspended in 1.5-2 ml of the cell growth medium.

- D. Add 100 μl of solution B, add 100 μl of solution A (the test compounds: SNF2, CPC and triclosan), add same amount of culture medium, add 100 μl of HEK-blue-TRL4 into a microfuge tube.
- E. Place the microfuge tube inside an incubator at 35° C. with gently shaking for 1 hours
- F. Centrifuge the microfuge tube at low speed (<1000 rpm) 1-2 min to precipitate cells
- G. Remove supernatant and add 1 ml of the assay buffer (1×HBSS, 20 mM HEPES, pH 7.4) to re-suspend the cell.
- H. Repeat procedures F and H once.
- I. Re-suspend the cells in 310 μl of the assay buffer
- J. Aliquot 100 μl of cells from the microfuge tube in procedure I into 96-well assay plate (Black wall and clear bottom).
- K. Perform imaging and analysis using ImageXpress and MetaXpress software (Molecular Devices, Sunnyvale, Calif.).

Results: Strong green fluorescence was observed on the surface of HEK-Blue-hTLR4 in the control samples (without test compounds) as shown in FIG. 36, indicating labeled LPS binding to the TLR4 receptors located on the cell surface. Stannous fluoride and CPC reduced fluorescence intensity significantly (TABLE 14).

TABLE 14

Inhibition of <i>E. coli</i> LPS binding to HEK-blue-TLR4 cells by SnF <sub>2</sub> and CPC.			
	Average fluorescence intensity (FI)	STDEV (FI)	% of control
Control	1027	68.5	100
500 μM SnF <sub>2</sub>	662	54.6	64.5
66 μM CPC	0	17.4	0
33 μM CPC	368	25.4	35.8
16.5 μM CPC	635	48.4	61.8
500 μM triclosan	1021	56.7	99.4

### Example 31

#### Lipopolysaccharides (LPS) and Bacteria Stimulated Production of Proinflammatory Cytokines in Human Peripheral Blood Mononuclear Cells (PBMC)

Human peripheral blood mononuclear cells (PBMC) were isolated from blood obtained from Gulf Coast Regional Blood Center, Houston, Tex., USA, using Histopaque 1077 (Sigma Aldrich Co., St. Louis, Mo., USA) and Leucosep tubes (Greiner Bio-One, Monroe, N. Carolina, USA). The cells were cultured in 200 μl of RPMI 1640 medium in each well of a 96-well plate (ThermoFisher Scientific, Inc., Grand Island, N.Y., USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics at 37° C. with a 5% CO<sub>2</sub> atmosphere. *P. gingivalis* LPS and *E. coli* LPS were purchased from InvivoGen (San Diego, Calif.). *P. gingivalis*, *P. pallens*, and *F. alocis* were grown as described in EXAMPLE 1.

The human PBMC cells were seeded onto 96-well at 200,000 cells per well in 200 μl of RPMI 1640 medium in each well (ThermoFisher Scientific, Inc., Grand Island, N.Y., USA) containing 10% fetal bovine serum and 1%

penicillin/streptomycin antibiotics, and treated with clinical bacteria and LPS. The cells were then incubated for 24 hours at 37° C. with a 5% CO<sub>2</sub> atmosphere. The conditioned media were harvested at the end of experiment and analyzed for proinflammatory cytokines in an ELISA kit following manufacturer's instruction (Meso Scale Diagnostics, Rockville, Md.). As shown in FIGS. 37A, B, C, D and E, all bacteria stimulated production of proinflammatory cytokines. Similarly, both *E. coli* LPS and *P. gingivalis* LPS 1690 also promoted production of proinflammatory cytokines in human PBMC (FIGS. 38A, B, C, D and E).

### Example 32

#### Application of Lipopolysaccharide Mitigation Chemistry to an Oral Tooth Strip

##### Methods of Use.

Methods of using the desensitizing delivery system set forth herein to treat the oral cavity (or portions thereof) before or after the use of a whitening delivery system (such as a commercially available whitening strip product like CREST WHITESTRIPS) are also contemplated herein.

Examples of lipopolysaccharide inactivation compositions applied to a tooth strip according to the present invention are found below in Tables 15A, 15B, 15C, and 15D. The compositions were compounded using either a Ross double planetary mixer LDM-2 or Ross double planetary mixer DPM-40. Glycerin was weighed and added to a clean Ross mix tank. Water was weighed and added to a separate stainless steel mix tank equipped with a lightning mixer and 4" diameter turbine mixing blade. The carboxymethyl cellulose and the CARBOPOL were weighed and added to a clean plastic polyethylene bucket. The lid was applied to the bucket and the powders were rotated by hand to blend the powders together for 10 minutes. The potassium sorbate, potassium benzoate and potassium oxalate were weighed and added to the water in the stainless steel mixing tank. The lightning mixer was then used to completely dissolve the added salts. The sodium hydroxide was weighed and added to the potassium sorbate/sodium benzoate/potassium oxalate solution in the stainless steel mix tank. The solution was then mixed until clear. The blended carboxymethyl cellulose and CARBOPOL powder mix was carefully added to the glycerin by hand adding to cover the surface of the glycerin uniformly in the Ross mix tank. The Ross mixer was started and ran at a speed setting of 5 for 30 minutes. The solution from the stainless steel tank was then added to the Ross mixer and mixing continued for 45 minutes at a speed setting of 5. After this mixing session, the composition was checked for visible lumps. If lumps were present, the composition was mixed for an additional 20 minutes at a speed setting of 5.

Once the compounding of the composition was complete, strip-type delivery systems were made by slot coating the composition onto a sheet of release liner made of fluoropolymer coated polypropylene film sold under the brand name SCOTCHPACK 9741 RELEASE LINER (commercially available from 3M (Minnesota, USA)) and then combined with a polyethylene film material, an HDPE/LDPE blend polyethylene film (commercially available from Clopay (Cincinnati, USA) as embossed polyethylene film—32 GSM Sof-flex) using a continuous lamination process. This resulting laminate was then run through rotary kiss cutting dies to cut the strip shape to 1 cm×3 cm. The polyethylene material outside of the strip perimeter was removed and the resultant release liner with 1 cm×3 cm strip spaced out on the web was

cut into individual release liners measuring 3 cm×9 cm. The individual release liners with strips were placed into foil laminate pouches and sealed with heat.

The resulting delivery systems were tested for release off the release liner and gel flow and the Complex Modulus and Delta were measured and calculated pursuant to the methods set forth herein. Some products were further tested for in vivo adhesion and the results included below. To determine release off the liner, the strip was removed from the foil laminate pouch and the strip and laminated composition were grasped between two fingers and pulled from the release liner at a rate of approximately 3 cm/sec. The percentage amount of composition remaining on the release liner was visually analyzed and recorded. In vivo adhesion was determined by a wear panel.

The delivery systems with the compositions shown in Table 15 are according to the invention set forth herein. As may be seen by the data tabulated in the Table below, compositions 15a through 15E, were found to perform acceptably in all categories tested.

TABLE 15

Desensitizing Oral Care Compositions					
Ingredient	15A Wt. %	15B Wt. %	15C Wt. %	15D Wt. %	15E Wt. %
Carboxymethyl Cellulose 7M8SF	5.0	5.0	4.5	4.5	4.0
Glycerin USP (99.7)	31.86	31.86	31.86	31.86	31.86
CARBOPOL 956	1.0	1.0	2.5	2.5	2.5
Sodium Benzoate, NF FCC	0.50	0.5	0.5	0.5	0.5
Potassium Sorbate	0.20	0.2	0.2	0.2	0.2
Sodium Hydroxide Solution 50	0.25	0.25	1.1	1.05	1.0
Myristyl- $\gamma$ -picolinium chloride	0.1				
potassium linoleate		0.3			
Hexaethylene glycol, monodecyl ether			0.4		
Palmitamide ethanolamine				0.4	
Stannous fluoride					0.3
Deionized water	QS	QS	QS	QS	QS

Carboxymethyl Cellulose 7M8SF, 9H, and 7H are part of the AQUALON series commercially available from Hercules.

CARBOPOL 956 is commercially available from Lubrizol.

\*Data point not collected for sample.

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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 SEQUENCE LISTING
 

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<210> SEQ ID NO 1

<211> LENGTH: 786

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

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Ile Arg Ile Gln Leu Ser Glu Glu Ser Glu Phe Leu Val Asp Arg Ser
20           25           30

Lys Asn Gly Leu Ile His Val Pro Lys Asp Leu Ser Gln Lys Thr Thr
35           40           45

Ile Leu Asn Ile Ser Gln Asn Tyr Ile Ser Glu Leu Trp Thr Ser Asp
50           55           60

Ile Leu Ser Leu Ser Lys Leu Arg Ile Leu Ile Ile Ser His Asn Arg
65           70           75           80

Ile Gln Tyr Leu Asp Ile Ser Val Phe Lys Phe Asn Gln Glu Leu Glu
85           90           95

Tyr Leu Asp Leu Ser His Asn Lys Leu Val Lys Ile Ser Cys His Pro
100          105          110

Thr Val Asn Leu Lys His Leu Asp Leu Ser Phe Asn Ala Phe Asp Ala
115          120          125

Leu Pro Ile Cys Lys Glu Phe Gly Asn Met Ser Gln Leu Lys Phe Leu
130          135          140

Gly Leu Ser Thr Thr His Leu Glu Lys Ser Ser Val Leu Pro Ile Ala
145          150          155          160

His Leu Asn Ile Ser Lys Val Leu Leu Val Leu Gly Glu Thr Tyr Gly
165          170          175

Glu Lys Glu Asp Pro Glu Gly Leu Gln Asp Phe Asn Thr Glu Ser Leu
180          185          190

His Ile Val Phe Pro Thr Asn Lys Glu Phe His Phe Ile Leu Asp Val
195          200          205

Ser Val Lys Thr Val Ala Asn Leu Glu Leu Ser Asn Ile Lys Cys Val
210          215          220

Leu Glu Asp Asn Lys Cys Ser Tyr Phe Leu Ser Ile Leu Ala Lys Leu
225          230          235          240

Gln Thr Asn Pro Lys Leu Ser Asn Leu Thr Leu Asn Asn Ile Glu Thr
245          250          255

Thr Trp Asn Ser Phe Ile Arg Ile Leu Gln Leu Val Trp His Thr Thr
260          265          270

Val Trp Tyr Phe Ser Ile Ser Asn Val Lys Leu Gln Gly Gln Leu Asp
275          280          285

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

His Gln Val Val Ser Asp Val Phe Gly Phe Pro Gln Ser Tyr Ile Tyr
305          310          315          320

Glu Ile Phe Ser Asn Met Asn Ile Lys Asn Phe Thr Val Ser Gly Thr
325          330          335

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Arg Met Val His Met Leu Cys Pro Ser Lys Ile Ser Pro Phe Leu His  
 340 345 350

Leu Asp Phe Ser Asn Asn Leu Leu Thr Asp Thr Val Phe Glu Asn Cys  
 355 360 365

Gly His Leu Thr Glu Leu Glu Thr Leu Ile Leu Gln Met Asn Gln Leu  
 370 375 380

Lys Glu Leu Ser Lys Ile Ala Glu Met Thr Thr Gln Met Lys Ser Leu  
 385 390 395 400

Gln Gln Leu Asp Ile Ser Gln Asn Ser Val Ser Tyr Asp Glu Lys Lys  
 405 410 415

Gly Asp Cys Ser Trp Thr Lys Ser Leu Leu Ser Leu Asn Met Ser Ser  
 420 425 430

Asn Ile Leu Thr Asp Thr Ile Phe Arg Cys Leu Pro Pro Arg Ile Lys  
 435 440 445

Val Leu Asp Leu His Ser Asn Lys Ile Lys Ser Ile Pro Lys Gln Val  
 450 455 460

Val Lys Leu Glu Ala Leu Gln Glu Leu Asn Val Ala Phe Asn Ser Leu  
 465 470 475 480

Thr Asp Leu Pro Gly Cys Gly Ser Phe Ser Ser Leu Ser Val Leu Ile  
 485 490 495

Ile Asp His Asn Ser Val Ser His Pro Ser Ala Asp Phe Phe Gln Ser  
 500 505 510

Cys Gln Lys Met Arg Ser Ile Lys Ala Gly Asp Asn Pro Phe Gln Cys  
 515 520 525

Thr Cys Glu Leu Gly Glu Phe Val Lys Asn Ile Asp Gln Val Ser Ser  
 530 535 540

Glu Val Leu Glu Gly Trp Pro Asp Ser Tyr Lys Cys Asp Tyr Pro Glu  
 545 550 555 560

Ser Tyr Arg Gly Thr Leu Leu Lys Asp Phe His Met Ser Glu Leu Ser  
 565 570 575

Cys Asn Ile Thr Leu Leu Ile Val Thr Ile Val Ala Thr Met Leu Val  
 580 585 590

Leu Ala Val Thr Val Thr Ser Leu Cys Ser Tyr Leu Asp Leu Pro Trp  
 595 600 605

Tyr Leu Arg Met Val Cys Gln Trp Thr Gln Thr Arg Arg Arg Ala Arg  
 610 615 620

Asn Ile Pro Leu Glu Glu Leu Gln Arg Asn Leu Gln Phe His Ala Phe  
 625 630 635 640

Ile Ser Tyr Ser Gly His Asp Ser Phe Trp Val Lys Asn Glu Leu Leu  
 645 650 655

Pro Asn Leu Glu Lys Glu Gly Met Gln Ile Cys Leu His Glu Arg Asn  
 660 665 670

Phe Val Pro Gly Lys Ser Ile Val Glu Asn Ile Ile Thr Cys Ile Glu  
 675 680 685

Lys Ser Tyr Lys Ser Ile Phe Val Leu Ser Pro Asn Phe Val Gln Ser  
 690 695 700

Glu Trp Cys His Tyr Glu Leu Tyr Phe Ala His His Asn Leu Phe His  
 705 710 715 720

Glu Gly Ser Asn Ser Leu Ile Leu Ile Leu Leu Glu Pro Ile Pro Gln  
 725 730 735

Tyr Ser Ile Pro Ser Ser Tyr His Lys Leu Lys Ser Leu Met Ala Arg  
 740 745 750



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Arg Val Lys Arg Ile Thr Val Glu Asn Ser Lys Val Phe Leu Val Pro  
 340 345 350

Cys Leu Leu Ser Gln His Leu Lys Ser Leu Glu Tyr Leu Asp Leu Ser  
 355 360 365

Glu Asn Leu Met Val Glu Glu Tyr Leu Lys Asn Ser Ala Cys Glu Asp  
 370 375 380

Ala Trp Pro Ser Leu Gln Thr Leu Ile Leu Arg Gln Asn His Leu Ala  
 385 390 395 400

Ser Leu Glu Lys Thr Gly Glu Thr Leu Leu Thr Leu Lys Asn Leu Thr  
 405 410 415

Asn Ile Asp Ile Ser Lys Asn Ser Phe His Ser Met Pro Glu Thr Cys  
 420 425 430

Gln Trp Pro Glu Lys Met Lys Tyr Leu Asn Leu Ser Ser Thr Arg Ile  
 435 440 445

His Ser Val Thr Gly Cys Ile Pro Lys Thr Leu Glu Ile Leu Asp Val  
 450 455 460

Ser Asn Asn Asn Leu Asn Leu Phe Ser Leu Asn Leu Pro Gln Leu Lys  
 465 470 475 480

Glu Leu Tyr Ile Ser Arg Asn Lys Leu Met Thr Leu Pro Asp Ala Ser  
 485 490 495

Leu Leu Pro Met Leu Leu Val Leu Lys Ile Ser Arg Asn Ala Ile Thr  
 500 505 510

Thr Phe Ser Lys Glu Gln Leu Asp Ser Phe His Thr Leu Lys Thr Leu  
 515 520 525

Glu Ala Gly Gly Asn Asn Phe Ile Cys Ser Cys Glu Phe Leu Ser Phe  
 530 535 540

Thr Gln Glu Gln Gln Ala Leu Ala Lys Val Leu Ile Asp Trp Pro Ala  
 545 550 555 560

Asn Tyr Leu Cys Asp Ser Pro Ser His Val Arg Gly Gln Gln Val Gln  
 565 570 575

Asp Val Arg Leu Ser Val Ser Glu Cys His Arg Thr Ala Leu Val Ser  
 580 585 590

Gly Met Cys Cys Ala Leu Phe Leu Leu Ile Leu Leu Thr Gly Val Leu  
 595 600 605

Cys His Arg Phe His Gly Leu Trp Tyr Met Lys Met Met Trp Ala Trp  
 610 615 620

Leu Gln Ala Lys Arg Lys Pro Arg Lys Ala Pro Ser Arg Asn Ile Cys  
 625 630 635 640

Tyr Asp Ala Phe Val Ser Tyr Ser Glu Arg Asp Ala Tyr Trp Val Glu  
 645 650 655

Asn Leu Met Val Gln Glu Leu Glu Asn Phe Asn Pro Pro Phe Lys Leu  
 660 665 670

Cys Leu His Lys Arg Asp Phe Ile Pro Gly Lys Trp Ile Ile Asp Asn  
 675 680 685

Ile Ile Asp Ser Ile Glu Lys Ser His Lys Thr Val Phe Val Leu Ser  
 690 695 700

Glu Asn Phe Val Lys Ser Glu Trp Cys Lys Tyr Glu Leu Asp Phe Ser  
 705 710 715 720

His Phe Arg Leu Phe Asp Glu Asn Asn Asp Ala Ala Ile Leu Ile Leu  
 725 730 735

Leu Glu Pro Ile Glu Lys Lys Ala Ile Pro Gln Arg Phe Cys Lys Leu  
 740 745 750



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Arg Lys Ile Met Asn Thr Lys Thr Tyr Leu Glu Trp Pro Met Asp Glu  
755 760 765

Ala Gln Arg Glu Gly Phe Trp Val Asn Leu Arg Ala Ala Ile Lys Ser  
770 775 780

<210> SEQ ID NO 3  
<211> LENGTH: 904  
<212> TYPE: PRT  
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Met Arg Gln Thr Leu Pro Cys Ile Tyr Phe Trp Gly Gly Leu Leu Pro  
1 5 10 15

Phe Gly Met Leu Cys Ala Ser Ser Thr Thr Lys Cys Thr Val Ser His  
20 25 30

Glu Val Ala Asp Cys Ser His Leu Lys Leu Thr Gln Val Pro Asp Asp  
35 40 45

Leu Pro Thr Asn Ile Thr Val Leu Asn Leu Thr His Asn Gln Leu Arg  
50 55 60

Arg Leu Pro Ala Ala Asn Phe Thr Arg Tyr Ser Gln Leu Thr Ser Leu  
65 70 75 80

Asp Val Gly Phe Asn Thr Ile Ser Lys Leu Glu Pro Glu Leu Cys Gln  
85 90 95

Lys Leu Pro Met Leu Lys Val Leu Asn Leu Gln His Asn Glu Leu Ser  
100 105 110

Gln Leu Ser Asp Lys Thr Phe Ala Phe Cys Thr Asn Leu Thr Glu Leu  
115 120 125

His Leu Met Ser Asn Ser Ile Gln Lys Ile Lys Asn Asn Pro Phe Val  
130 135 140

Lys Gln Lys Asn Leu Ile Thr Leu Asp Leu Ser His Asn Gly Leu Ser  
145 150 155 160

Ser Thr Lys Leu Gly Thr Gln Val Gln Leu Glu Asn Leu Gln Glu Leu  
165 170 175

Leu Leu Ser Asn Asn Lys Ile Gln Ala Leu Lys Ser Glu Glu Leu Asp  
180 185 190

Ile Phe Ala Asn Ser Ser Leu Lys Lys Leu Glu Leu Ser Ser Asn Gln  
195 200 205

Ile Lys Glu Phe Ser Pro Gly Cys Phe His Ala Ile Gly Arg Leu Phe  
210 215 220

Gly Leu Phe Leu Asn Asn Val Gln Leu Gly Pro Ser Leu Thr Glu Lys  
225 230 235 240

Leu Cys Leu Glu Leu Ala Asn Thr Ser Ile Arg Asn Leu Ser Leu Ser  
245 250 255

Asn Ser Gln Leu Ser Thr Thr Ser Asn Thr Thr Phe Leu Gly Leu Lys  
260 265 270

Trp Thr Asn Leu Thr Met Leu Asp Leu Ser Tyr Asn Asn Leu Asn Val  
275 280 285

Val Gly Asn Asp Ser Phe Ala Trp Leu Pro Gln Leu Glu Tyr Phe Phe  
290 295 300

Leu Glu Tyr Asn Asn Ile Gln His Leu Phe Ser His Ser Leu His Gly  
305 310 315 320

Leu Phe Asn Val Arg Tyr Leu Asn Leu Lys Arg Ser Phe Thr Lys Gln  
325 330 335

Ser Ile Ser Leu Ala Ser Leu Pro Lys Ile Asp Asp Phe Ser Phe Gln  
340 345 350

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Trp Leu Lys Cys Leu Glu His Leu Asn Met Glu Asp Asn Asp Ile Pro  
 355 360 365

Gly Ile Lys Ser Asn Met Phe Thr Gly Leu Ile Asn Leu Lys Tyr Leu  
 370 375 380

Ser Leu Ser Asn Ser Phe Thr Ser Leu Arg Thr Leu Thr Asn Glu Thr  
 385 390 395 400

Phe Val Ser Leu Ala His Ser Pro Leu His Ile Leu Asn Leu Thr Lys  
 405 410 415

Asn Lys Ile Ser Lys Ile Glu Ser Asp Ala Phe Ser Trp Leu Gly His  
 420 425 430

Leu Glu Val Leu Asp Leu Gly Leu Asn Glu Ile Gly Gln Glu Leu Thr  
 435 440 445

Gly Gln Glu Trp Arg Gly Leu Glu Asn Ile Phe Glu Ile Tyr Leu Ser  
 450 455 460

Tyr Asn Lys Tyr Leu Gln Leu Thr Arg Asn Ser Phe Ala Leu Val Pro  
 465 470 475 480

Ser Leu Gln Arg Leu Met Leu Arg Arg Val Ala Leu Lys Asn Val Asp  
 485 490 495

Ser Ser Pro Ser Pro Phe Gln Pro Leu Arg Asn Leu Thr Ile Leu Asp  
 500 505 510

Leu Ser Asn Asn Asn Ile Ala Asn Ile Asn Asp Asp Met Leu Glu Gly  
 515 520 525

Leu Glu Lys Leu Glu Ile Leu Asp Leu Gln His Asn Asn Leu Ala Arg  
 530 535 540

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

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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

<210> SEQ ID NO 4  
 <211> LENGTH: 798  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

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

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

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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

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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

<210> SEQ ID NO 5  
 <211> LENGTH: 858  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<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  
                   130                                  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  
                   210                                  215                                  220

Asn Met Val Leu Glu Ile Leu Asp Val Ser Gly Asn Gly Trp Thr Val  
 225                                  230                                  235                                  240

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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  
 290 295 300  
 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  
 370 375 380  
 Lys Leu Gln Thr Leu Asp Leu Arg Asp Asn Ala Leu Thr Thr Ile His  
 385 390 395 400  
 Phe Ile Pro Ser Ile Pro Asp Ile Phe Leu Ser Gly Asn Lys Leu Val  
 405 410 415  
 Thr Leu Pro Lys Ile Asn Leu Thr Ala Asn Leu Ile His Leu Ser Glu  
 420 425 430  
 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

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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
      850                      855

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

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<400> SEQUENCE: 6

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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

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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 Phe Glu Lys Cys Ser Thr Leu Val Lys Leu Glu Thr Leu Ile Leu  
 370 375 380

Gln Lys Asn Gly Leu Lys Asp Leu Phe Lys Val Gly Leu Met Thr Lys  
 385 390 395 400

Asp Met Pro Ser Leu Glu Ile Leu Asp Val Ser Trp Asn Ser Leu Glu  
 405 410 415

Ser Gly Arg His Lys Glu Asn Cys Thr Trp Val Glu Ser Ile Val Val  
 420 425 430

Leu Asn Leu Ser Ser Asn Met Leu Thr Asp Ser Val Phe Arg Cys Leu  
 435 440 445

Pro Pro Arg Ile Lys Val Leu Asp Leu His Ser Asn Lys Ile Lys Ser  
 450 455 460

Val Pro Lys Gln Val Val Lys Leu Glu Ala Leu Gln Glu Leu Asn Val  
 465 470 475 480

Ala Phe Asn Ser Leu Thr Asp Leu Pro Gly Cys Gly Ser Phe Ser Ser  
 485 490 495

Leu Ser Val Leu Ile Ile Asp His Asn Ser Val Ser His Pro Ser Ala  
 500 505 510

Asp Phe Phe Gln Ser Cys Gln Lys Met Arg Ser Ile Lys Ala Gly Asp  
 515 520 525

Asn Pro Phe Gln Cys Thr Cys Glu Leu Arg Glu Phe Val Lys Asn Ile  
 530 535 540

Asp Gln Val Ser Ser Glu Val Leu Glu Gly Trp Pro Asp Ser Tyr Lys  
 545 550 555 560

Cys Asp Tyr Pro Glu Ser Tyr Arg Gly Ser Pro Leu Lys Asp Phe His  
 565 570 575

Met Ser Glu Leu Ser Cys Asn Ile Thr Leu Leu Ile Val Thr Ile Gly  
 580 585 590



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Ala Thr Met Leu Val Leu Ala Val Thr Val Thr Ser Leu Cys Ile Tyr  
595 600 605

Leu Asp Leu Pro Trp Tyr Leu Arg Met Val Cys Gln Trp Thr Gln Thr  
610 615 620

Arg Arg Arg Ala Arg Asn Ile Pro Leu Glu Glu Leu Gln Arg Asn Leu  
625 630 635 640

Gln Phe His Ala Phe Ile Ser Tyr Ser Glu His Asp Ser Ala Trp Val  
645 650 655

Lys Ser Glu Leu Val Pro Tyr Leu Glu Lys Glu Asp Ile Gln Ile Cys  
660 665 670

Leu His Glu Arg Asn Phe Val Pro Gly Lys Ser Ile Val Glu Asn Ile  
675 680 685

Ile Asn Cys Ile Glu Lys Ser Tyr Lys Ser Ile Phe Val Leu Ser Pro  
690 695 700

Asn Phe Val Gln Ser Glu Trp Cys His Tyr Glu Leu Tyr Phe Ala His  
705 710 715 720

His Asn Leu Phe His Glu Gly Ser Asn Asn Leu Ile Leu Ile Leu Leu  
725 730 735

Glu Pro Ile Pro Gln Asn Ser Ile Pro Asn Lys Tyr His Lys Leu Lys  
740 745 750

Ala Leu Met Thr Gln Arg Thr Tyr Leu Gln Trp Pro Lys Glu Lys Ser  
755 760 765

Lys Arg Gly Leu Phe Trp Ala Asn Ile Arg Ala Ala Phe Asn Met Lys  
770 775 780

Leu Thr Leu Val Thr Glu Asn Asn Asp Val Lys Ser  
785 790 795

<210> SEQ ID NO 7  
 <211> LENGTH: 1049  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

Met Val Phe Pro Met Trp Thr Leu Lys Arg Gln Ile Leu Ile Leu Phe  
1 5 10 15

Asn Ile Ile Leu Ile Ser Lys Leu Leu Gly Ala Arg Trp Phe Pro Lys  
20 25 30

Thr Leu Pro Cys Asp Val Thr Leu Asp Val Pro Lys Asn His Val Ile  
35 40 45

Val Asp Cys Thr Asp Lys His Leu Thr Glu Ile Pro Gly Gly Ile Pro  
50 55 60

Thr Asn Thr Thr Asn Leu Thr Leu Thr Ile Asn His Ile Pro Asp Ile  
65 70 75 80

Ser Pro Ala Ser Phe His Arg Leu Asp His Leu Val Glu Ile Asp Phe  
85 90 95

Arg Cys Asn Cys Val Pro Ile Pro Leu Gly Ser Lys Asn Asn Met Cys  
100 105 110

Ile Lys Arg Leu Gln Ile Lys Pro Arg Ser Phe Ser Gly Leu Thr Tyr  
115 120 125

Leu Lys Ser Leu Tyr Leu Asp Gly Asn Gln Leu Leu Glu Ile Pro Gln  
130 135 140

Gly Leu Pro Pro Ser Leu Gln Leu Leu Ser Leu Glu Ala Asn Asn Ile  
145 150 155 160

Phe Ser Ile Arg Lys Glu Asn Leu Thr Glu Leu Ala Asn Ile Glu Ile  
165 170 175

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Leu Tyr Leu Gly Gln Asn Cys Tyr Tyr Arg Asn Pro Cys Tyr Val Ser  
 180 185 190  
 Tyr Ser Ile Glu Lys Asp Ala Phe Leu Asn Leu Thr Lys Leu Lys Val  
 195 200 205  
 Leu Ser Leu Lys Asp Asn Asn Val Thr Ala Val Pro Thr Val Leu Pro  
 210 215 220  
 Ser Thr Leu Thr Glu Leu Tyr Leu Tyr Asn Asn Met Ile Ala Lys Ile  
 225 230 235 240  
 Gln Glu Asp Asp Phe Asn Asn Leu Asn Gln Leu Gln Ile Leu Asp Leu  
 245 250 255  
 Ser Gly Asn Cys Pro Arg Cys Tyr Asn Ala Pro Phe Pro Cys Ala Pro  
 260 265 270  
 Cys Lys Asn Asn Ser Pro Leu Gln Ile Pro Val Asn Ala Phe Asp Ala  
 275 280 285  
 Leu Thr Glu Leu Lys Val Leu Arg Leu His Ser Asn Ser Leu Gln His  
 290 295 300  
 Val Pro Pro Arg Trp Phe Lys Asn Ile Asn Lys Leu Gln Glu Leu Asp  
 305 310 315 320  
 Leu Ser Gln Asn Phe Leu Ala Lys Glu Ile Gly Asp Ala Lys Phe Leu  
 325 330 335  
 His Phe Leu Pro Ser Leu Ile Gln Leu Asp Leu Ser Phe Asn Phe Glu  
 340 345 350  
 Leu Gln Val Tyr Arg Ala Ser Met Asn Leu Ser Gln Ala Phe Ser Ser  
 355 360 365  
 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

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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 His His Asn Arg  
 770 775 780  
 Phe Leu Cys Thr Cys Asp Ala Val Trp Phe Val Trp Trp Val Asn His  
 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  
 865 870 875 880  
 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  
 945 950 955 960  
 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

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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
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
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 Leu Asn Ile Thr Asp Gly Ala Phe Leu Asn Leu Lys Asn Leu Arg		
115	120	125
Glu Leu Leu Leu Glu Asp Asn Gln Leu Pro Gln Ile Pro Ser Gly Leu		
130	135	140
Pro Glu Ser Leu Thr Glu Leu Ser Leu Ile Gln Asn Asn Ile Tyr Asn		
145	150	155
Ile Thr Lys Glu Gly Ile Ser Arg Leu Ile Asn Leu Lys Asn Leu Tyr		
165	170	175
Leu Ala Trp Asn Cys Tyr Phe Asn Lys Val Cys Glu Lys Thr Asn Ile		
180	185	190
Glu Asp Gly Val Phe Glu Thr Leu Thr Asn Leu Glu Leu Leu Ser Leu		
195	200	205
Ser Phe Asn Ser Leu Ser His Val Pro Pro Lys Leu Pro Ser Ser Leu		
210	215	220
Arg Lys Leu Phe Leu Ser Asn Thr Gln Ile Lys Tyr Ile Ser Glu Glu		
225	230	235
Asp Phe Lys Gly Leu Ile Asn Leu Thr Leu Leu Asp Leu Ser Gly Asn		
245	250	255
Cys Pro Arg Cys Phe Asn Ala Pro Phe Pro Cys Val Pro Cys Asp Gly		
260	265	270
Gly Ala Ser Ile Asn Ile Asp Arg Phe Ala Phe Gln Asn Leu Thr Gln		
275	280	285
Leu Arg Tyr Leu Asn Leu Ser Ser Thr Ser Leu Arg Lys Ile Asn Ala		
290	295	300
Ala Trp Phe Lys Asn Met Pro His Leu Lys Val Leu Asp Leu Glu Phe		
305	310	315
Asn Tyr Leu Val Gly Glu Ile Ala Ser Gly Ala Phe Leu Thr Met Leu		
325	330	335

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Pro Arg Leu Glu Ile Leu Asp Leu Ser Phe Asn Tyr Ile Lys Gly Ser  
                   340                                  345                                  350

Tyr Pro Gln His Ile Asn Ile Ser Arg Asn Phe Ser Lys Leu Leu Ser  
                   355                                  360                                  365

Leu Arg Ala Leu His Leu Arg Gly Tyr Val Phe Gln Glu Leu Arg Glu  
                   370                                  375                                  380

Asp Asp Phe Gln Pro Leu Met Gln Leu Pro Asn Leu Ser Thr Ile Asn  
 385                                  390                                  395                                  400

Leu Gly Ile Asn Phe Ile Lys Gln Ile Asp Phe Lys Leu Phe Gln Asn  
                                   405                                  410                                  415

Phe Ser Asn Leu Glu Ile Ile Tyr Leu Ser Glu Asn Arg Ile Ser Pro  
                   420                                  425                                  430

Leu Val Lys Asp Thr Arg Gln Ser Tyr Ala Asn Ser Ser Ser Phe Gln  
                   435                                  440                                  445

Arg His Ile Arg Lys Arg Arg Ser Thr Asp Phe Glu Phe Asp Pro His  
                   450                                  455                                  460

Ser Asn Phe Tyr His Phe Thr Arg Pro Leu Ile Lys Pro Gln Cys Ala  
 465                                  470                                  475                                  480

Ala Tyr Gly Lys Ala Leu Asp Leu Ser Leu Asn Ser Ile Phe Phe Ile  
                                   485                                  490                                  495

Gly Pro Asn Gln Phe Glu Asn Leu Pro Asp Ile Ala Cys Leu Asn Leu  
                   500                                  505                                  510

Ser Ala Asn Ser Asn Ala Gln Val Leu Ser Gly Thr Glu Phe Ser Ala  
                   515                                  520                                  525

Ile Pro His Val Lys Tyr Leu Asp Leu Thr Asn Asn Arg Leu Asp Phe  
                   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

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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
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

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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  
                                   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

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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				
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		



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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

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What is claimed is:

1. A method for identifying agents for detoxifying oral virulence factors comprising:

- a) providing a reporter cell that expresses at least one of TLR2 or TLR4;
- b) providing an oral virulence factor derived from dental plaque;
- c) providing a potential detoxifying agent;
- d) combining oral virulence factor and potential detoxifying agent with a reporter cell;
- e) measuring binding of potential detoxifying agents using at least one of BODIPY method or LAL method of low EC50 virulence factor;
- f) determining EC50 of virulence factor;
- g) distinguishing healthy gingivae from an inflamed site suffering from the symptoms of gingivitis.

2. The method of claim 1, wherein the oral virulence factor is lipopolysaccharide.

3. The method of claim 2, wherein the lipopolysaccharide is from a Gram negative bacterium.

4. The method of claim 1, wherein the oral virulence factor is lipoteichoic acid.

5. The method of claim 4, wherein the lipoteichoic acid is from a Gram positive bacterium.

6. The method of claim 1, wherein the potential detoxifying agent is at least one of myristyl-gamma-picolinium chloride; potassium linoleate; sodium hexadecyl sulfate; fenugreek; steapyrium chloride; alpha-glycerol monooloate; stearyldiethanolamine; dimethylaminopropyl laurylamide; stearylamidopropyl dimethylamine; linoleamide; N-lauryldiethanolamine; steartrimonium chloride; hexaethylene glycol, monodecyl ether; laureth-6; laureth-9; quaternium-17; bromoenol lactone; oleyl amine; cetrimonium chloride; or palmitamide monoethanolamine.

7. A method for identifying agents for detoxifying oral virulence factors comprising:

- a) providing a reporter cell that expresses at least one of TLR2 or TLR4;
- b) providing oral matter comprising at least one oral virulence factor derived from dental plaque;
- c) providing a potential detoxifying agent;
- d) combining oral matter and potential detoxifying agent with reporter cell;
- e) measuring binding of potential detoxifying agents using at least one of BODIPY method or LAL method of low EC50 virulence factor; and
- f) determining EC50 of each virulence factor;
- g) distinguishing healthy gingivae from an inflamed site suffering from the symptoms of gingivitis.

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