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REVIEW

Lipopolysaccharide and the gut microbiota: considering structural variation

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Systemic inflammation is associated with chronic disease and is purported to be a main pathogenic mechanism underlying metabolic conditions. Microbes harbored in the host gastrointestinal tract release signaling byproducts from their cell wall, such as lipopolysaccharides (LPS), which can act locally and, after crossing the gut barrier and entering circulation, also systemically. Defined as metabolic endotoxemia, elevated concentrations of LPS in circulation are associated with metabolic conditions and chronic disease. As such, measurement of LPS is highly prevalent in animal and human research investigating these states. Indeed, LPS can be a potent stimulant of host immunity, but this response depends on the microbial species' origin, a parameter often overlooked in both preclinical and clinical investigations. Indeed, the lipid A portion of LPS is mutable and comprises the main virulence and endotoxic component, thus contributing to the structural and functional diversity among LPSs from microbial species. In this review, we discuss how such structural differences in LPS can induce differential immunological responses in the host.

Keywords: gut barrier; gut microbiome; immunity; inflammation; lipopolysaccharide binding protein; lipopolysaccharide variant; lipopolysaccharides; metabolic endotoxemia; obesity

Obesity and obesogenic diets have been correlated with negative changes in intestinal flora composition and function [1–3]. Such alterations have been established to promote increased gut permeability, translocation of bacterial products, and interactions with gut-associated lymphoid tissue (GALT), which together promote systemic low-grade, chronic inflammation [4–6]. Termed 'meta-inflammation,' this state is considered part of the pathogenic mechanisms underlying insulin resistance, atherosclerosis, and other metabolic diseases [7–10]. Disruptions to gut barrier integrity may spur an increased influx of microbial

Abbreviations
BA, bile acid; Caco-2, colorectal adenocarcinoma cells; CD14, cluster of differentiation 14; CID, collision-induced dissociation; CVD, cardiovascular disease; DC, dendritic cells; FITC, fluorescein isothiocyanate; GALT, gut-associated lymphoid tissue; GI, gastrointestinal; GM, gut microbiota; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HEK, human embryonic kidney; IBD, inflammatory bowel disease; Ig, immunoglobulin; IL, interleukin; INF, interferon; iNOS, nitric oxide synthase; JNK, c-Jun N-terminal protein kinase; LAL, limulus amoebocyte lysate; LBP, lipopolysaccharide binding protein; LDL, low-density lipoprotein; LPS, lipopolysaccharides; MD-2, myeloid differentiation protein-2; MS, mass spectrometry; MyD88, myeloid differentiation primary response gene 88; NAFLD, non-alcoholic fatty liver disease; NF-κB, nuclear factor kappa-beta; PAMPs, pathogen-associated molecular patterns; PBMCs, peripheral blood mononuclear cells; PGE2, prostaglandin E2; PRR, pattern recognition receptors; QQQ, triple quadrupole; T2D, type 2 diabetes; TLR-4, Toll-like receptor-4; TNF-α, tumor necrosis factor-alpha; TRIF, TIR domain-containing adapter-inducing interferon-β; VLDL, very low-density lipoprotein.

subcomponents, chiefly lipopolysaccharides (LPS), and other inflammatory mediators and cytokines, into the host systemic circulation [11,12]. Defined as metabolic endotoxemia, elevations in circulating LPS are observed in obesity and have become a prevalent outcome examined in animal and human studies [12–14]. This interest is warranted as metabolic endotoxemia has been associated with several chronic diseases in humans including type 2 diabetes (T2D), nonalcoholic fatty liver disease (NAFLD), and Alzheimer's disease [15–17]. Therefore, LPS may represent an important potential target for therapeutic intervention in disease prevention and treatment associated with meta-inflammation.

Lipopolysaccharide has been identified as an 'alarm molecule' sensed by the body's innate immunity, warning of a potential threat of invasion by pathogens [18]. Consequently, LPS is generally thought of as a potent stimulant of host immune response and thus a promoter of proinflammatory cytokine secretion. However, this response appears to be dependent on the microorganism (i.e., commensal or pathogenic) from which LPS originates and, consequently, on the LPS structure [19]. For example, LPS derived from *Escherichia coli* (*E. coli*) induces an increase in the levels of interleukin (IL)-1B, IL-6, IL-8, and tumor necrosis factor-alpha (TNF-α), much higher than *Pseudomonas aeruginosa* (*P. aeruginosa*)-derived LPS, which also stimulates cytokine production, and that of chemokine monocyte chemoattractant protein-1 (MCP-1) via toll-like receptor-4 (TLR-4) [20]. These differences in the host response to LPS highlight important features of LPS that are often neglected in gut microbiota (GM)-focused research.

Lipopolysaccharide is a large amphipathic glycoconjugate that is composed of a lipid domain, an oligosaccharide core, and a distal polysaccharide [21]. The lipid domain, lipid A, is an endotoxin and the main LPS virulence factor [22]. The oligosaccharide core, O-antigen, has a variable number of repeated subunits and is responsible for the antigenicity and serotype-specific immunogenicity of LPS [22,23]. Importantly, this domain varies among different microbes, although it is essential in imparting specific pathogenic features to bacteria. Therefore, variation in LPS structure contributes to different serotypes and ensuing immunological responses in the host. Serotypes can be grouped by microbial species with similar structural components, including LPS. Because LPS is a major structural component of Gram-negative bacteria, it follows that the intestinal tract represents a substantial and dynamic source of endotoxins [24].

Defined as the community of microorganisms colonizing the gastrointestinal (GI) tract, the human GM is highly individualized and harbors a vast array of taxonomically distinct bacteria in addition to various archaea, fungi, and viruses, which together play a large role in host health [25]. Diet can alter the abundance of pathogenic bacteria and intestinal epithelial barrier function, which may ultimately modulate LPS structure and abundance in the host [11–13,26,27]. An often-cited example is the Western diet, which is composed of high fat/protein and low fiber intake. Such a diet pattern may imbalance the gut flora which, in turn, would promote increased LPS translocation from the intestinal lumen into host circulation (via transcellular and/or paracellular mechanisms) [28,29]. As an immune stimulant, LPS may support a continued state of meta-inflammation in individuals following a Western diet, thus playing a role in obesity and associated chronic diseases. However, many studies investigating this possible connection usually rely on two assays, Limulus amoebocyte lysate (LAL) test and, less frequently, rabbit pyrogen test (US Pharmacopeia rabbit test) [30]. The LAL test is not without its shortcomings, providing only endotoxin-general activity as opposed to structural, functional, and/or distribution profiles of LPS [30,31]. The wide use of these assays in preclinical and clinical research leaves a gap in our understanding of how alterations in the GM specifically alter responses in the host via LPS. In this review, we raise key issues regarding LPS structural variation and how future research might better elucidate its biochemical specificity.

The gut microbiota as a diverse reservoir of LPS

Lipopolysaccharide is a structural component of Gram-negative bacteria that consists of long chains of carbohydrate moieties that are connected to lipids by covalent bonds [32]. These chains form a dense protective network that shields the outside from bacteria, keeping a moist and slightly charged surface to ward off potentially damaging particles and compounds from the cell (though it is loose enough for nutrient passage) [32]. Moreover, the length of these carbohydrate chains, and thus the length of LPS, can be modified by the bacterium in a process termed 'phase-shift regulation' [33]. Throughout the life cycle of Gram-negative bacteria, modification of its LPS may be vital for survival by conserving energy (shortening chain length), and modifying pathogenicity and presentation to the immune system of host organisms (shortening and lengthening) [33–35]. Similarly, variation of the lipid A domain of LPS is one of the main survival methods used by Gram-negative bacteria. This serves to provide

resistance to innate immune system elements and evade detection by TLR-4 [23]. LPS fragments are released by bacteria in a live state as this layer is under continual renewal to maintain structural integrity [32].

Similar to the microbes that create them, LPS can display specificity, which suggest important distinctions in relation to molecular structure. Termed serotype, this classification has long been established to classify bacterial subspecies by their variability in carbohydrate surface antigens [36–38]. Indeed, there can be thousands of different serotypes found in a particular species of bacteria. Common in molecular biology, differences in structure often result in differences in function. For example, in the brain, oral, and GI environment, these differences can induce varying responses on the molecules and cells they may interact with [33,39–41]. Under healthy conditions, nonspecific LPS concentrations in the gut range from 0 to 1.0 ng·mL^{-1} (1.8 μg·mL^{-1} in the rat distal ileum) [42–44], and LPS does not penetrate the intestinal epithelium [44]. At these physiologically relevant levels, LPS does not promote intestinal epithelial destruction yet contributes to the modulation of tight junction proteins as seen with *in vitro* [colorectal adenocarcinoma cells (Caco-2)] and *in vivo* models of inflammation through the increase of enterocyte membrane TLR-4 expression [44]. However, prior studies examining intestinal permeability conditions such as inflammatory bowel disease (IBD) and necrotizing enterocolitis and the effects of prolonged high-fat diet consumption show that a defective epithelial tight junction barrier allows for LPS translocation and systemic circulation via chylomicrons [45,46]. The former is physiologically advantageous as it may reduce LPS toxicity via hepatic clearance [47,48]. Though the excessive formation of chylomicrons from high-fat diets can increase the risk of extrahepatic exposure to LPS, over time [45].

Diet, along with other environmental and host factors can heavily influence the microbial community composition and function. Importantly, different microbial taxa within the GM have different abundance and may localize in a diverse manner. For example, during periods of significant energy restriction or dietary fasting, some taxa may congregate proximal to the intestinal epithelium, feeding off host-derived mucin [49]. Conversely, during periods of increased nutrient intake, there may be less competition between microbes for energy substrate and less encroachment. In relation to the epithelium, such differential abundance or localization patterns could increase the propensity for different LPS variants from more insidious (or innocuous) bacteria to enter portal circulation. Work investigating the dynamics and implications of these factors on LPS structure and movement into the host is scant.

The host can provide a mechanism to handle LPS inside the intestinal lumen, including the production of intestinal phosphatase, which acts to 'detoxify' lipid A via the removal of phosphate [50]. However, translocation across the brush border is much more problematic. Indeed, the extensive presence of the immune system (i.e., GALT) exemplifies the long and intricate relationship the GM and host have shared over their co-evolution. Not surprisingly, LPS can be a potent immunostimulant, and once passed, the gut barrier and associated GALT can interact with tissues much less equipped to handle high concentrations of this pyrogen (a fever-inducing compound). To alert the host and spur immune signal transmission, immune cells use TLR-4 to recognize LPS variants and emit cytokines that trigger an inflammatory response [50]. These immune cells are highly responsive to LPS in circulation as the bloodstream could rapidly transport bacteria throughout the body, which could lead to septic shock and, potentially, death of the host [51].

LPS variants and the gut barrier

As an internalized external environment, the components and products of the gut flora must cross through several defensive barriers to enter circulation. This includes the mucus layer lining the gut wall and the gut wall itself, composed of specialized epithelial cells [52]. The mucosal layer and GALT work in tandem with immune responses promoted by commensal bacteria incursions [53]. The utility of these incursions is the triggering and effective priming of the innate immune system of the host through bacterial interactions with dendritic cells (DC) [54]. Another critical component of gut barrier defense is pattern recognition receptors (PRR), which enable the gut epithelium to sense commensal or pathogenic microbes by employing TLRs, which can trigger proinflammatory responses, alerting the host to potential infection [55]. More specifically, TLR-4 with the assistance of cluster of differentiation 14 (CD14) as a cofactor can recognize gut-derived bacterial LPS and other bacterial components [56,57]. Additionally, LPS can interact with other structures on epithelial cells, which can lead to the movement of LPS to the systemic circulation via paracellular transport [57]. This interaction is seen in the activation of the canonical nuclear factor kappa-beta (NF-κB) and the production of proinflammatory cytokines, such as TNF-α, IL-6, IL-8, and IL-1B [58], further increasing intestinal permeability [59]. Though, other cytokines like IL-10 may serve as a

protective agent for the intestinal barrier [60]. Studies of Caco-2 cells have also suggested that LPS exhibits a dose- and time-dependent increase in permeability by activating myosin light-chain kinase expression and kinase activity [61].

Within the realm of the GI tract, scant research has addressed how host-associated tissues might differentially respond to LPS variants. So-called 'gut dysbiosis' (better stated as 'disease-associated' GM) is a reported characteristic of many metabolic and GI diseases such as IBD and T2D [62]. Interestingly, the gut microbial communities during these inflammatory conditions also promote the production of a variety of LPS variants that have a wide range of effects on the production of proinflammatory cytokines associated with intestinal barrier defects [33]. Past studies have shown that Gram-negative bacteria promote distinct LPS strains implicated in the modulation of proinflammatory cytokines and immune responses [63]. For instance, Stephens and von der Weid [33] recently explored the effects of five major species of Gram-negative bacteria associated with IBD progression and evaluated their pathogenicity through LPS. These included *Klebsiella pneumoniae*, *E. coli* (O127:B8), *Salmonella enterica*, *P. aeruginosa*, and *Serratia marcescens*. The activity of LPS from these species was assessed through the use of TLR-4 transfected human embryonic kidney (HEK) 293 cells. Normalizing to *E. coli* (positive control), LPS from *P. aeruginosa* induced significantly less NF-κB and IL-8 production, whereas *S. enterica* and *S. marcescens* induced a significantly higher amount of IL-8 production. In addition, utilizing a model of intestinal epithelial permeability (i.e., Caco-2 cell monolayers), 24-h LPS stimulation induced a significantly unique cytokine signature between each serotype. For instance, *S. macescens* induced TNF-α at a greater proportion, whereas *K. pnemoniae* induced substantially more IL-10 than any of its respective serotype counterparts. Of note, the induction of IL-6 and IL-8 were generally stable between serotypes. Moreover, anti-inflammatory factors, like IL-4 and IL-10, were more apt to be differentially expressed. Stephens and von der Weid [33] speculated that this skew could be an important driver of inflammation at the gut barrier. Finally, in the examination of Caco-2 permeability via fluorescein isothiocyanate (FITC)-dextran flux assay, all LPS variants significantly increased permeability over 24 h. Specifically, *S. marcescens* and *E. coli* had the most pronounced impact on permeability (~2× greater than the others). The flux correlated with decreased zonula occludens-1, which appeared to promote a complete disruption in the monolayer. In comparison, *P. aeruginosa*, *K. pnemoniae*, and *S. enterica* had reduced permeability at 24 h, displaying a larger response at 6 h. Overall, these findings from Stephens and von der Weid [33] suggest that LPS variants differentially affect TLR-4 activation, altering epithelial barrier function, spurring local cell activity, and ultimately promoting disease through localized inflammatory mechanisms.

Innate immune cells also reportedly distinguish between different LPS variants and, as a result, deploy-specific responses. This was noted by Stephens and colleagues (2021) in human macrophages with LPS derived from *P. aeruginosa*, *E. coli*, and *S. enterica* [41]. Specifically, in classically activated macrophages, *P. aeruginosa* LPS signaling from TLR-4 on the membrane to P65 (a key transcription factor in regulating NF-κB) was dependent on the intracellular signaling proteins TAK1 and TBK1. *E. coli* LPS signaling also relied on TAK1 and TBK1 as intermediaries to induce P65, and the interferon regulatory factor, IRF3. In contrast, *S. enterica* induced P65 and IRF3 phosphorylation through signaling via an endosomal mechanism. These findings outline distinct signaling pathways by which innate immune cells can discern structural differences in LPS and promulgate specialized responses through TLR-4 signaling. Thus, cytokine-differentiated macrophages have the capability to discern and respond accordingly to different LPS variants.

As prevalent conditions, IBDs like colitis are hallmarked by recurrent intestinal inflammation. In mice, LPS has been shown to determine the outcome of inflammation for experimental colitis [64]. The GM composition is important as certain microbes like *Bacteroides vulgatus* mpk have been reported to modulate immune responses, which may help prevent colitis induction in mice [65–67]. Steimle et al. [68] isolated LPS from *B. vulgatus* mpk and stimulated murine CD11c+ cells *in vitro* noting noninduction of proinflammatory cytokine expression. More intriguingly, this stimulation appeared to induce a dulled responsiveness toward subsequent LPS stimuli in these cells. In mice with experimental colitis, administration of this purified LPS also helped 'rebalance' immune homeostasis in the GI tract [68]. These apparent health-promoting effects were speculated by Steimle and colleagues to be attributed to the weak agonistic properties of this LPS on the MD-2/TLR-4 receptor pathway. Di Lorenzo et al. [69] likewise evaluated the impact of *B. vulgatus*-derived LPS on HEK cell lines, human macrophages, and DCs. In the human macrophages comparing this LPS to *E. coli*-derived LPS in activating the macrophages, *B. vulgatus* LPS showed a

significantly weaker ability to elicit production of proinflammatory cytokines. In addition, LPS derived from *B. vulgatus* was able to stimulate production of the anti-inflammatory cytokine, IL-10. This body of work suggests that LPS derived from *B. vulgatus* could play a potential therapeutic role in buffering against excessive inflammation in IBD and other GI-relevant conditions.

LPS variants beyond the gut: antagonistic vs. agonistic roles in inflammation?

A common hypothesis in clinical conditions like obesity is that LPS from the gut lumen enters circulation, spurring an inflammatory response, which alters metabolic function in peripheral adipose tissue. Broadly, LPS in circulation is recognized by TLR-4 along with LBP, the CD14 co-receptor of TLR-4, and the myeloid differentiation protein-2 (MD-2) [70]. After recognition of certain LPS variants, TLR-4 can undergo oligomerization and recruit its downstream adaptor molecules, TIR domain-containing adapter-inducing interferon-β (TRIF) and myeloid differentiation primary response gene 88 (MyD88) [70]. This leads to the activation of downstream signaling pathways, such as NF-κβ and mitogen-activated protein kinase (MAPK) (Fig. 1) [47,71]. The translocation of NF-κB to the nucleus promotes the activation of genes that encode for proteins involved in the inflammatory response, such as TNF-α, IL-1β, IL-6, IL-8, inducible nitric oxide synthase (iNOS), and MCP-1 [72]. For MAPK, activated signaling pathways include c-Jun N-terminal protein kinase (JNK), p38 MAPK, and extracellular signal-regulated kinases [73]. Chronic stimulation of these pathways via low-dose administration of *E. coli*-derived LPS increases body and liver weight, subcutaneous and visceral fat, and postprandial glucose in mice [13]. In humans, acute *E. coli*-derived LPS administration disrupts insulin sensitivity [74–76]. In addition, TLR-4 has been shown to be increased in diabetic humans and animal models of obesity and metabolic syndrome [77,78]. This forms the model of metabolic endotoxemia, broadly grouping LPS as simply a proinflammatory, deleterious agent. Unfortunately, such generalities have obscured some of the important nuances of LPS.

Beneficial roles of different LPS variants

In animal and human research, LPS from *E. coli* has been preferentially used, yet certain LPS variants may impart inert or even beneficial effects on the host. In one of the largest studies of its kind, Vatenen et al. (2016) tracked the GM development from birth until age 3 in a cohort of infants (n = 222) and found low *Bacteroides* species abundance in some, but dominant in other individuals [79]. Those with low *Bacteroides* abundance had a greater proportion of *E. coli*. Upon a more detailed examination, the *Bacteroides* species produced a structurally and functionally distinct form of LPS. After purifying LPS from fecal samples, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MS) was employed to determine the structure of the lipid A domain from *Bacteroides dorei* and *E. coli*. *B. dorei* contained tetra- and penta-acylated lipid A structures, compared with the hexa-acylated lipid A in *E. coli*. In terms of functionality, *B. dorei*-derived LPS inhibited immune stimulation (via luciferase activity in TLR-4-NFκB reporter cells) and inflammatory cytokine responses to *E. coli*-derived LPS in human peripheral blood mononuclear cells (PBMCs) (via NFκB-dependent cytokines TNFα, IL-1β, IL-6, and IL-10). In the context of the study population, these more inert forms of LPS were perceived to be detrimental as they could not provide the same 'education' of the immune system as more toxic forms of LPS. Training the immune system during infancy through GM assembly is likely critical and is a tenet of the hygiene hypothesis. However, these findings have important implications in the context of the GM for adults and those with chronic inflammatory conditions.

d'Hennezel et al. [80] extracted total LPS from fecal samples collected from of a group healthy adult humans and found that the LPS was not immunogenic and in fact prevented TLR-4-induced production of cytokines. Briefly, human primary PBMCs were stimulated with the extracted and purified LPS and cytokines downstream of NF-κB activation (TNF-α, IL-12p70, IL-1b, IL-6, IL-8, and IL-10) were assessed in comparison with *E. coli*-derived LPS. LPS from the fecal samples of healthy adults showed ≥ 2-fold lower stimulatory potency than purified *E. coli*-derived LPS with some samples being immunologically 'silent' all the way up to the largest dose tested. Next, d'Hennezel and colleagues co-treated PBMCs with the extracted LPS from healthy adults then stimulated them with *E. coli* LPS. The fecal LPS had a potent inhibitory effect on cytokine (IL-6, IL-1b) production elicited by *E. coli* LPS, showcasing fecal LPS from healthy donors as an inhibitor of TLR-4 stimulation. Using metagenomic sequencing on the fecal samples, they found that numerous members of the order *Bacteroidales* appeared to produce antagonistic forms of LPS. d'Hennezel et al. [80] speculated that structural

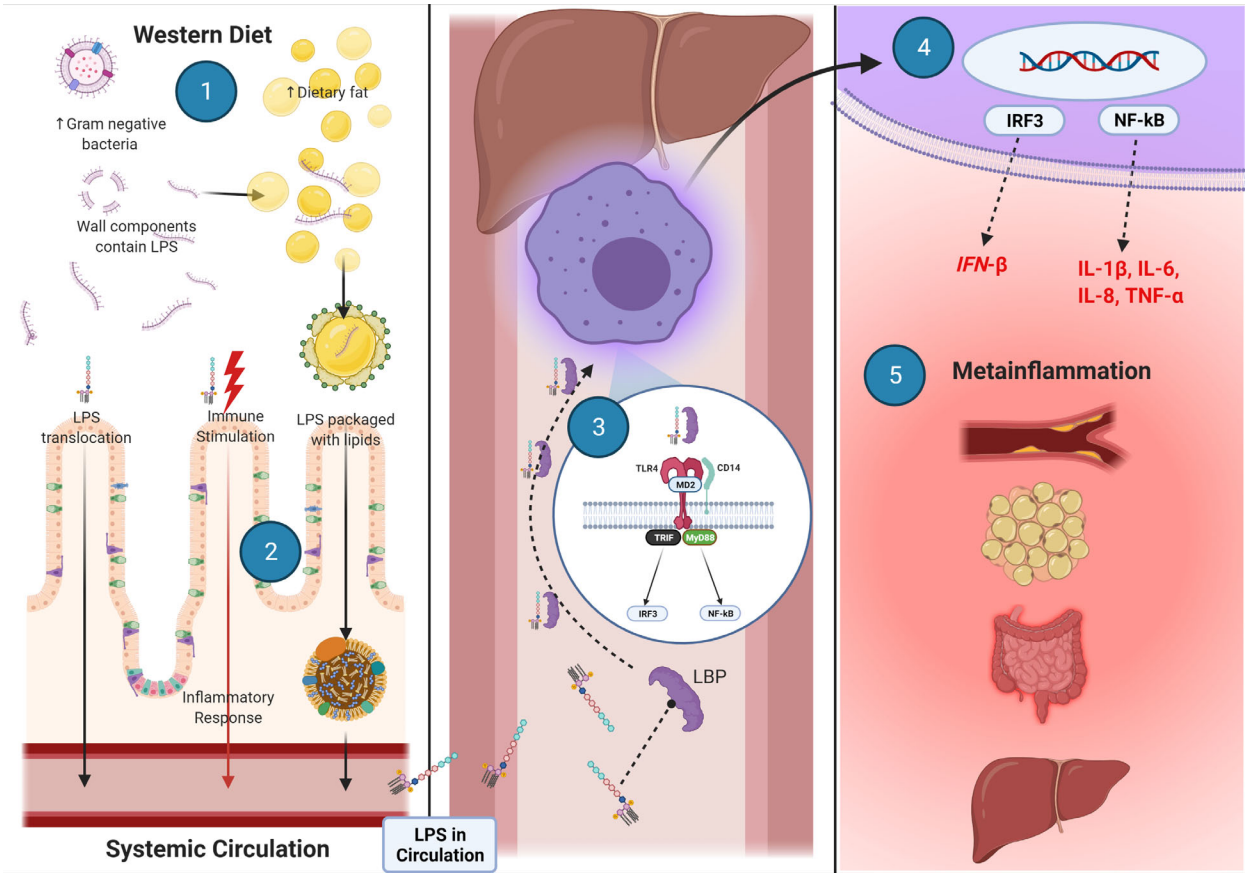

Fig. 1. Classic model of metabolic endotoxemia in which LPS may enter systemic circulation and spur chronic inflammation. The classic model of metabolic endotoxemia outlines that (1) a Westernized diet may increase populations of LPS-producing bacteria and increase fat absorption. (2) LPS may act locally and spur an inflammatory response and also enter systemic circulation via translocation at the brush border and inside chylomicrons. (3) Derived primarily from the liver, LBP binds to the lipid A portion of LPS in circulation. On the outer membrane of various cells, the protein CD14 along with LBP aids the presentation of LPS to TLR-4. As a transmembrane protein, TLR-4 is an LPS receptor located on the surface of monocytes, macrophages, neutrophils, and DCs. As a dimer, TLR-4 requires MD-2 to detect LPS. Once activated via LPS, TLR-4 recruits MyD88 and TRIF. (4) This in turn activates other molecules, including IRF3 and NF-κB. (5) This results in turning off/on genes of inflammation.

features of the lipid A domain from several species within this order interfered with TLR-4-MD-2 signaling through competitive inhibition. This translated to the overall immune silencing effect that was observed. Therefore, these findings suggest that variants of freely circulating LPS originating from the gut may actually prevent inflammation, rather than promote it like the case of *E. coli*.

While less likely to be consumed as part of the diet, some Gram-negative microbes, such as photosynthetic prokaryotes, can be ingested through other means. For instance, cyanobacterial blooms are increasing globally and may contaminate water or food products by releasing toxins, such as LPS [81]. While generally considered toxic, cyanobacteria are diverse in physiology, metabolism, and morphology and have long been reported to exhibit reduced toxicity compared with LPS from other bacterial variants like *E. coli* [82]. For example, in comparison with *E. coli*-derived LPS, those derived from cyanobacteria have a different lipid A structure or oligosaccharide core that modulates the TLR-4-dependent immune response [83]. Murine B cells exposed to *Oscillatoria* sp. LPS have low IgM production in comparison with *E. coli*. This difference in IgM production is due to incomplete TLR-4 signaling, as IRF-3 was not induced in B cells after stimulation with *Oscillatoria* sp. [84]. *In vitro* exposure of rat neonatal microglia to *Oscillatoria* sp. LPS resulted in a concomitant proinflammatory (less than *E. coli* as a positive control) and anti-

inflammatory mediator release [85]. These findings suggest that activation of rat brain microglia by this LPS could result in an interplay between neuroinflammation and neural tissue repair in the central nervous system. In another study, administration of LPS from the cyanobacterium Spirulina suppressed tumor growth in C3H/HeN mice by reducing IL-17 and IL-23 and increasing IFN-γ levels mediated by T-cells [86]. This effect was not observed in TLR-4-mutant C3H/HeJ mice. Finally, *in vitro* experiments showed that this Spirulina-derived LPS impaired the antigen-presenting function that promotes the generation of IL-17-producing cells in a TLR-4-dependent manner [86].

Other cyanobacteria-derived LPS, like *Geitlerinema* sp., have been reported to activate human monocytes (via increases in activation markers and phagocytosis) and induce the production of proinflammatory cytokines to a significantly lower level compared with *E. coli* LPS [87]. Comparing these two variants, *Geitlerinema* sp. LPS lack the 12–14 fatty acid side chains that are agonistic in *E. coli* and contain longer fatty acid side chains that appear to have reduced agonistic abilities. Taken together, these data show that *Geitlerinema* sp. LPS is similar to other LPS molecules with limited TLR-4 signaling capacity. This finding appears to be consistent with other cyanobacteria in terms of biochemistry and inflammatory activity. LPS isolated from *Microcystis aeruginosa*, the most prevalent cyanobacteria in water blooms, exhibited a significantly lower ability to activate blood phagocytes, select signaling pathways, ERK1/2, NF-κB, and p38, and proinflammatory cytokines, compared with *E. coli* in human blood phagocytes based on *in vitro* tests [88]. In contrast to the observed biological activity, when this LPS was analyzed by the PyroGene test (Lonza, Basel, Switzerland), pyrogenicity levels were almost 2x greater than *E. coli* LPS [88]. This fluorescent assay measures endotoxin activity in a similar conceptual fashion as the LAL test. Importantly, this result demonstrates that the pyrogenicity of different LPS does not necessarily translate to negative biological effects.

LPS and cardiovascular disease (CVD) progression

Circulating LPS has also been investigated for its potential role in the progression of CVD. For example, studies focusing on heart failure have implicated LPS in the activation of proinflammatory cytokines and LPS has been proposed as a biomarker for the detection of atherosclerosis [89]. Yet, here too, structural variation is important. Yoshida et al. [90] reported the genus *Bacteroides* is negatively associated with fecal LPS concentrations in patients with CVD. In comparison with *E. coli*-derived LPS, endotoxicity as determined by the LAL test and levels of proinflammatory cytokine production via *in vitro* stimulation of macrophages (RA 264.7) were significantly lower from *Bacteroides*-derived LPS. Importantly, *Bacteroides* species do not express lpxM (an acyltransferase), which helps form the lipid A structure of LPS derived from *E. coli* [79,80]. Instead, the lipid A structure in these species is known to elicit reduced TLR-4 responses [79,80], which may provide a blunted inflammatory response compared with *E. coli*-derived LPS. Thus, increased abundance of *Bacteroides* in the GM may be an important consideration in mitigating inflammatory diseases. As a proof of concept, Yoshida et al. [90] used a gut model, composed of anaerobic culturing vessels, fermenting the CVD patient fecal samples with probiotics (1×10^8 cells each of *B. vulgatus* and *B. dorei*), and found a significant increase in the *Bacteroides* abundance compared with control, suggesting that the GM could potentially be manipulated toward modulating fecal LPS types and levels. Indeed, work in preclinical models previously showed next-generation probiotic strains from the *Bacteroides* genus significantly blunted endotoxemia followed by a decrease in GM-derived LPS production and suppression of proinflammatory cytokines compared with control [91,92]. Novel probiotic preparations such as these may be considered for therapeutic purposes against inflammatory conditions in humans in future. Though, much preclinical and pilot work is still required.

This body of research requires continued efforts, but it is clear that the underappreciated structural variation of GM-derived LPS has tremendous health implications once beyond the gut. Indeed, the structure and function of the GM and the immunogenicity of certain microbes may even impact immune responses to vaccinations [93] and behavioral disorders like autism spectrum disorder [94]. Native or engineered LPS (through the lipid A moiety) from certain GM-derived microbes, such as *Bacteroidetes*, have been suggested as immune-modulating agents for the prevention and treatment of inflammation-related diseases [95]. Clearly, the heterogeneity of LPS has not been well characterized, though different lipid A moieties have been exemplified from the above (Table 1). Research efforts should seek to define these lipid A structures, particularly for bacteria that are predominant in the gut and/or certain disease states. Such analyses of lipid A from each bacterium may lead to a better understanding of variations in phenotypes.

Table 1. Examples of differential effects of LPS variants on cell cultures *in vitro*, animals, and humans. ALDH, aldehyde dehydrogenase; ALT, alanine aminotransferase; AST, aspartate transaminase; AUC, area under the curve; CD11b, cluster of differentiation molecule 11b; CD4, cluster of differentiation 4; CD66b, cluster of differentiation 66b; CD8, cluster of differentiation 8; CD86, cluster of differentiation 86; CINC-1/CXCL-1, cytokine-induced neutrophil chemoattractant 1; cLP, colonic lamina propria; CRP, C-reactive protein; CXCL-10, C-X-C motif chemokine ligand 10; CXCL-8, C-X-C motif chemokine ligand 8; DCs, dendritic cells; ERK 1/2, extracellular signal-regulated protein kinase 1/2; FFA, free fatty acid; HbA1c, hemoglobin A1c; HMG-B1, high mobility group-B1; IFN-γ, interferon γ; IgM, immunoglobulin M; IKK, IκB kinase; IL-1, interleukin-1; IL-10, interleukin-10; IL-12, interleukin-12; IL-17, interleukin-17; IL-1α, interleukin 1α; IL-1β, interleukin-1-beta; IL-23, interleukin-23; IL-5, interleukin-5; IL-6, interleukin-6; IL-8, interleukin-8; IP-10/CXCL-10, human interferon-inducible protein 10; IRF3, interferon regulatory factor 3; LBP, lipopolysaccharide binding protein; LD_{50}, median lethal dose; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MCP-1/CCL-2, monocyte chemoattractant protein-1; MHC II, major histocompatibility complex; MIF, macrophage migration inhibition factor; MIP-1α/CCL-3, macrophage inflammatory protein-1α; MIP-2/CXCL-2, macrophage inflammatory protein-2; MMP-9, metallopeptidase 9; NF-κB, nuclear factor kappa B; OGTT, oral glucose tolerance test; PAI-1, plasminogen activator inhibitor-1; RANTES/CCL-5, regulated on activation normal T expressed and secreted/CC ligand 5; ROS, reactive oxygen species; TAK1, transforming growth factor-β-activated kinase 1; TBARS, thiobarbituric acid–reacting substances; TBK1, TANK-binding kinase 1; Th1, T helper type 1; Th17, T helper type 17; TLR2, toll-like receptor-2; TLR-4, toll-like receptor-4; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue.

Study	LPS variant/dose/route of administration	Subject/sample	Diet	Duration	Outcome(s)
In vitro					
Díaz-Zúñiga et al., 2014 [39]	*Aggregatibacter actinomycetemcomitans*–derived LPS (serotypes a–c) Increasing multiplicity of infection of 10^{-1}–10^{2} (bacteria/DCs ratio)	Human DCs	-	2-day incubation	• ↑ IL-1β, IL-6, IL-12, IL-23, IFN-γ and TNF-α in DCs stimulated by *A. actinomycetemcomitans* serotype b, compared with serotypes a and b
Mayer et al., 2016 [85]	*Cyanobacterium Oscillatoria* sp.–derived LPS 0.1–100 000 ng·mL^{-1}	Sprague Dawley rat neonatal microglia	–	17-h incubation	Treatment with 0.1–10 000 ng·mL^{-1} *Oscillatoria* sp.: • Minimal LDH release • Concentration-dependent O_2^- generation, MMP-9, TNF-α, IL-6, MIP-2/CXCL-2, IP-10/CXCL-10, MIP-1α/CCL-3, MCP-1/CCL-2, RANTES/CCL-5, and IL-10 Treatment with 100 000 ng·mL^{-1} *Oscillatoria* sp.: • Significant LDH release • ↓ cytokines and chemokines investigated (exception of IL-1α & CINC-1/CXCL1)
Vatanen et al., 2016 [79]	*E. coli* vs. *Bacteroides dorei* –derived LPS	Primary human PBMCs; *in vitro* differentiated monocyte-derived dendritic cells or HEK293-NFκB reporter cells expressing hTLR4	–	18- to 20-h incubation	PBMCs: • *E. coli*: ↑ IL-10, TNFα, IL-1β, and IL-6 • *B. dorei*: No response Dendritic cells: • *E. coli*: ↑ NFκB-luciferase activity • *B. dorei*: ↓ NFκB-luciferase activity

Table 1. (Continued).

Study	LPS variant/dose/route of administration	Subject/sample	Diet	Duration	Outcome(s)
Swanson-Mungerson et al., 2017 [99]	*Oscillatoria* sp. vs. *E. coli* (positive control)-derived LPS 0–100 000 ng·mL^{-1}	B cells from c57Bl/6 mice	–	48-h to 5-day incubation	• ↑B cell proliferation, upregulation of MHC II and CD86, enhance antigen uptake, and induce IgM production at low levels, vs. *E. coli* LPS • Incomplete TLR-4 signaling at lower concentration, vs. *E. coli* LPS
Rojas et al., 2018 [76]	*A. aactinomycetemcomitans*-derived *LPS (serotypes a-c) vs. E. coli*-derived *LPS (positive control), noninduced DCs (control)* Multiplicity of infection = 100 LPS concentration, 50 ng·mL^{-1} 10 ng·mL^{-1} of *E. coli* LPS	Human dendritic cells	–	2-day incubation	• ↑ Th1 and Th17-type cytokine expression in DCs stimulated with LPS serotype b, compared with serotypes a or c • In LPS co-infected DC's, Th1- and Th17-type cytokine expression ↓ compared with serotype b alone • ↑ IL-5 (Th2-type cytokine) expression in LPS a + c stimulated DCs
Moosová et al., 2019 [103]	*Experiment 1* LPS isolated from water bloom sample (*Microcystis aeruginosa* dominant) vs. *E. coli* (positive control)-derived LPS (026:B6) LPS concentration of *E. coli* and Water Bloom, 0.01–1.0 μg·mL^{-1} *Experiment 2* LPS from laboratory cultures (axenic and non-axenic *M. aeruginosa* strains) vs. LPS from water bloom (positive control) 0.1–10 μg·mL^{-1} 0.1 μg·mL^{-1} LPS from water bloom (positive control)	Human blood based *in vitro* tests of blood phagocyte monocytes and polymorphonuclear leukocytes (PMNL) Human blood based *in vitro* tests of blood phagocyte monocytes and polymorphonuclear leukocytes (PMNL)	–	30 min to 24-h incubation 6 to 24-h incubation	LPS from water bloom: • ↑ TNF-α, IL-6, IL-1β to lesser extent than *E. coli* LPS • ↑ expression of CD11b in blood phagocytes, similar to *E. coli*; ↑ expression of CD66b in PMNL, similar to *E. coli* LPS • ↑ phosphorylation of ERK1/2, NF-κB, p38 in PMNL at 1 μg·mL^{-1}, similar to *E. coli* LPS LPS from laboratory cultures: • ↑ IL-1β, IL-6, IL-8, TNF-α at 10 μg·mL^{-1} • ↑ expression of CD66b, CD11b in PMNL, and ↑ expression of CD11b in monocytes at 10 μg·mL^{-1}

Table 1. (Continued).

Study	LPS variant/dose/route of administration	Subject/sample	Diet	Duration	Outcome(s)
Swanson-Mungerson et al., 2019 [87]	*Geitlerinema* sp. vs. *E. coli*-derived LPS (positive control) 0–100 000 ng·mL^{-1}	Human monocytes	-	30-min to 72-h incubation	• ↓ monocyte proliferation vs. *E. coli* LPS • Similar levels of phagocytosis in monocytes as *E. coli* LPS • ↓ IL-1β, IL-6, TNF-α, and CD86 vs. *E. coli* LPS • Lipid A of *Geitlerinema* sp. LPS → longer fatty acid side chains vs. *E. coli* LPS, potential mechanism for ↓ agonistic capacity
Di Lorenzo et al., 2020 [69]	*Bacteroides vulgatus*–derived LPS (LPS_{BV}), and its isolated lipid A portion vs. *E. coli* (O111:B4)-derived LPS and synthetic antagonist tetra-acylated lipid IV_A (controls) Pam_3CSK_4 (500 ng·mL^{-1}), the positive control for TLR2 ligand (positive controls) 0–100 ng·mL^{-1}	Human TLR-4, TLR2 transfected human embryonic kidney (HEK) 293 cell lines, and human peripheral blood-monocyte-derived macrophages and dendritic cells	–	6- to 12-h incubation	Peripheral blood-monocyte-derived macrophages: • LPS_{BV} induced ↓ TNF-α, IL-6, IL-10, and chemokine IP-10 release vs. *E. coli* LPS, though similar IL-6, IL-10 release at highest LPS concentrations Peripheral blood-monocyte-derived dendritic cells: • LPS_{BV} induced ↓ TNF-α, IL-6, IL-10 release vs. *E. coli* LPS, though similar IL-6, IL-10 release at highest LPS concentrations • LPS_{BV}-induced low TNF-α compared with IL-10 release (contrast to *E. coli* LPS ↑ TNF-α release) HEK Cell Lines: • In HEK293 hTLR4, LPS_{BV} ↓ NF-κB activation vs. *E. coli* LPS, and no CXCL-8 release • In HEK293 hTLR2 LPS_{BV} ↑ NF-κB activation vs. untreated cells, ↑ CXCL-8 vs. *E. coli* LPS (no activation of NF-κB and no CXCL-8 release with *E. coli* LPS and lipid IV_A) • In HEK293 hTLR4 + hTLR2, LPS_{BV} induced ↓ NF-κB activation CXCL-8 release vs. *E. coli* LPS
Stephens and von der Weid, 2020 [33]	*Klebsiella pneumoniae*–, *Salmonella enterica*–, *Pseudomonas aeruginosa*–, *Serratia marcescens*–derived LPS normalized to *E. coli* (O127:B8)-derived LPS 1 ng·mL^{-1}	TLR-4 transfected human embryonic kidney (HEK) 293 cells, human intestinal colonic epithelial cell monolayers (Caco-2)	–	24-h incubation	HEK Cells: • Normalizing to *E. coli*, LPS from *P. aeruginosa* ↓ NF-κB and IL-8, whereas *S. enterica* and *S. marcescens* ↑ IL-8 Caco-2: • Normalizing to *E. coli*, *S. macescens* ↑ TNF-α vs. other LPS, *K. pnemoniae* ↑ IL-10 vs. other LPS • LD_{50} of *S. marcescens* was > 30× higher than the other LPS

Table 1. (Continued).

Study	LPS variant/dose/route of administration	Subject/sample	Diet	Duration	Outcome(s)
					• *S. marcescens* and *E. coli* induced greatest ↑ in monolayer permeability, and all LPS ↑ tight-junction disruption (TLR-4-dependent) at 24 h
Stephens et al., 2021 [41]	*Salmonella enterica* (serotype Typhimurium), *Pseudomonas aeruginosa* (serotype 10.22), and *E. coli* (O127:B8)-derived LPS 1 ng·mL^{-1}	Human THP-1 monocytic cell lines (M1 and M2 phenotypes)	–	30-min to 24-h incubation	• All LPS ↑ proinflammatory cytokine production • *E. coli* LPS signals, dependent or independent of the endosome, utilize both TAK1- and TBK1-signaling to induce P65 and IRF3 inducible genes and cytokines • *S. enterica* LPS induced P65 and IRF3 phosphorylation through signaling via the endosome • *P. aeruginosa* LPS signaled via cell surface TLR-4-driven response, independent of the endosome, to p65 dependent on TAK1 and TBK1 signaling
Animals					
Cani et al., 2007 [13]	*Experiment 1* *Escherichia coli* (055:B5) Subcutaneous minipump 300 μg·kg^{-1}·day^{-1}	Male C57bl6/J mice	Chow diet OR high-fat diet: 72% fat (corn oil and lard), 28% protein, and <1% carbohydrate as energy content	2–4 weeks	• Endotoxemia ↑2.7X, vs. control • ↑body and liver weight, subcutaneous and visceral fat, postprandial glucose, and liver triglyceride, vs. control
–	*Experiment 2* *E. coli* (055:B5) Infusion 0.5 mg·kg^{-1}·h^{-1}	CD14 mutant and C57bl6 male mice (control)	–	3-h	• Blunted ↑ IL-6, PAI-1, and IL-1 mRNA concentrations in the subcutaneous adipose depot vs. control • Blunted ↑ NF-κB and IKK forms in the liver vs. control
Márquez-Velasco et al., 2007 [113]	*Escherichia coli* (055:B5) vs. 100 μL of RPMI orally (control) OR nontreated (sham) group 50 μg/every 4 days, 5× (diluted in 100 μL of RPMI culture medium) Intraperitoneal injection (IP) or oral delivery (O)	Male BALB/c mice Cecal ligation and puncture (CLP)-induced sepsis following LPS pretreatment	–	Pretreatment: Every 4 days for a total of 5 times CLP 1 week after pretreatment, survival studied up to 2 weeks	• Following CLP, O significantly ↑ survival to 87% vs. 50% in the control group • O ↓ % Kupffer cells in the liver, ↓ pulmonary edema vs. IP and control • O ↓ IFN-γ vs. IP at 4-hr post-CLP • O ↑ IgM anti-LPS antibodies vs. IP, control, and sham

Table 1. (Continued).

Study	LPS variant/dose/route of administration	Subject/sample	Diet	Duration	Outcome(s)
Okuyama et al., 2017 [86]	*Experiment 1* *Spirulina pacifica* vs. *E. coli* (0111:B4)-derived LPS (positive control), and saline (negative control) Intraperitoneal injection 0–100 $\mu g \cdot day^{-1}$ (days 6, 13, and 20) *Experiment 2* *S. pacifica* LPS vs. saline Intraperitoneal injection 100 $\mu g \cdot day^{-1}$ (days 8, 13, and 20) *Experiment 3* *Spirulina pacifical* vs. *E. coli* (0111:B4)-derived LPS (positive control) 200 μg Intraperitoneal injection	Female C3H/HeN mice, female TLR-4-mutant C3H/HeJ Intradermal injections of 1 × 106 MH134 cells in the backs (MH134 hepatoma model) Tumor-bearing C3H/HeN mice treated with anti-CD4 and/or anti-CD8 or rat IgG antibodies (control) on days −1, 0, and 3 Normal Female C3H/HeN mice, female TLR-4-mutant C3H/HeJ	–	Days 6, 13, 20 Days 8, 13, 20 4 h	• Suppressed tumor growth (TLR-4-dependent), with antitumor effect involving CD4, CD8 T-cells • ↑ resistance to reimplanted MH134 tumors vs. saline and *E. coli* LPS • ↑ serum IFN-γ levels mediated by CD4 T-cells, and ↓ serum IL-17 and IL-23 • ↓ IL-6 and IL-23 vs. *E. coli* but ↑ CD4 T-cell-dependent IL-12
Kobayashi et al., 2018 [122]	*Pantoea agglomerans* (*P. agglomerans*)–derived LPS Oral delivery 0.3 or 1 $mg \cdot kg^{-1}$ body weight per day	Male apoE-deficient mice (BALB/c.KOR/StmSlc-Apoeshl), aged 10–12 weeks	HFD (21.9 $kJ \cdot g^{-1}$, 35% fat, 0.03% cholesterol) High-fat diet (HFD)-induced atherosclerosis model	16 weeks	• ↓ HFD-induced weight gain, epididymal WAT, liver weight and atherosclerotic lesions vs. control • Improved glucose metabolism (↓ glucose response to OGTT, AUC from OGTT, plasma insulin, and HbA1c), lipid profiles, and liver function (↓ AST, ALT) vs. control • ↓ proinflammatory cytokine expression (plasma MCP-1, colonic TNF-α, IL-6), oxidative-burst activity, and *E. coli* phagocytosis vs. control
Steimle et al., 2019 [68]	*Bacteroides vulgatus mpk* (BVMPK)–derived LPS vs. viable BVMPK 5×10^8 viable BVMPK per mL drinking water 160 $mg \cdot mL^{-1}$ LPS in the drinking water (~ 1 mg per mouse)	Rag1-deficient mice with dysbiotic microbiota transplanted with naive wild-type $CD4^+$ $CD62L^+$ $CD45Rb^{hi}$ T-cells T-cell-transplanted mice with no BVMPK (positive controls) Experimental Colitis model	-	8 weeks (total experiment) Live BVMPK, BVMPK LPS administration began at 4 weeks	• Mice treated with live BVMPK, BVMPK LPS ↓ colonic inflammation vs. positive control • Mice treated with live BVMPK, BVMPK LPS expressed ↓ IL-17 in cLP T-cells, ↑ ALDH mRNA expression in colonic scrapings

Table 1. (Continued).

Study	LPS variant/dose/route of administration	Subject/sample	Diet	Duration	Outcome(s)
Humans					
Agwunobi et al., 2000 [76]	*E. coli* 20 U·kg^{-1} Intravenous bolus (baseline)	Healthy adult male and female humans; 33.7 ± 1.7 years; $n = 6$	Weight-maintaining diet w/≥ 200 g carbohydrate per day	8-h post-LPS phase	• ↑insulin resistance, IL-6, TNF-α, vs. saline control
Dandona et al., 2010 [74]	*E. coli* 2 ng·kg^{-1} Intravenous bolus (baseline)	Healthy adult male humans; 26 ± 3 years; $n = 19$	–	24-h post-LPS phase	• ↑ nitric oxide metabolites, TBARS, ROS, polymorphonuclear leukocytes, plasma FFAs, TNF-α, IL-6, MCP-1, MIF, CRP, resistin, visfatin, LBP, HMG-B1, and myoglobin vs. baseline
Mehta et al., 2010 [75]	*E. coli* 3 ng·kg^{-1} Intravenous bolus (baseline)	Healthy adult male and female humans; 27.3 ± 4.8 years; $n = 20$	–	24-h post-LPS phase	• ↑ insulin resistance (via frequently sampled intravenous glucose tolerance testing) • Adipose: ↓insulin receptor substrate-1 and ↑ IL-6, TNF, MCP-1, and CXCL-10

The liver as an important recipient of LPS

As a bidirectional pathway, liver-derived products can greatly modulate the GM and gut barrier integrity [96,97]. This two-way street between the GM and liver, comprising the 'gut-liver axis,' is emerging as an important player in the progression of NAFLD [98,99]. Though, there are multiple intricacies with this relationship and many open questions remain unresolved (Fig. 2). Conceivably, the liver receives a larger influx of endotoxin in comparison with other tissues due to the portal vein. For instance, LPS concentration in the portal system has been reported to be 10 times greater versus concentrations in peripheral blood [100]. Though LPS is also absorbed with lipids, it effectively binds to lipoproteins in chylomicrons due to the action of LBP [101]. Through this mechanism, LPS can traffic through the lymphatic system and enter the bloodstream via the subclavian vein. Moreover, LPS has a binding affinity with circulating lipoproteins, including very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) [102] and can be transferred between these carriers via LBP and phospholipid transfer protein [103]. HDL is the main acceptor of LPS and conditions where HDL is reduced may free up LPS, spurring further inflammation [104]. Regardless, the vast majority of bound and unbound LPS in circulation passes through the liver, interacting with Kupffer cells (liver-associated macrophages) [105]. The liver contains 80–90% of all tissue/resident macrophages (Kupffer cells) in the body [106]. Though, LPS bound to HDL can be distributed to other tissues, including adipose and kidney [105]. LPS that accumulate in the liver is inactivated by acyloxyacyl hydroxylase produced by Kupffer cells regardless of whether it is free or bound to HDL [105]. However, this interaction produces reactive oxygen species and proinflammatory cytokines [107]. Thus, elevated LPS in circulation can accelerate lipid accumulation [108] and promote liver injury [109].

Despite the known importance of the gut-liver axis and the liver's role in receiving LPS, little research is available specifically investigating this organ in the context of LPS variation. Generally, and perhaps not surprisingly, research has implicated increased circulating levels of LPS with negative outcomes for liver health. In a longitudinal study of 6727 adults, serum LPS was measured by the LAL assay for an average of 16.3 years and was found to be positively correlated with hospitalization, liver cancer, and death related to liver disease [110]. In a serotype-specific study,

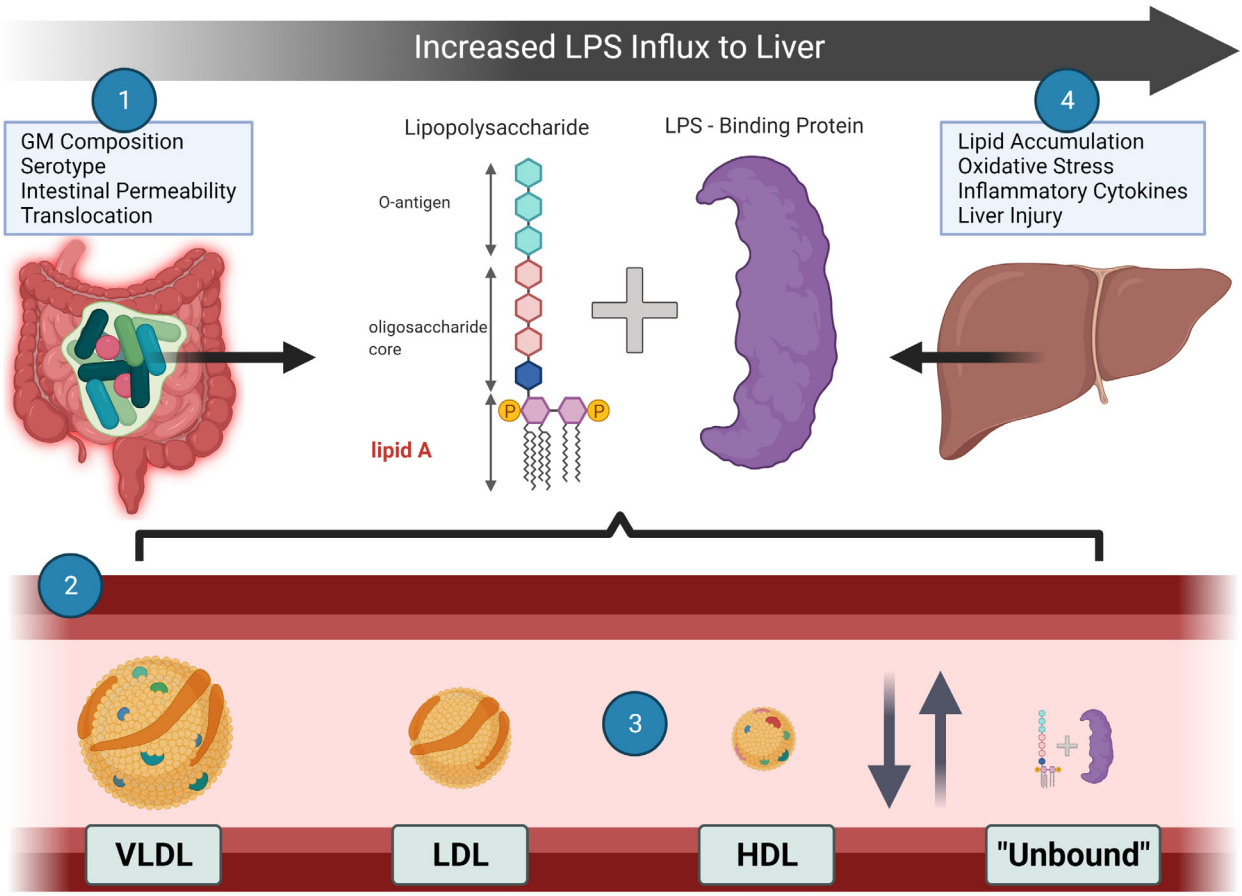

Fig. 2. A depiction of the 'gut-liver axis' considering lipopolysaccharide variants. (1) The GM can produce different variants that may have varying effects on permeability and movement of microbial byproducts through the gut wall. (2) The liver receives an increased LPS load via the portal vein. (3) LPS in circulation has binding affinity for LBP and lipoproteins, including VLDL, LDL, and HDL, with the latter being the main acceptor. (4) Elevated LPS in circulation may be damaging to the liver, depending on serotype.

administration of LPS serotype O55:B5 (*E. coli*-derived LPS) for 4 weeks resulted in hepatocyte necrosis and upregulated gene expression of proinflammatory cytokines in broiler chickens [111]. Paradoxically, LPS has also been explored for use as a liver health-promoting agent [112]. For example, Márquez-Velasco et al. [113] reported that oral administration of *E. coli*-derived LPS serotype (O55:B5) for four days significantly increased survival rate while reducing inflammatory responses and IgM anti-LPS antibodies in the liver of mice that underwent cecal ligation and puncture. Additionally, prior work suggests that LBP gene therapy can restore innate immunity in LBP knockout mice inoculated with *Klebsiella pneumoniae*, a bacterium responsible for the onset of respiratory pneumonia [114]. Together, these variations in the response to *E. coli* LPS and the preventative properties of LBP gene therapy indicate the need for more research on LPS treatment for liver conditions that include the examination of immunoregulatory responses and the role of LBP in LPS recognition and disease severity.

Impact of diet

In seminal work by Cani et al. [13], elevated plasma levels of LPS after a fat-enriched diet were identified as a triggering agent for metabolic endotoxemia in mice. In addition, subcutaneously infused LPS-induced obesity and insulin resistance in mice fed a normal diet. Conversely, knockout of the CD14 (a coreceptor of TLR-4) effectively stopped the ability of LPS to promote those effects. Cani and colleagues noted that inflammation spurred by this type of chronic LPS infusion was the critical element for the development of obesity and insulin resistance in these animals. Further, a high-fat, obesogenic diet is in part, responsible for this mechanism by promoting increased gut permeability and enhanced LPS absorption [11]. These

findings have been corroborated with overfeeding studies in humans. For instance, a crossover study of healthy participants consuming a Western diet (40% kcal fat) for one month showed increased plasma LPS concentrations by 71% [115]. Similarly, in an 8-week over-feeding trial (+760 kcal·day^{-1}; 70 g fat) with 18 healthy males, there was a significant increase in postprandial accumulation of endotoxins [116]. Furthermore, LPS has also been noted to increase after acute high-fat feeding (50 g) [117].

Over the course of human evolution, chronic high-fat consumption was likely a seldom occurrence and positive selection for the advantages of high caloric intake is likely [118]. LPS associated with lipoproteins in circulation can be effectively delivered to adipose tissue, which (depending on the LPS structure) promotes lipogenesis and inflammation [118]. This event likely encourages the change of macrophages in adipose tissue during hypertrophy by a high-fat diet, changing the polarity from an M2 (anti-inflammatory) to M1 (proinflammatory) phenotype [119]. Macrophage change from the M2 to M1 phenotype in adipose tissue is a result of increased LPS delivery into the tissue during lipid accumulation and storage [118]. In the body, this may be a refined adaptation where LPS from the GI tract is packaged into chylomicrons to effectively control its binding, which is utilized to regulate delivery and intestinal absorption of LPS and lipid disposition in target tissues after high-fat intake [118]. The purpose of this may be to prepare the body for potential infections and sepsis, where adipose tissue could function as an 'endotoxin sink' to clear excess LPS from the circulation. While such an adaptation is reasonable under sporadic conditions to prime the body for potential infections, humans consuming a Western diet are likely not equipped to deal with the consequences of chronic stimulation by continuous high-fat feeding.

The issue of LPS structural variation and the influence of diet remain to be elucidated. As noted previously, oral administration of LPS has been explored for potential therapeutic effects. A prime example includes LPS derived from Gram-negative *P. agglomerans*. This bacterium has been observed to have a symbiotic relationship with a wide range of plants and has been employed as a biological protective agent against bacterial diseases on some fruits [120,121]. In senescence-accelerated mice fed a high-fat diet, oral administration of *P. agglomerans*-derived LPS at 0.3 or 1 mg·kg^{-1} body weight a day for 18 weeks significantly improved glucose and lipid metabolism [122]. The LPS treatment also reduced liver weight, inflammatory factors, and oxidative stress. Similar outcomes were noted in apoE-deficient mice who were fed a high-fat diet and provided drinking water containing *P. agglomerans*-derived LPS (0.3 or 1 mg·kg^{-1} body weight per day) [123]. In comparison with studies that injected LPS (*E. coli*) as described earlier [13], these results indicated that this LPS (provided orally) decreased body weight, improved glucose and lipid metabolism, and decreased markers of inflammation. In detail and within the context of atherosclerosis, supplemented mice displayed reduced plaque deposition, plasma LDL, oxidized LDL, and oxidative stress. Taken together, oral supplementation of *P. agglomerans*-derived LPS appeared to act as a protective agent, buffering against high-fat, diet-induced metabolic disorder and propagation of constitutional factors involved in atherosclerosis.

Other factors, such as bile acid (BA) modification, can act to change the composition of the GM and potentially alter what type of LPS ultimately interacts with the host. This is dependent on nutritional substrate type/availability, GM composition, and microbial competition. Such considerations are of particular importance in the context of obesity and during the formation and development of the gut flora [124,125]. van Best et al. [125] analyzed the effect of liver function and metabolism on GM development, noting that BAs are potent drivers of early GM maturation in mice. Conjugated primary BAs dominated during the postnatal period. After weaning, these BAs were present at high concentrations in the small intestine. Orally supplementing the two major conjugated BAs (tauro-cholic acid or β-tauro-murocholic acid) to newborn mice accelerated postnatal microbiota maturation. β-tauro-murocholic acid, in particular, reduced the abundance of *Escherichia*, while increasing the abundance of *lactobacilli*. Importantly, this action inverted the *Lactobacillus* : *Escherichia* ratio, a proxy metric for GM maturation. BA influence on the microbiota composition, acting through LPS and other mechanisms, can play a large role in the maturation of mucosal immunity during development [126,127].

In the context of diet, LPS has generally been implicated in various metabolic diseases. However, many of these studies utilize LAL assays, which are not specific to serotype. It may be that certain diet types and/or food components promote the proliferation of LPS variants that are more apt to spur meta-inflammation or vice versa. Much research is to be done in this area, particularly investigating gut-derived vs. orally ingested LPS, as noted from the paradoxical findings of *P. agglomerans* above. In addition, investigations of whether different metabolic signatures and/or levels of adiposity modulate these potential responses are warranted.

Impact of obesity: a larger LPS reservoir?

Adipose tissue acts as a signaling platform of the innate immune system with TLRs eliciting a rapid immune defense reaction, leading to cytokine release and inflammation [118]. As in an obese state, this platform can be greatly expanded, amplifying the immune response and subsequent inflammation via stimulation by LPS. Moreover, obese individuals exhibit elevated levels of circulating LPS after high-fat meals and, during fasting, higher IL-6 parallel to LBP levels compared with normal weight individuals [128].

Obesity is often associated with hyperinsulinemia [129], which may act to amplify the inflammatory response generated by macrophages. Indeed, elevated insulin levels have been reported to promote LPS-elicited increases of inflammatory markers in mice [130] and healthy humans [131–133]. Insulin may 'synergize' with LPS to promote proinflammatory cytokine and prostaglandin E2 (PGE2) expression [134]. By stimulating human macrophages with *E. coli*-derived LPS, insulin, and PGE2, LPS-dependent expressions of IL-1β and IL-8 were significantly enhanced. Moreover, combining all three stimuli induced an even greater expression than the duo of LPS plus insulin or LPS plus PGE2. These findings highlight the relevance of hyperinsulinemia in (directly and indirectly) enhancing LPS-dependent cytokine expression. Highly pertinent in the context of obesity is the potential attenuation of the body's ability to respond to these signals over time. For example, *E. coli*-derived LPS-treated (i.p. injection) diabetic Zucker rats did not show impaired metabolic responses and were less sensitive to stress compared with lean littermates [135]. There also appears to be an adaptation over time based on exposure. For instance, in mice receiving a high-fat diet (60% kcal) with dietary LPS (O5:B55 *E. coli* 20.4 $\mu g \cdot g^{-1}$ LPS) for 20 weeks, circulating levels of LPS and glycated hemoglobin (HbA1c) increased [136]. While there was a slower uptake in blood glucose in the LPS supplemented mice once an obese state was reached, such differences dissipated after a 20-week period. It may be that certain LPS variants promote disordered glucose metabolism in a particular time frame until a certain threshold of fat disposition is obtained (e.g., via ectopic fat deposits). However, the mechanism by which different variants of LPS affect this is unknown as the aforementioned experiments used *E. coli*-derived LPS.

As with diet, what remains unclear is how an obese-associated GM impacts structural differences in LPS, and, in turn, how these products then impact the host. Clearly, diet is innately tied to obesity status, and adoption of a healthful eating pattern and weight loss alter GM community dynamics. Conversely, obesogenic diets, higher in fat, offer increased opportunity for LPS to enter host circulation regardless of their potential pathogenic or beneficial properties. Obesity seems to be associated with increased pathogenic and disease-associated Gram-negative bacteria. Once in circulation, such derived LPS have an expansive platform of various tissues and immune cells to interact with. Might some LPS derived from more pathogenic bacteria 'over-ride' signals from some inert or beneficial LPS variants? As these studies are limited in their scope due to the broad testing techniques, future studies should seek to uncover some of these complexities.

LPS detection and defining serotype specificity

For LPS detection, the LAL reactivity assay is widely used, though is susceptible to capturing several non-specific activators and inhibitors in circulation making accurate quantification of LPS challenging, particularly at lower concentrations [137,138]. For this reason, the LAL assay has recently been regarded as inappropriate for metabolic endotoxemia studies [139]. Moreover, detecting differences in LPS structure with the LAL test is not possible. Increased levels of circulating endotoxemia could be a result of greater concentrations of LPS or a lower concentration of LPS with greater endotoxic activity due to their structure [140]. Therefore, there are apparent limitations in terms of this assay's utility. In contrast, its binding protein, LBP, is regarded as an endotoxemia marker [141]. Indeed, circulating LBP is highly correlated with LPS levels [142] and has been noted as a reliable systemic biomarker [143]. This is especially evident for LPS detection in healthy individuals who have generally lower concentrations of circulating endotoxin [144]. However, LBP binds to components of both Gram-positive and Gram-negative bacteria, which may relegate its utility to a more generalized survey of bacterial exposures depending on the research context [143]. Therefore, utilizing both measures may be a prudent practice depending on the study population, intervention factors, and research question.

Lipopolysaccharide binding protein is present in circulation at much greater concentrations compared with LPS (approximately 2–20 $\mu g \cdot mL^{-1}$) [145,146]. As an acute-phase reactant predominantly produced and released from the liver, LBP binds to the lipid A portion of LPS and interacts with the TLR-4/MD-2/CD14 protein complex to induce downstream immune signaling pathways, as previously noted [47,147]. LBP

concentrations increase markedly after inflammatory affronts, such as bacterial sepsis [145]. Moreover, acute-phase LBP may be a potential defense mechanism against infection by inhibiting LPS-mediated cytokine release [148]. Both *in vitro* and *in vivo* work have shown LBP production occurs rapidly upon contact with LPS, inducing liver production of LBP within ~ 15–30 min [149]. Maximum levels in circulation appear to occur after 24–48 h [150]. Importantly, LBP significantly accelerates LPS binding to CD14 thereby increasing the sensitivity of cells to LPS [151]. The physiological role of LBP in the innate immune system is to aid macrophages in their response to LPS, increase the LPS-CD14 interaction, and elevate overall LPS recognition in the host [152,153]. At present, LBP has been suggested as one of the preferred, surrogate markers of endotoxemia but is likely not indicated/appropriate in acute instances (i.e., minutes/hours) [139]. Furthermore, if the LAL assay is used, researchers should calculate and report correlations with other metrics like LBP or another alternative method of analysis as a quality measure [139].

Lipopolysaccharides and LBP (indirectly, as a surrogate) assays can be useful for general detection and quantification purposes but are not able to profile LPS variation or capture specific serotype endotoxicity (unless strains are individually assessed). Rapala et al. [154] implemented the LAL assay to test 26 cyanobacterial strains noting endotoxicity was at least five orders of magnitude lower in comparison with *E. coli*-derived LPS. Moreover, several strains were below the detection limits of the assay. Clearly, lipid A structures of different LPS variants are variable enough to impart different reactivities with the LAL assay. Therefore, these types of methodologies are limited. Indeed, to the best of our knowledge, no commercially available assay at this time is capable of distinguishing structural differences of LPS that may impart protective, inert, or inflammatory effects once in circulation. If the aforementioned LPS and LBP assays are used, caution is warranted if attempting to connect GM-derived LPS and systemic inflammation. In more detailed (and labor-intensive) examinations, fecal LPS could be recovered and purified from stool samples as previously described [155,156]. The immunostimulatory potency of each sample of fecal LPS can then be assessed by stimulating different *in vitro* cells, such as human primary PBMCs.

For more feasible examinations, perhaps paired with LPS and LBP assays, the structure of intact lipid A could be identified with matrix-assisted laser desorption/ionization MS [157–160] and direct infusion-electrospray ionization MS [161,162]. There are also a few examples of MS coupled with separation methods, such as reverse-phase liquid chromatography (LC), which can be employed for elucidating the structure of intact lipid A as well [163,164]. The difficulty in the analysis of intact lipid A by LC-MS is due to the diverse physical properties arising from different numbers of acyl chains and phosphate groups [164]. Recently, Okahashi et al. [165] created a method for analyzing lipid A using LC-quadrupole time-of-flight MS, which was developed and used in the analyses of *E. coli* and *Bacteroidetes* strains. Briefly, an ammonia-containing alkaline solvent was used to produce sharp lipid A peaks with high sensitivity. Comparison of the lipid A structures, such as acyl side chains, and total amount across the tested strains revealed a specific association between lipid A signatures and phylogenetic classifications of the strains. While pairing more general assessments like the LAL (LPS) and LBP assays with these more specific/qualitative MS-based measures have been done in the case of sepsis [166], these dual measurements are lacking in other areas of research. Pairing these methods in future research is important as quantitative and qualitative analyses of lipid A in each bacterium are expected to help understand the strain-specific phenotypes [79]. We acknowledge the current limitations of assays like the LAL test and the feasibility issues discerning different serotypes. Therefore, at the very least, researchers who publish in this area should acknowledge the issue of structural differences of LPS and state their lack of measurement of it as a limitation.

Using MS, researchers may accurately profile LPS structure with increased sensitivity. Triple quadrupole (QQQ) instruments with soft ESI have been a mainstay technology for the detection of LPS. Every stage of fragmentation offers valuable information helping elucidate chemical structure [31]. MS^1 analysis of lipid A renders satellite peaks of 80 and 123 Da differences indicating the loss of P and PEtN groups, respectively, from diphosphorylated species, whereas B1 oxonium ions (only in positive-ion mode) with 80 Da difference indicate 4′-phosphate substitution. Further disassociation in the second quadrupole produces ions at m/z 79 and 97 corresponding to loss of $[PO_3]^-$ and $[H_2PO_4]^-$, respectively, from mono and/or diphosphorylated species. MS^2 scans also reveal ions at m/z 159 and m/z 177 ($[HP_2O_6]^-$ and $[H_3P_2O_7]^-$, respectively) indicating pyrophosphorylated lipid A moieties. With MS^n experiments, serial release of fatty acids in each MS stage is observed along with diagnostic cross-ring and inter-ring fragment of selected ions; the loss of N-linked fatty acid at position 2 might occur at higher MS stages such as MS^5. Figure 3 depicts a conceptual schema of typical LPS analysis via ESI-QQQ.

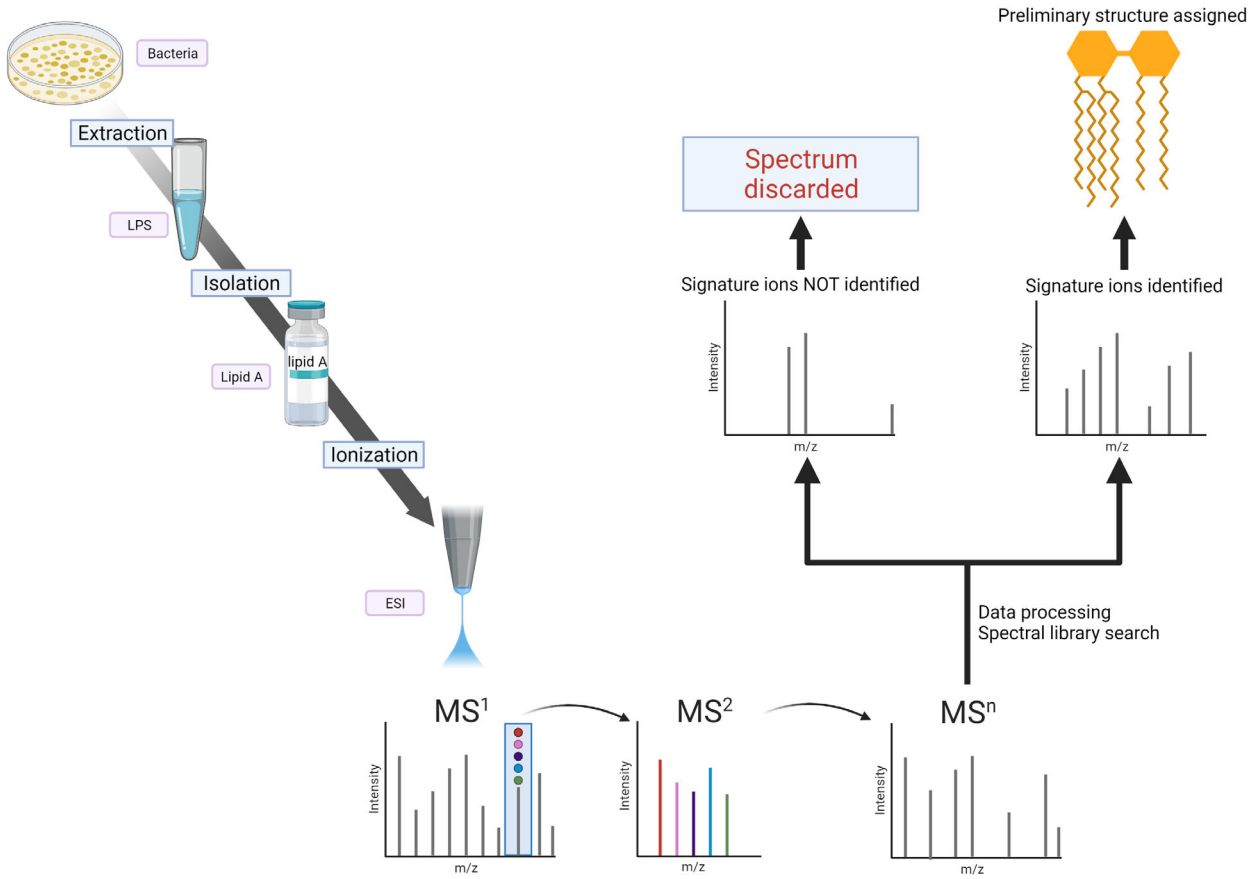

Fig. 3. Overview of hierarchical ESI-QQQ analysis of LPS from bacterial isolate. Lipid A is subjected to MS^n to acquire tandem mass spectra of each precursor ion and all fragment ions. The acquired spectra are queried against theoretical signatures using spectral libraries for structural assignment.

Manipulation of MS operating parameters allows for enhanced qualitative and quantitative assessment of LPS. Low-energy collision-induced dissociation (CID)-MS/MS permits selective cleavage of C-O, C-N, and C-C bonds, together with glycosidic C-O and cross-ring cleavages, affording excellent structural analysis of a heterogeneous lipid A mixture derived from Gram-negative *Aeromonas hydrophila* [167]. Additionally, in a study of LPS host specificity, low-energy CID was harnessed in conjunction with Fourier-transform ion cyclotron resonance (FT-ICR)-MS/MS to accurately determine the fatty acid acylation positions on the H_2 PO_3→4-O'-β-D-GlcpN-(1→6)-α-D-GlcpN disaccharide backbones of lipid A [167]. In conjunction with database searches, careful manipulation of instrument parameters can allow researchers to identify specific LPS signatures beyond what is possible with traditional reactivity assays.

Given the structural similarity of LPS species, more advanced MS instrumentation is increasingly utilized for routine analysis. LPS species contain a distribution of isotopes that produce a series of condensed isotopic clusters less than one m/z apart. Given the almost identical m/z, due to the multiple charges assumed during ionization, the high resolving power of FT-ICR [168] or TOF [169,170] is a prerequisite to the quantitation of LPS from complex biological samples. High-resolution MS methods have also been employed in the analysis of LPS via the surrogate LBP. Using a highly sensitive Orbitrap platform in conjunction with FT, Kim et al. [171] screened and quantified specific LBPs given their interaction with various LPS species with known immunogenic effects. Indeed, the combination of soft ionization techniques such as ESI, gentler fragmentation methods, and high-resolution instrumentation allows for the accurate chemical characterization and quantitation of LPS.

Conclusions

In this review, we covered many topics that impact LPS-focused research. While there are many

unknowns, the structural diversity observed among different LPSs is clearly important, though often neglected, component that warrants investigation beyond *in vitro* work. Nearly 20 years ago, the spuriousness of assuming LPS derived from taxonomically distinct microbes would impose similar biological effects was conveyed [172]. As Netea et al. [172] eloquently stated, 'structural and functional differences between LPS across species are the rule rather than the exception'. This is not trivial and, as highlighted throughout this review, research over more than a decade has shown that LPS/LBP is positively correlated with increased dietary fat intake, obesity, metabolic disturbances, and inflammation. Furthermore, inflammatory signaling spurred by LPS appears to contribute to the pathogenesis of multiple metabolic diseases. The complex axes that link the microbial composition of the GM to inflammatory states and metabolically important tissues like the adipose, liver, and muscle have yet to be fully delineated. However, there is experimental support for the notion that the grade of metabolic endotoxemia may be dependent on the (commensal or pathogenic) microorganism that produces LPS and, consequently, on LPS structure. How do different LPS variants impact important protective structures at the luminal interface, specifically GALT and the functioning of the brush border cells? As discussed, the type of LPS variant is important in determining TLR signaling and downstream inflammatory response, depending mostly on lipid A and O-antigen moieties. Moreover, not all LPS variants are injurious and potentially could benefit host health. How might this be leveraged for diseases like T2D, NAFLD, IBD, CVD, obesity, and other chronic inflammatory conditions? How might diet modulate serotype composition and these disease states? These are questions that deserve consideration in future work. While there is no current clear-cut, uncomplicated way to answer these, combined methodologies of clinical research, *in vitro* testing, and perhaps more sophisticated MS-based technologies can be deployed.

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Author contributions

AEM and MC involved in conceptualization; AEM, MC, PJ, SM, and KLS involved in methodology; AEM, MC, PJ, SF, and KLS involved in investigation; AEM, MC, PJ, and KLS involved in writing—original draft preparation; AEM, MC, PJ, SM, and KLS involved in writing—review and editing; AEM and PJ involved in visualization; KLS involved in supervision; AEM and KLS involved in project administration.

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