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Aluminum-induced generation of lipopolysaccharide (LPS) from the human gastrointestinal (GI)-tract microbiome-resident *Bacteroides fragilis*

PN Alexandrov[1], **JM Hill**[2,3], **Y Zhao**[2,4], **T Bond**[2], **C Taylor**[3], **ME Percy**[5], **W Li**[2,6], **WJ Lukiw**[1,3,7,8]

[1]Russian Academy of Medical Science, Moscow 113152, RUSSIAN FEDERATION

[2]LSU Neuroscience Center, LSU Health Sciences Center, New Orleans LA, 70112 USA

[3]Department of Microbiology, Immunology and Parasitology, LSUHSC, New Orleans LA, 70112 USA

[4]Department of Cell Biology and Anatomy, LSU Health Sciences Center, New Orleans LA, 70112, USA

[5]Departments of Neurogenetics, University of Toronto, Toronto ON M5S 1A8, CANADA

[6]Department of Pharmacology, Jiangxi University of TCM, Nanchang, Jiangxi 330004 CHINA

[7]Department of Neurology, LSU Health Sciences Center, New Orleans LA, 70112, USA

[8]Department of Ophthalmology, LSU Health Sciences Center, New Orleans LA, 70112, USA.

Abstract

Gram-negative bacteria of the human gastrointestinal (GI) tract microbiome: **(i)** are capable of generating a broad-spectrum of highly neurotoxic, pro-inflammatory and potentially pathogenic molecules; and **(ii)** these include a highly immunogenic class of amphipathic surface glycolipids known as lipopolysaccharide (LPS). *Bacteroides fragilis* (*B. fragilis*), a commensal, Gram negative, non-motile, non-spore forming obligatory anaerobic bacillus, and one of the most

*Communicating author: Walter J. Lukiw, BS, MS, PhD, Professor of Neuroscience, Neurology and Ophthalmology; Bollinger Professor of Alzheimer's disease; LSU Neuroscience Center, Louisiana State University Health Sciences Center, 2020 Gravier Street, Suite 904, LA 70112 USA; TEL 504-599-0842 wlukiw@lsuhsc.edu.

Author Contributions

PNA, JMH and WJL performed all bacterial culture work and neurotoxin, including LPS and other neurotoxic protein extraction from *B. fragilis,* and antibody-based detection of *B. fragilis* endotoxins, exotoxins, lipooligosaccahride (LOS), lipopolysaccharide (LPS), bacterial amyloids, and small non-coding RNAs (sncRNA). PNA, JMH, YZ, TB, CT, MEP, WL, WJL collected, analyzed, performed bioinformatics analysis and summarized the data and reviewed the current neurologically-relevant miRNA literature; YZ, WL and WJL performed the experiments and were involved in data extraction and bioinformatics; WJL wrote the article.

Conflict of Interest/disclosure/ethics statement

Declaration of interest for all authors including financial and personal relationships with other people or organizations: none. We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. This work was reviewed and approved by the Institutional Biosafety Committee/Institutional Review Board (IBC/IRB) at the LSU Health Sciences Center, New Orleans LA 70112 USA.

abundant bacteria found in the human GI tract, produces a particularly pro-inflammatory and neurotoxic LPS (BF-LPS). BF-LPS: **(i)** is known to be secreted from the B. *fragilis* outer membrane into the external-medium; **(ii)** can damage biophysiological barriers via cleavage of zonula adherens cell-cell adhesion proteins, thereby disrupting both the GI-tract barrier and the blood-brain barrier (BBB); **(iii)** is able to transit GI-tract barriers into the systemic circulation and cross the BBB into the human CNS; and **(iv)** accumulates within CNS neurons in neurodegenerative disorders such as Alzheimer's disease (AD). This short communication provides evidence that the incubation of B. *fragilis* with aluminum sulfate [$Al_2(SO_4)_3$] is a potent inducer of BF-LPS. The results suggest for the first time that the pro-inflammatory properties of aluminum may not only be propagated by aluminum itself, but by a stimulation in the production of microbiome-derived BF-LPS and other pro-inflammatory pathogenic microbial products normally secreted from human GI-tract-resident microorganisms.

GRAPHICAL ABSTRACT—Gram-negative bacteria such as *B. fragilis* of the human gastrointestinal (GI) tract microbiome generate pro-inflammatory glycolipids known as lipopolysaccharide (LPS) and other neurotoxic species, and are amongst the most pro-inflammatory molecules known. Incubation of aluminum sulfate [$Al_2(SO_4)_3$] with *B. fragilis* in culture resulted in a significant increase in the release of LPS.

Keywords

aluminum; Alzheimer's disease; *B. fragilis*; dysbiosis; fragilysin; BF-LPS; inflammation; lipopolysaccharide; microbiome

Overview

The human gastrointestinal (GI)-tract contains a complex and dynamic microbiome consisting primarily of anaerobic, Gram-negative bacteria, with archaea, fungi, microbial eukaryotes, protozoa, viruses, and other microorganisms making up the remainder [1–5]. Together with human host cells the GI-tract microbiome comprises an entire community of interacting biological entities referred to as the meta-organism where symbiotic associations and microbiome-host interactions are critical to human health and disease [5–12]. These disorders include lethal, progressive, age-related, inflammatory neurodegenerative disorders of the human CNS such as Alzheimer's disease (AD) [3–13].

Of the 52 currently recognized divisions of bacteria, humans have co-evolved with just 2 dominant phyla: *Bacteroidetes,* representing about ~24% of all human GI-tract resident bacteria, and *Firmicutes* (~72%), with *Actinobacteria* (~3%), *Proteobacteria* (~1%) and *Verrucomicrobia* (~0.1%) making up the remainder [1–4,9]. These four major bacterial phyla represent the *'bacterial-core'* of the human GI-tract microbiome [1–4,9–15]. The vast majority of all GI-tract microbiota consists of Gram-negative anaerobic bacteria, and *Bacteroidetes* species represent the most abundant Gram-negative anaerobes, outnumbering *Escherichia coli* (E.coli) in abundance by about 100 to 1 in some parts of the GI-tract [1–5,11–15]. Certain strains of *Bacteroidetes* species such as *Bacteroides fragilis (B. fragilis),* as a normal commensal microbe of the human GI-tract, are thought to be ordinarily beneficial to human health due to their multiple capabilities: **(i)** to biosynthesize useful metabolic co-factors and products such as polysaccharides, transport proteins, volatile fatty

acids and other nutrients [9–11]; **(ii)** to cleave dietary fiber into digestible short-chain fatty acids (SCFAs) that include acetate, propionate, and butyrate [9,10,15]; **(iii)** to function in the maintenance, development and homeostasis of the host-immune system [15–19]; (iv) to support immunomodulation and protection against pathogens including potentially pathogenic GI-tract bacteria [9,15]; and **(v)** to support glucose homeostasis [9,20,21]. However, when stressed, *B. fragilis* release an extensive and complex array of highly neurotoxic, pro-inflammatory and potentially pathogenic molecules that promotes the establishment in the GI-tract microbiome of bacterial dysbiosis [3,6,7,23,24–29]. Secreted neurotoxins of *B. fragilis* comprise six major classes of secreted molecules and include, in order of abundance, lipopolysaccharide (LPS), lipooligosaccharide (LOS; consisting of smaller versions of full sized LPS), bacterial amyloids, endotoxins (such as fragilysin), exotoxins, and small non-coding RNA (sncRNA) [5–8,23–26] (**Figure 1 and 2**). The most prevalent non-spore forming, Gram-negative GI-tract bacterial phylum, *Bacteroides,* makes up around one quarter of the cells in a typical Western GI-tract microbiome; these cells harbor as much as ~250 mg of LPS, making BF-LPS one of the highest-abundance microbial-derived amphipathic, neurotoxic molecules in the human GI-tract [11,16,26–31]. LPS, also known as lipoglycan, bacterial endotoxin, bacterial sugar-lipid or glycolipid, are 50–100 kDa self-aggregating, thermostable components consisting of a lipid and a polysaccharide composed of an O-antigen, an outer core and an inner core covalently linked, and are the most densely-packed surface molecules found within the outer membrane of Gram-negative bacteria (Figure 1). Typically LPS stimulates the release of tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β), gamma interferon (IFN_{γ}), interleukin 8 (IL-8), CXC ligand 8 (CXCL8) and other inflammatory cytokines and chemokines in various cell types, leading to an acute inflammatory response towards these bacterial molecular pathogens, which in the host orchestrates a robust anti-infectious, innate-immune response [22,25–33].

The neurobiological effects of environmentally abundant, neurotoxic metals on the growth and behavior of Gl-tract resident bacteria such as *B. fragilis* and their secreted lipopolysaccharides such as BF-LPS and other neurotoxic exudates are not well understood and incompletely characterized. In this communication we report for the first time the significant induction of BF-LPS by aluminum (sulfate). Interestingly, the pro-inflammatory effects of aluminum [26–28] may be supplemented via the actions of aluminum-induced neurotoxic glycolipids such as BF-LPS which along with fragilysin are known: **(i)** to disrupt normal bio-physiological barriers [26,30–33]; and **(ii)** to stimulate innate-immune signaling and support the pro-inflammatory neurodegeneration of central nervous system (CNS) tissues [26–30].

Bacteroides fragilis (B. fragilis; ATCC 23745; American Type Culture Collection, Manassas VA, USA) [26,2934] were propagated in ATCC® Medium 1490 (modified chopped meat medium; www.atcc.org/~/media/85260BB7A69A4640A5BB1042498807E4.ashx; https://www.atcc.org/~/media/EB141471E3D04ED9B6E940B3A505BE4C.ashx; last accessed 7 October 2019) under anaerobic conditions at 37°C (under Biosafety Level 2; BSL-2; https://www.vumc.org/safety/basics-biosafety-level-2; last accessed 7 October 2019) using either broth tubes or blood agar plates according to the supplier's instructions [ATCC; ATCCusers/

downloads/ 23745%20(1).pdf; last accessed 7 October 2019] and as previously described [26,31–35]; ATCC Medium 1490 was supplemented with degassed solutions containing metal sulfates [Na_2SO_4; $MgSO_4$; $In_2(SO_4)_3$; $Ga_2(SO_4)_3$; or aluminum sulfate [$Al_2(SO_4)_3$]; see details below] and made up to 0 (control), 50, 100 and 500 nM using sodium-, magnesium-, indium-, gallium- or aluminum-sulfate in ultrapure water (Invitrogen-ThermoFisher Scientific UltraPure™ DNase/RNase-Free Distilled Water; cat no. 10977015 or equivalent); these cultures were incubated anaerobically at 37°C for 48 hrs with or without metal sulfate additives; total fragilysin, LPS, bacterial amyloid and sncRNA were isolated as previously described, and/or characterized by our group or our collaborators, or were purchased from commercial sources for use as control markers [4–8,34–37]. Cultures of *B. fragilis* were incubated in parallel with aluminum sulfate [$Al_2(SO_4)_3$; ultrapure reagent ~99.99 %; CAS Number 10043–01-3; Sigma-Aldrich 202614, St Louis MO, USA; https://www.sigmaaldrich.com/catalog/product/aldrich/202614?lang=en&region=US&gclid=EAlalQobChMIIathY244wIV6R-Bh1niQ6DEAMYASAAEgJkffDBwE; last accessed 7 October 2019] or sodium sulfate (Na_2SO_4; anhydrous, granular, free-flowing, Redi-Dri™, ACS reagent, >99.9%; CAS Number 7757–82-6; Sigma Aldrich 1066370500); magnesium sulfate ($MgSO_4$; anhydrous, *ReagentPlus™,* >99.5%; CAS Number 7487–88-9; Sigma Aldrich M7506); indium sulfate [$In_2(SO_4)_3$; CAS Number 304655–87-6; Sigma Aldrich 288721]; gallium sulfate [$Ga_2(SO_4)_3$; >99.9%; CAS Number 13494–91-2; Sigma Aldrich 254207; last accessed 7 October 2019] as metal sulfate controls. Stock solutions of 0.1 to 0.5 M of these metal sulfates were made up in ultrapure water and added to the ATCC® *B. fragilis* incubation Medium 1490 to a ambient concentration of 0 (control), 50, 100 and 500 nM; virtually identical results were obtained when stock metal sulfate solutions were directly added to *B. fragilis* cultures. LPS was extracted from B. *fragilis* according to established standard methods hot phenol-water methods or as previously described with some modifications [34–37]; alternately LPS was purchased from commercial sources and used according to the supplier's instructions (LPS L8–274; Sigma Aldrich; https://www.sigmaaldrich.com/Graphics/COfAInfo/SigmaSAPQM/SPEC/L8/L8274/L8274-BULKSIG MA.pdf). All protein concentrations were quantified using a Qubit Fluorometric Protein Assay Kit (Cat Number Q33212; sensitivity 12.5 μg/ml to 5 mg/ml) and/or by using antibodies specific for LPS, fragilysin, bacterial amyloid and sncRNA according to the supplier's instruction and as previously described [4–8,14,26,30–37].

As mentioned earlier, the human Gl-tract microbiome-resident Gram-negative bacillus *B. fragilis* produces a notable assortment of soluble neurotoxins that are shed from the bacterial surface into their immediate environment (**Figure 1 and 2**); all of these Gl-tract microbiome-derived neurotoxins, and especially LPS, were found to be induced by nM levels of aluminum sulfate in *B. fragilis* cultures; for example in *B. fragilis* cultures just 50 nM aluminum sulfate after 24 hrs increased BF-LPS to levels 8.1-fold over control and the results are highly significant (Figure 3). Four other monovalent-, divalent- or trivalent-metal sulfates - sodium sulfate (Na_2SO_4), magnesium sulfate ($MgSO_4$); indium sulfate [$In_2(SO_4)_3$] or gallium sulfate [$Ga_2(SO_4)_3$] displayed an inability to induce BF-LPS to the extent observed with aluminum sulfate [$Al_2(SO_4)_3$] addition at any concentration tested. Interestingly: **(i)** the increase in BF-LPS abundance at 50 nM aluminum sulfate is not linearly proportionate to increases in LPS at 100 nM and 500 nM aluminum sulfate

suggesting that the system may become rapidly saturated at the relatively lower concentrations of applied aluminum sulfate; **(ii)** an excess of aluminum sulfate added may be reacting with other components of the system under study; and **(iii)** at higher concentrations of aluminum sulfate (~100 nM and 500 nM) in this experimental test system other *B. fragilis-secreted* neurotoxins become induced above basal levels and the results are again highly significant (Figure 3). For example, at 500 nM [$Al_2(SO_4)_3$] the neurotoxins LPS, fragilysin, bacterial amyloid and sncRNA were increased, respectively to 13.5-fold, 2.1-fold, 4.5-fold and 3.5-fold over control (Figure 3). While aluminum itself is pro-inflammatory, as measured by its ability to induce the pro-inflammatory transcription factor NF-kB (p50/p65) complex and up-regulate pathogenic microRNAs (miRNAs) such as miRNA-146a that support inflammation [25,28], aluminum-mediated induction of LPS and other neurotoxins such as LPS, fragilysin, bacterial amyloid and sncRNA may also contribute to the pro-inflammatory actions of aluminum-sulfate in the human GI-tract and the CNS [25–28]. Aluminum-induced up-regulation of microbiome-derived LPS may also contribute to systemic inflammation, a potential precursor to the development of AD [17,45,46,50], however this pathological mechanism is not well understood and requires additional study (Figure 3).

One of the highest abundance Gram-negative bacteria-derived neurotoxins in the human microbiome, BF-LPS, is also the most abundant pro-inflammatory glycolipid in the human GI-tract [11,14,16,26,27,31,29,36–39] (Figure 1 and 2). Besides BF-LPS, *B. fragilis* also secretes neurotoxins in less abundance and these include the metalloproteinase fragilysin, a variety of different types of bacterial amyloid and sncRNA, as well as other as yet poorly characterized secreted microbial molecules [5–9,11,26,33,38], (Figure 2). Both BF-LPS shed from the exterior membrane surface of and the *B. fragilis* endotoxin fragilysin have been shown to cleave the zonula adherens protein, E-cadherin and thus disrupt normally homeostatic biophysiological membrane barriers [11–14,16–18,26,38,39]. When secreted neurotoxins of enterotoxigenic strains of *B. fragilis* leak through normally protective bio-physiological-mucosal barriers they can cause substantial inflammatory pathology systemically that can contribute to significant mortality and morbidity [38,39]. Dietary intake of fiber may have a determining role in regulating the composition, organization and stoichiometry of the GI-tract microbiome; for example *Bacteroidetes* species proliferate in porcine models fed high-fat diets that are deprived of sufficient dietary fiber and the presence of aluminum may potentiate these effects [38,40; unpublished observations]. Interestingly, based on the evolution of the NF-kB (p50/p65) pro-inflammatory transcription factor, BF-LPs has been recently shown to be the most inflammation-inducing glycolipid compared to TNFα, Aβ40 peptide, Aβ42 peptide, IL-1β, the combination of Ap42 peptide and IL-1β together or *E. coli* LPS (EC-LPS) [26]. Importantly, aluminum may represent an important ingested dietary factor capable of inducing pro-inflammatory signaling in the human GI-tract, systemic circulation and CNS via the initial up-regulation of neurotoxic glycolipids such as BF-LPS.

Lastly, aluminum is a pervasive neurotoxic element in our biosphere that is being increasingly mobilized both into our environment and into multiple aspects of our daily life through the air, the food we eat and the water we drink [42–45]. This is well above and

beyond the contribution of alum [as $KAl(SO_4)_2 \cdot 12H_2O_4$] added to drinking water supplies worldwide to produce a clear, 'finished' water product [42–45]. Aluminum sulfate-induced up-regulation of LPS and other bacterial-derived neurotoxins may make a significant contribution: **(i)** via selective cadherin cleavage to biophysiological barrier disruption to allowing other neurotoxins access via systemic circulation into CNS compartments [11–14,16–19,39]; **(ii)** towards systemic inflammation, sometimes referred to as a precursor to inflammatory neurodegenerative diseases such as AD [45–51]; **(iii)** to the accumulation of pro-inflammatory LPS within the human brain parenchyma and neuronal cytoplasm [5–8,49–52]; and/or **(iv)** to the LPS-mediated disruption of homeostatic genetic activities involving brain gene transcription in CNS neurons [4–8;48–53]. Ingested aluminum particularly over the long term might contribute chronically to AD-type change and promote AD as a disease transformation rather than a disease state where epigenetics may play a role in both cause and eventual treatment [54–56]. If dietary aluminum crosses GI-tract barriers to access aluminum-sensitive Gram-negative bacterial species such as *B. fragilis,* to produce increased amounts of BF-LPS, aluminum may ultimately increase LPS abundance in the systemic circulation and eventually cross the BBB into the CNS (57–62). There is recent evidence that LPS: **(i)** can both disrupt and transverse the BBB to gain access to the brain parenchyma, associate with senile plaques (characteristic lesions of the AD brain) and interact with the nuclear envelope of neurons [6,8,48,49,52,60,61]; **(ii)** functions to increase blood-brain barrier permeability to Thioflavin-S (MW ~319), to ^{14}C-sucrose (MW ~342) and to ^{99m}Tc-albumin (MW 66,500)' in experimental mouse models [59,60]; and **(iii)** this suggests that LPS-mediated BBB-disruption may allow the entry of other pathogenic GI-tract-derived neurotoxins such as fragilysin (MW ~20,600), LOS (MW <10,000), bacterial amyloid (CsgA-His; MW~13,900), and/or sncRNA (MW~14,100) into the brain. We speculate that combinations of environmentally abundant metals or other ingested 'dietary' factors which stimulate the profusion of neurotoxins derived from anaerobic Gram-negative bacteria and other constituents of the GI-tract microbiome may significantly contribute to the initiation, development and/or propagation of inflammatory neurodegeneration and related neurological disease processes with an inflammatory component.

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Abbreviations

AD	Alzheimer's disease
BBB	blood brain barrier
BF	*Bacteroides fragilis (B. fragilis)*
BF-LPS	LPS from *B. fragilis*
CNS	central nervous system
GI	gastrointestinal
LOS	lipooligosaccharide
LPS	lipopolysaccharide
miRNA	microRNA
NF-kB (p50/p65)	nuclear factor for kappa B p50/p65 subunit
sncRNA	small, non-coding RNA

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HIGHLIGHTS

- the human gastrointestinal (GI)-tract microbiome contains an abundance of *Bacteroides fragilis;*
- *Bacteroides fragilis* generates pro-inflammatory glycolipid lipopolysaccharide (BF-LPS); aluminum sulfate [$Al_2(SO_4)_3$] significantly induces the generation of BF-LPS;
- this may contribute to human systemic inflammation and neuro-inflammatory disease.

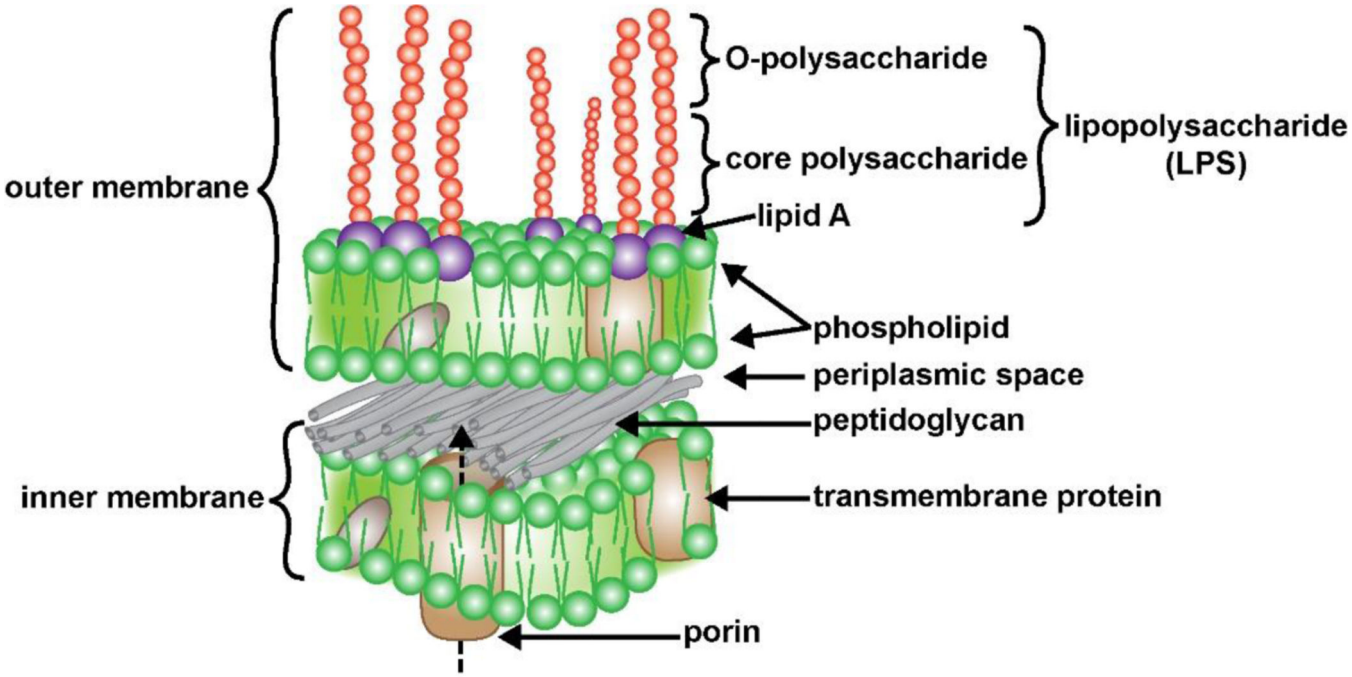

FIGURE 1 -

Typical structure and organization of an anaerobic Gram-negative bacterial cell membrane and its containment of a lipopolysaccharide (LPS) coated surface (purple and red spheres); the two horizontal layers include an external (outer) and an internal (inner) membrane, both layers contain both integral (gray) and transmembrane (beige) globular proteins; the membranes are separated by an interwoven peptidoglycan layer and a periplasmic space; transmembrane protein complexes such as *porin* that transverse the inner membrane facilitate molecular communication and LPS transport between the bacterial cell interior to the bacterial outer membrane surface (dashed arrow) but the mechanisms are not well understood [20,47,57–59]. The outer surface of the external membrane contains a dense layer of LPS with lipids anchored in the membrane (purple spheres), and long core polysaccharide and O-polysaccharide side chains extending outward (red spheres); the externally facing LPS are highly thermostable, neurotoxic, pathogenic, extremely pro-inflammatory and a potent trigger of robust antigenic responses within the human immune system; LPS are constantly shed into the external environment where they may find their way past the GI-tract barrier into the systemic circulation and past the BBB into the brain parenchyma [1–5,9,15–18,39,50,57–62]; the mechanism of the induction of LPS and other GI-tract microbiome-derived neurotoxins by aluminum sulfate is not known; (source; figure adapted from https://www.dreamstime.com/stock-illustration-structure-gram-negative-bacteria-cell-wall-labeled-d-illustration-image84181743; last accessed 7 October 2019).

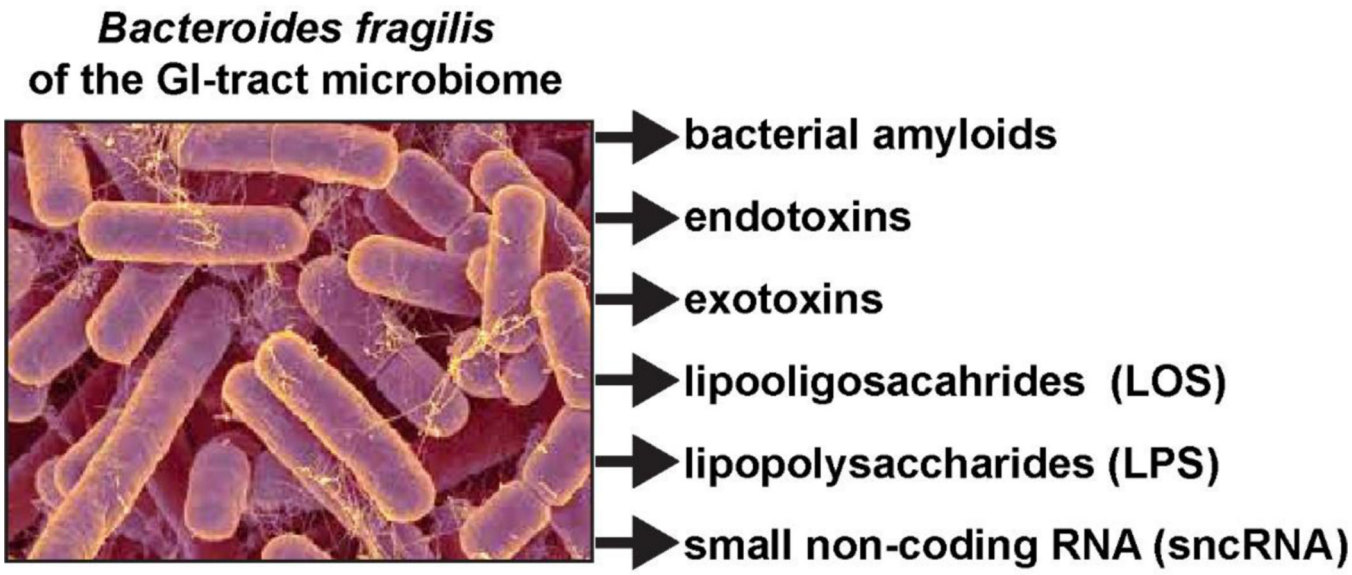

FIGURE 2 -

Comparable to all other anaerobic Gram-negative bacilli, the gastrointestinal (GI) tract abundant *Bacteroides fragilis* is capable, when stressed, of releasing a broad spectrum of highly neurotoxic, pro-inflammatory and potentially pathogenic molecules; these comprise six major classes of secreted molecules and include bacterial amyloids, endotoxins, exotoxins, lipooligosacahride (LOS; consisting of smaller isoforms of LPS), lipopolysaccharide (LPS; in this photo yellowish filamentous structures associated with some *B. fragilis* bacillus rods) and small non-coding RNAs (sncRNA; some similar in size to microRNAs). For example, the human GI tract-abundant *B. fragilis* secretes the endotoxin-LPS *B. fragilis* LPS (BF-LPS) which has been shown to be strongly pro-inflammatory and extremely neurotoxic toward human CNS neurons in primary culture; BF-LPS may be the most pro-inflammatory bacterial-derived glycolipid known [5,6,26,51–53]. While the phyla *Bacteriodetes* (representing about ~24% of all GI-tract bacteria), *Firmicutes* (~72% of all GI-tract bacteria), *Actinobacteria, Proteobacteria,* and *Verrumicrobia* (together, typically ~4% of all GI-tract bacteria), are the most common microbes in the human GI tract microbiome it should be kept in mind that other microbes including fungi, protozoa, viruses, and other commensal microorganisms may also contribute neurotoxic exudates which are highly toxic, pro-inflammatory and detrimental to the homeostasis of CNS neurons; (micrograph of *B. fragilis* shown; the original photo is shown courtesy of Rosa Rubicondior; http://rosarubicondior.blogspot.com/2014/11/evolving-cooperation-but-for-who-or-what.html; last accessed 7 October 2019).

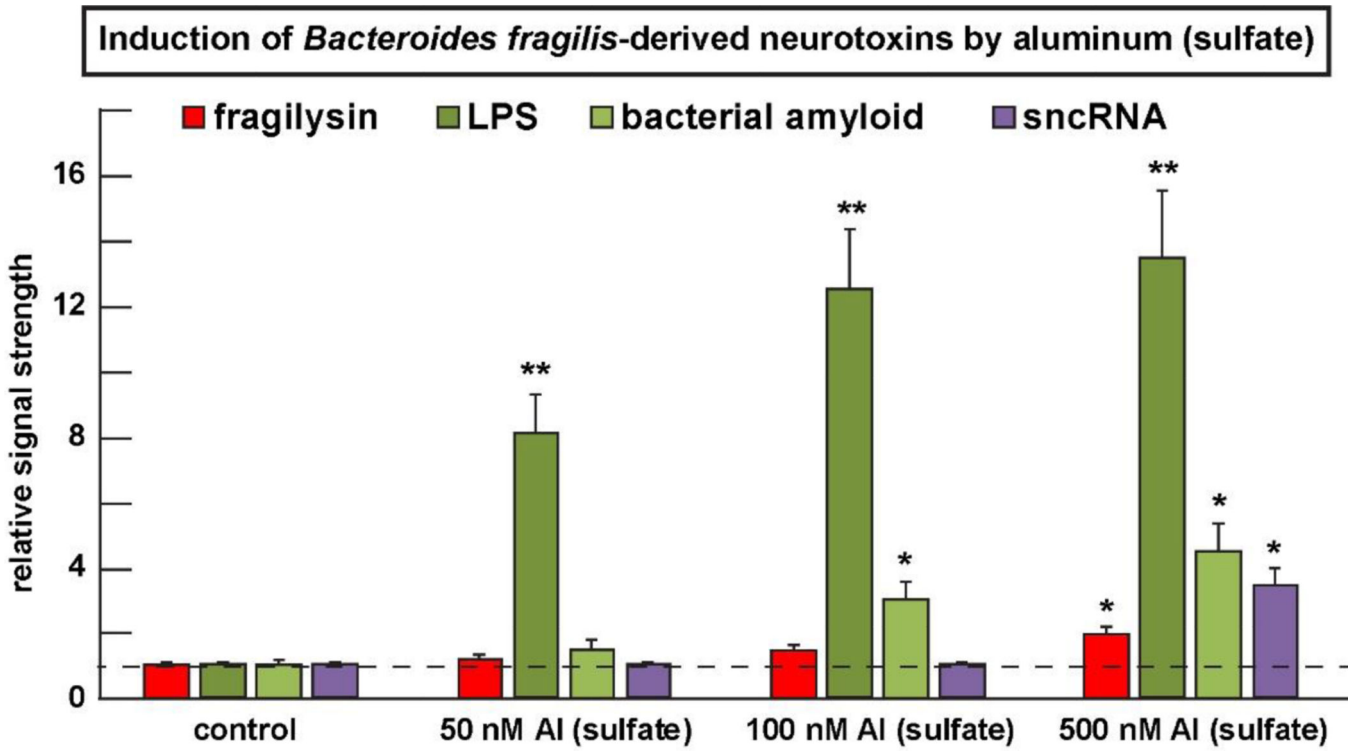

FIGURE 3 -

The human GI-tract microbiome-resident *Bacteroides fragilis (B. fragilis)* produces an array of soluble neurotoxins (such as fragilysin, LOS, LPS, bacterial amyloid, sncRNA and others) that are secreted into their immediate environment (see text); many of these neurotoxins are known to cross both the GI-tract intestinal barrier into the systemic circulation and induce a systemic inflammation; some of these neurotoxins may cross the blood brain barrier (BBB) to access the brain parenchyma in the aging human BBB or in transgenic murine models for AD and other neurodegenerative disease states [39,51,57–62]. All of these GI-tract microbiome-derived neurotoxins and especially lipopolysaccharide (LPS) are induced by nM levels of aluminum sulfate in *B. fragilis* cultures; interestingly **(i)** the increase in LPS at 50 nM aluminum sulfate is not proportionate to increases in LPS at 100 nM and 500 nM aluminum sulfate suggesting that the system may become rapidly saturated at the relatively lower concentrations of applied aluminum sulfate; and **(ii)** at higher concentrations of aluminum sulfate (~500 nM) in this system other *B. fragilis-secreted* neurotoxins such as fragilysin, bacterial amyloid and sncRNA become induced above basal levels and the results are significant; while aluminum itself is pro-inflammatory as measured by its ability to induce the pro-inflammatory transcription factor NF-kB (p50/p65) complex [26–28], aluminum-mediated induction of LPS and other inflammation-supporting neurotoxins such as LPS may also contribute to the pro-inflammatory actions of aluminum-sulfate in both the human GI-tract and the human CNS [42,43,56]. Aluminum-induced up-regulation of microbiome-derived LPS may also contribute to systemic inflammation but this pathological mechanism is not well understood and requires further study; in Figure 3 a dashed horizontal line at 1.0 is included for ease of comparison; N=3 to

5 experiments per determination; data in the bar graph represents the mean and one standard deviation of that mean; *$*p<0.05$; **$p<0.001$* (ANOVA).