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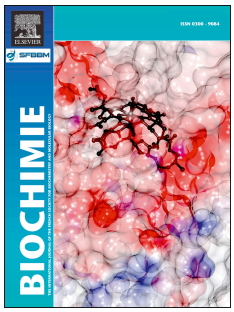

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With regard to *in vitro* pro-inflammatory activities, TNF-α and IL-6 inducing capacities were compared on THP-1 cells treated with LPSs isolated from the three *Ralstonia*. *R. pickettii,* with low inflammatory capacities, and recently involved in nosocomial outcomes, could explain the low inflammatory level reported in previous studies on diabetic patients and animals. In addition, transmission electron microscopy was performed on the three *Ralstonia* species. It showed that the *R. pickettii* under-acylated LPSs, with a higher level of phosphate substitution had the capacity of producing more outer membrane vesicles (OMVs). The latter could facilitate transfer of LPSs to the blood and explain the increased low-grade inflammation in obese/diabetic patients.

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Structure function relationships in three lipids A from the *Ralstonia* genus rising in obese patients

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Running title: *Ralstonia lipid A structures and inflammatory activity in relation to obesity*

Abbreviations: AraN, 4-amino-4-deoxy-l-arabinose; AOAH, acyloxyacyl hydrolase; DHB, 2,5-dihydroxybenzoic acid; EA, ethanolamine; FA, fatty acid; GlcN, glucosamine; IL, Interleukin; LPS, lipopolysaccharide; NAC, N-acetyl-cystein; PBS, phosphate buffer saline; PEA, phosphoethanolamine; PS, polysaccharide; TLC, thin-layer chromatography; TNF, tumor necrosis factor

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Highlights

- Characterization of the lipid A structures of *Ralstonia eutropha*, *R. mannitolilytica* and *R. pickettii* was performed in connection to the observed increase of Ralstonia in obese patients.
- The pro-inflammatory activities of the three corresponding *Ralstonia* LPS were tested and compared, explaining the low-grade inflammation level observed in obese patients.
- Transmission electron microscopy performed on the three *Ralstonia* species helps understanding LPS contribution to the bacterial membrane and OMV formation.

Abstract

The identification of a functional molecular moiety relating the lipopolysaccharides (LPSs) to their capacity to induce inflammation-mediated metabolic diseases needed to be performed. We previously described a proportional increase in the relative abundance of the 16SrDNA bacterial gene from the genus *Ralstonia,* within the microbiota from the adipose tissue stroma vascular fraction of obese patients, suggesting a causal role of the bacteria. Therefore, we first characterized the structures of the lipids A, the inflammatory inducing moieties of LPSs, of three *Ralstonia* species: *Ralstonia eutropha*, *R. mannitolilytica* and *R. pickettii*, and then compared each, in terms of *in vitro* inflammatory capacities. *R. pickettii* lipid A displaying only 5 Fatty Acids (FA) was a weaker inducer of inflammation, compared to the two other species harboring hexa-acylated lipids A, despite the presence of 2 AraN substituents on the phosphate groups.

With regard to *in vitro* pro-inflammatory activities, TNF-α and IL-6 inducing capacities were compared on THP-1 cells treated with LPSs isolated from the three *Ralstonia*. *R. pickettii,*

with low inflammatory capacities, and recently involved in nosocomial outcomes, could explain the low inflammatory level reported in previous studies on diabetic patients and animals. In addition, transmission electron microscopy was performed on the three *Ralstonia* species. It showed that the *R. pickettii* under-acylated LPSs, with a higher level of phosphate substitution had the capacity of producing more outer membrane vesicles (OMVs). The latter could facilitate transfer of LPSs to the blood and explain the increased low-grade inflammation observed in obese/diabetic patients.

1. Introduction

Although LPSs share a common architecture, their structural details exert a strong influence on the biological activities they induce. The number of fatty acids and the length of their carbon chains, the phosphate groups and their possible substitution with various decorations, are crucial for the regulation of endotoxic activities. In some examples, there is a direct relationship between LPSs structures and bacterial niches (1). In mice, a fat-enriched diet (HFD) impacts gut microbiota (2, 3) leading to an increased ratio of Gram-negative to Gram-positive bacteria, thereby increasing the probability of LPSs diffusion into the blood. Furthermore, the fat component of the diet favors the intestinal absorption and the transport of LPSs through the intestinal epithelium into the blood stream and towards the tissues (4,5). Eventually, in type 2 diabetic patients we observed an impaired distribution of LPS within lipoprotein associated with a reduced lipoprotein turnover, therefore putatively triggering inflammation in such patients (6).

We recently observed an increased frequency of Gram-negative bacteria from the *Ralstonia* genus in the stroma vascular of obese patients (7). This tissue microbiota, and notably the corresponding LPS, could be triggering adipose tissue expansion and metabolic inflammation through the binding of CD14/TLR4 as previously shown (8).

Ralstonia is a genus of Proteobacteria previously included in the Pseudomonadaceae. These bacteria were mainly known as plant pathogens, but the first documented case of *Ralstonia* bacteremia and death was reported in 1968. At that time, the pathogen was reported as an unclassified Gram-negative bacterium which was later identified as *Ralstonia pickettii* (9).

More recent outbreaks of *R. pickettii* infections are documented as nosocomial outbreaks related to the use of contaminated medical solutions (saline, sterile water, disinfectants, etc.) used in patient care (9). The presumptive ability of *Ralstonia* to persist in sterile solutions is thought to be associated with its ability to survive within a wide range of temperatures (15°C–42°C). These bacteria pass through both 0.45 and 0.2 μm filters, used to sterilize medical solutions. *R. pickettii* is well-known for contaminating waters and information on its corresponding LPS structure and inflammatory capacities was lacking. The present study will therefore also be relevant in the context of *Ralstonia* infections.

Metabolic diseases induced by a high-fat diet, like diabetes and obesity, are characterized by a low-tone inflammation (10). The origin of inflammation could be related to the metabolic endotoxemia observed in these patients at risk of developing diabetes (11). The continuous low-rate infusion of LPS induces hyperglycemia, and when mice deprived of LPS receptors are fed with a high-fat diet they are less prone to insulin resistance and obesity (12). After eating, cholesterol and triglycerides are absorbed into the intestines and are packaged into particles called chylomicrons, and then released into the blood circulation (13). These particles can transport LPS across the intestinal epithelium and other lipoproteins can transport LPS towards targeted tissues, such as liver and adipose tissues (4,5). Another mean by which bacterial LPS could reach tissues is related to an increased translocation of bacteria from the intestinal mucosa towards tissues, as previously described in type 2 diabetic mice by Amar et al (14). Eventually, the LPSs when reaching adipose tissue induce macrophage and pre-adipocyte proliferation leading to cytokine production and insulin resistance (8). LPS can also be modified by a host enzyme, acyloxyacyl hydrolase (AOAH), which removes branched fatty acids (FA) from lipid A decreasing its cytokine induction capacities.

Based on the above reported evidences, there was a need to chemically characterize the LPS molecular moieties relating the role of LPSs to metabolic inflammation.

2. Material and Methods

2.1.Bacterial strains and growth conditions

Three strains of *Ralstonia* (Gram negative, Order Burkholderiales, Family *Ralstoniaceae*) were obtained from the Pasteur Institute. *R. pickettii* and *R. mannitolilytica* were spread on TCS (triptone- caseine -soja) agar and *R. eutropha* (*Cupriavidus necator* in the International Code of Nomenclature of Bacteria) on Columbia agar. All three strains were liquid cultured in Brain Heart Infusion (BHI) complete medium at 30°C under agitation (150 rpm) and stored in 30% glycerol at -80°C. Once at confluence, bacteria were centrifuged (3000 rpm x 10 min) and the pellet washed with PBS and lyophilized for further analysis or purification.

2.2.LPS and lipid A preparation

The *Ralstonia* LPSs were extracted by the Isobutyric acid/1M ammonium hydroxide method (15). LPSs were purified by enzymatic treatments to remove DNA, RNA and proteins, as already described. They were also extracted with a mixture of solvents to remove phospholipids and lipoproteins. (16). The degree of purity of each sample, to be used for biological studies, was checked by UV spectrometry, SDS-PAGE, TLC and amino acid analyses for the detection of amino acid components as markers of peptides and proteins, and for Muramic acid as a marker for eventual peptidoglycan contaminants.

Lipid A was obtained by direct hydrolysis of the lyophilized bacteria (17). Briefly; 1-5 mg of lyophilized bacteria were suspended in 50-400 µl of isobutyric acid and 1M ammonium hydroxide (5:3, v:v), heated 2 hours at 100°C with stirring, cooled to 4°C, and centrifuged. The supernatant was diluted with water (1:1, v:v) and lyophilized. The material obtained was then washed twice with 100-400 µl of methanol and centrifuged (2000 g for 15 min). Finally, the insoluble lipid A was extracted in a 50 to 200 µl mixture of chloroform: methanol: water (3:1.5:0.25, v: v: v).

Monophosphorylated lipids A were obtained by HCl 0.1M hydrolysis of the lipids A for 10 min at 100°C, neutralised and recovered by ultracentrifugation.

2.3.Identification of glycose absolute configurations.

Lipids A (4 mg) were hydrolyzed with 0.5 ml of 4M HCl at 100°C for 2 hours. After cooling and extraction of fatty acids with chloroform, residual solutions were brought to neutrality by repeated evaporation under reduced pressure. After N-acetylation**,** the residue was treated with trifluoroacetic acid R-(-)-2-butanol, per-acetylated, and analyzed by gas chromatography (GC) on a BP10 capillary (Scientific Glass Engineering) column using a program 160°C (1 min) to 220°C, 5°C min^{-1} at 0.6 kPa.

2.4.Sequential liberation of ester-linked fatty acids by mild alkali treatment

This treatment was used to establish the lipid A acylation patterns (18). For the first-step liberation of primary ester-linked fatty acids, lipid A (200 µg) was suspended at 1 mg/ml in 28% ammonium hydroxide and stirred for 5 hours at 50°C. The solutions were dried under a stream of nitrogen, the residues taken up in a mixture of chloroform: methanol: water (3: 1.5: 0.25, v: v) followed by MALDI-MS analysis. In order to liberate the secondary ester-linked fatty acids more resistant due to steric hindrance, the resulting lipid A was suspended in 200 µl of 41% methylamine and stirred for 5 h at 37°C. The resulting samples were dried under a stream of nitrogen, and the residues were taken up in a mixture of chloroform, methanol, and water (3: 1.5: 0.25, v: v:v) and followed by MALDI-MS analyses.

2.5.Chemical analyses

Fatty acids were analyzed as already described (19). Briefly, LPS (0.5 mg) were submitted to strong acid treatment, 4M HCl, 2 hours at 100°C with 20:0 (10 µg) as internal standard, extraction with ethyl acetate and esterification with diazomethane. GC-MS analysis was performed using a Finnigan Mat 95S mass spectrometer.

2.6. SDS–polyacrylamide gel analysis of LPSs

Fifteen percent acrylamide gel was used, and 0.2 µg of LPS was loaded onto the 4% starting gel. The LPS preparation, electrophoresis process and the Tsai and Frash silver nitrate coloration were performed as previously described (20).

2.7. Mass spectrometry

MALDI negative-ion mass spectrometry analyses were performed on a PerSeptive Voyager-DE STR model time-of-flight mass spectrometer (Applied Biosystem) (I2BC, Université de Paris Sud Orsay), in linear mode with delayed extraction, and alternatively with a Shimadzu Axima Performance system in the same mode (21).

The ion-accelerating voltage was set at 20 kV. Dihydroxybenzoic acid (DHB) (Sigma chemical Co., St Louis) suspended in 0.1M citric acid was used as a matrix (22). A few micrograms of lipid A were dissolved in a mixture of chloroform: methanol: water (3:1.5:0.25, by vol.) at 1 µg/ µl and desalted with a few grains of ion-exchange resin (Dowex 50W-X8) (H^+), in an Eppendorf tube. A 1 µl aliquot of the solution (50 µl) was deposited on the target and covered with the same amount of the matrix suspended at 10 mg/ml in the same mixture of solvents (23). Different ratios between the samples and DHB were tested when necessary. *B. pertussis* lipid A was used as an external standard.

2.8. *In vitro* pro-inflammatory activity of LPSs

Purified LPSs were incubated with THP-1 cells (human monocyte cell line). Supernatants were harvested after 6 or 30 hours of stimulation, and cytokines were measured by ELISA. Purified *Escherichia coli* J5 LPS was used as a control.

2.9. Transmission Electron Microscopy

Lyophilized bacteria were suspended in PBS and centrifuged. The pellets were fixed with glutaraldehyde 2.5%, PFA 1% in CaCo 0.1M pH 7.4 buffer for 1h. Then, they were post-fixed with 1% osmium for 1h, dried in successive baths from ethanol 10% to absolute ethanol and included in resin (Epon). After polymerization, thin slices were cut and observed with a JEOL 1400 station.

3. Results

3.1. Total fatty-acid composition of the three *Ralstonia* lipids A

R. eutropha lipid A fatty acids composition was characterized by GC/MS and found to correspond to three to four 3-hydroxytetradecanoic acid (14:0(3-OH)), one tetradecanoic acid (14:0), and a small amount of 2- hydroxytetradecanoic acid (14:0(2-OH)).

R. mannitolilytica lipid A fatty acid composition was characterized by GC/MS and found to correspond to four 14:0(3-OH), one 14:0, one 16:0/16:0(2-OH).

R. pickettii lipid A fatty acid composition was characterized by GC/MS and found to correspond to three 14:0(3-OH), one 14:0, and traces of 14:0(2-OH).

3.2. Molecular heterogeneity and distribution of the fatty acids between the two D-glucosamine

3.2.1. MALDI-Mass spectrometry analysis of the three *Ralstonia* lipids A

The three lipids A were obtained by direct hydrolysis of the bacteria (17), purified, deionized and tested in the negative-ion mode after being deposited on the target and covered with the DHB matrix suspended in 0.1M citric acid solution.

R. eutropha lipid A spectrum, presented in Figure 1A displayed a major peak at *m/z* 1599 corresponding to a bis-phosphorylated di-GlcN penta-acyl molecular species with three

14:0(3-OH) and two 14:0. A satellite peak at *m/z* 1615 indicated some heterogeneity at the 14:0 with hydroxylation at position 2. Two peaks, at plus and minus 226u for 14:0(3-OH), appeared respectively at *m/z* 1825 at *m/z* 1373, and showed fatty acid substitution heterogeneity due to lipid A biosynthesis. Other peaks were representative of molecular species carrying one AraN at *m/z* 1504, 1730 and 1957 respectively for the tetra-, penta-, and hexa-molecular species.

R. mannitolilytica lipid A spectrum, presented in Figure 1B, showed a major peak at *m/z* 1627 corresponding to a bis-phosphorylated di-GlcN penta-acyl molecular species with three 14:0(3-OH), one 14:0 and one 16:0. A satellite peak was observed at plus 16 u corresponding to a molecular species containing 16:0(2-OH) instead of 16 :0. Two other doublet peaks were observed at *m/z* plus 131 and plus 226u, i.e. at *m/z* 1758/1774 and 1889/1905 corresponding respectively to the addition of one and two AraN residues. Minor peaks observed at *m/z* 1854, 1985 and 2116 were attributed to bi-phosphorylated hexa-acyl (four 14:0(3-OH), one 14 :0, and one 16 :0) unsubstituted with AraN, and substituted respectively with 1 and 2 AraN residues.

R. pickettii lipid A spectrum, presented in Figure 1C displayed a major peak at *m/z* 1599 corresponding to a bis-phosphorylated di-GlcN penta-acyl molecular species with three 14:0(3-OH) and two 14:0. Other peaks were representative of molecular species carrying one and two AraN residues and observed respectively at *m/z* 1730 and 1861.

3.2.2. Kinetics of sequential liberation of ester-linked fatty acids from the three *Ralstonia* species

- Ester-linked fatty acid liberation under NH_4-OH treatment.

As we previously demonstrated (18), under these conditions only the primary ester-linked fatty acids were released. In the case of *R. eutropha* and *R. mannitolilytica,* the release of two 14:0(3-OH) was confirmed by a shift at minus 452u leading to peaks of respective tetra-acyl

molecular species (see Table). *R. pickettii* lipid A being only penta-acylated, a shift at minus 226u (14:0(3-OH) was observed (see Table).

- Ester-linked fatty acid liberation under Methylamine treatment

All three lipids A led to peaks at *m/z* 952 after release of secondary ester-linked fatty acids; with of course some heterogeneity observed at plus one or two arabinosamine residues (131u) (see Table displaying calculated MW). The observed *m/z* shifts were consistent with fatty acid residues characterized by GC-MS analysis, i.e., secondary 14 :0 and 14 :0(2-OH) for *R. eutropha* and *R. pickettii* and 14:0, 16:0 and 16:0(2-OH) for *R. mannitolilytica.*

Bacterial Species: *R. eutropha*										
Native Sample Molecular Species			NH_4OH, 5h treatment				CH_3NH_3OH, 5h treatment			
Acylation	AraN Presence	*m/z* [M-H] -	*m/z* [M-H] -	ΔM	Primary FA		*m/z* [M-H] -	ΔM	Secondary FA	
					C3	C3'			C2	C2'
4FA		1372,7	1372,7	0	Free	Free	952	420,7	14:0	14:0
4FA		1388,7	1388,7	0	Free	Free	952	436,7	14:0(2-OH)	14:0
4FA	1AraN	1503,9	1503,9	0	Free	Free	1083,1	420,8	14:0	14:0
5FA		1599,1	1372,7	226,4	Free	14:0(3-OH)	952	420,7	14:0	14:0
5FA		1615,1	1388,7	226,4	Free	14:0(3-OH)	952	436,7	14:0(2-OH)	14:0
5FA	1AraN	1730,2	1503,9	226,3	Free	14:0(3-OH)	1083,1	420,8	14:0	14:0
6FA		1825,4	1372,7	452,7	14:0(3-OH)	14:0(3-OH)	952	420,7	14:0	14:0
6FA		1841,4	1388,7	452,7	14:0(3-OH)	14:0(3-OH)	952	436,7	14:0(2-OH)	14:0
6FA	1AraN	1956,6	1503,9	452,7	14:0(3-OH)	14:0(3-OH)	1083,1	420,8	14:0	14:0

Bacterial Species: *R. mannitolilytica*										
Native Sample Molecular Species			NH_4OH, 5h treatment				CH_3NH_3OH, 5h treatment			
Acylation	AraN Presence	*m/z* [M-H] -	*m/z* [M-H] -	ΔM	Primary FA		*m/z* [M-H] -	ΔM	Secondary FA	
					C3	C3'			C2	C2'
5FA		1627,1	1400,8	226,3	Free	14:0(3-OH)	952	448,8	16:0	14:0
5FA		1643,1	1416,8	226,3	Free	14:0(3-OH)	952	464,8	16:0(2-OH)	14:0
5FA	1AraN	1758,3	1531,9	226,4	Free	14:0(3-OH)	1083,1	448,8	16:0	14:0
5FA	1AraN	1774,3	1547,9	226,4	Free	14:0(3-OH)	1083,1	464,8	16:0(2-OH)	14:0
5FA	2AraN	1889,4	1663	226,4	Free	14:0(3-OH)	1214,2	448,8	16:0	14:0
5FA	2AraN	1905,4	1679	226,4	Free	14:0(3-OH)	1214,2	464,8	16:0(2-OH)	14:0
6FA	1AraN	1984,6	1531,9	452,7	14:0(3-OH)	14:0(3-OH)	1083,1	448,8	16:0	14:0
6FA	2AraN	2115,8	1663	452,8	14:0(3-OH)	14:0(3-OH)	1214,2	448,8	16:0	14:0

Bacterial Species: *R. pickettii*										
Native Sample Molecular Species			NH_4OH, 5h treatment				CH_3NH_3OH, 5h treatment			
Acylation	AraN Presence	*m/z* [M-H] -	*m/z* [M-H] -	ΔM	Primary FA		*m/z* [M-H] -	ΔM	Secondary FA	
					C3	C3'			C2	C2'
5FA		1599,1	1372,7	226,4	Free	14:0(3-OH)	952	420,7	14:0	14:0
5FA		1615,1	1388,7	226,4	Free	14:0(3-OH)	952	436,7	14:0(2-OH)	14:0
5FA	1AraN	1730,2	1503,9	226,3	Free	14:0(3-OH)	1083,1	420,8	14:0	14:0
5FA	2AraN	1861,3	1635	226,3	Free	14:0(3-OH)	1214,2	420,8	14:0	14:0

3.2.3. Fragmentation of *Ralstonia* lipid A molecules in the positive-ion mode

Fragmentation in the positive-ion mode (data not showed) allowed identification of the distribution of the different fatty acids on each of the two GlcN residues representing the core of the lipid A moiety. Fragmentation occurs at the level of the glycosidic bonds of the GlcN,

the distal GlcN carrying its phosphate group and corresponding fatty acid residues. A peak at *m/z* 904 appeared in all three lipids A spectra in the positive-ion mode. It was interpreted as corresponding to one phosphate, one GlcN, two 14:0(3-OH) and one 14:0. This determined that 16:0 and 16:0(2-OH), when present, as in *R. mannitolilytica,* were located on the reducing GlcN as well as 14:0(2-OH), when present, as in *R. eutropha* and *R. pickettii*.

3.2.4 The anomeric configuration of the GlcN-I glycosidic phosphate

The anomeric bond was determined to be α by the kinetics of phosphate release compared to the α- and ß-P anomers of synthetic GlcN-3-hydroxymyristic references. The glycose absolute configuration of D-GlcN was determined by GC-MS of L- and D-GlcN reference samples (23).

3.3.Comparison of IL-6 and TNF-α production after LPSs stimulation of THP-1 cells

The pro-inflammatory response to stimulation by all three *Ralstonia* LPS samples was lower than with *E. coli* J5 LPS as shown in Figure 2. All batches showed differences in TNF-α and IL-6 responses. Both cytokine production, as displayed by TNF-α, and IL-6 production respected the same ranking while trending to almost no induction with *R. pickettii*. It was followed by *R. eutropha*, then by *R. mannitolilytica* presenting the closest inducing capacities compared to *E. coli* J5 LPS.

According to the various described structure-activity relationships that we have previously established on other bacterial genera (24, 19, 25-27), we anticipated the lipids A with molecular species close to 6 FA to be the more active, like the *E. coli* lipid A reference is known to be. This is what was observed here with *R. eutropha* and *R. mannitolilytica*, while *R. pickettii* displaying only penta-acyl lipid A molecular species, was a weak inducer. *R. mannitolilytica* presents more of the hexa-acyl molecular species and this explained its higher inducing capacity. The presence of free aminated sugars on the phosphate groups is likely to reduce the important charge interactions between free Phosphate group molecules and the

receptor, and to modulate positively the activity. This would make sense, according to the present data, as the more active lipid A is the one with more substitution on the phosphate groups. This increased activity has already been described by us for *Bordetella pertussis* LPSs (25).

3.4.SDS-PAGE analysis

The three *Ralstonia* LPS profiles were compared after SDS-PAGE electrophoresis in Figure 3A. They were compared to a Smooth-type *E. coli* LPS and to the *B. pertussis* lipo-oligo-saccharide (LOS) as size references. *R. eutropha* LPS migrated at the level of the *E. coli* 0119 LPS with two well-defined specific regions, first with R-type LPSs migrating slightly under those of *B. pertussis* taken as a reference, migrating at 4kDa, and S-type LPSs limited to a few molecular species localized at about 10kDa.

R. mannitolilytica presented different molecular species at the Rough- and Semi-Rough level followed by LPSs with increasing O-chain elements of up to a dozen, with a maximum size at 10kDa. The more intense LPS molecular species were detected at the Semi-Rough level.

R. pickettii LPS molecular species were limited to Rough-type and Semi-Rough type LPSs with no molecular species over 5kDa.

3.5.Transmission Electron Microscopy

At a first glance, two types of membranes can be observed. A thick and dark membrane was found for *R. eutropha* and *R. mannitolilytica,* while *R. pickettii* displayed a thin and light-colored membrane. In addition, much more Outer Membrane Vesicles (OMV) were present between these bacteria and even at their surface, in the process of being generated. The thickness of the membrane was proportional to the size of the corresponding PS present in the LPS molecular species.

R. pickettii also possessed the lipid A molecular species with less FA, which has recently been correlated to the formation of OMVs (27). Another important physico-chemical trait of

these LPS molecules was the fact that most of the lipid A phosphate groups were substituted with AraN, thus reducing the stability of two vicinal molecules via Mg++ bivalent cations. Therefore, the membrane was less stable and more conducive to OMV formation which was observed in Figure 3B.

The present work further demonstrates the importance of the fine structural characterization of LPSs, in this case those of three bacterial species belonging to the *Ralstonia* genus, for the interpretation of biological activities. Three important species of the Ralstonia genus where selected due to the important increase of this genus in the stroma vascular of obese patients (7).

4. **Discussion**

The identification of the precise structure of LPSs is important to support the role of gut microbiota dysbiosis in the low grade inflammation-induced metabolic disease. Although the role of LPSs in the triggering of metabolic disease has been largely described in rodents and humans, several hypotheses could be raised regarding their mode of action (33). The low grade inflammation induced by LPS would depend upon several mechanisms favoring the excessive absorption of these molecules and upon their structure to inflammatory relationships (34). First, LPSs could be absorbed within chylomicrons released by the intestine to the lymph and then to the blood (35). During lipoprotein metabolism they would be exchanged between lipoproteins and released within the blood (36). LPS-binding proteins and sCD14 would prevent LPS from binding to the surface membrane receptor TLR4 that triggers a cascade of intracellular molecular mechanisms leading to the release of proinflammatory cytokines notably, but also by innate immune cells (37). Within the stroma vascular fraction of adipose depots, such cells, as well as others such as endothelial cells, therefore contribute to the low grade inflammation (38). Secondly, through an intestinal bacterial translocation process where bacteria are phagocytized by different intestinal cells

that remain intracellular, mostly within lysosomes, the prokaryotes reach the adipose depots (39). The Gram-negative bacteria can release LPSs to interact with TLR4 triggering, as well as inflammation. Therefore, from this pathway two rate limiting steps can be identified and related to the structure of the LPSs. First, LPS could be involved in the destruction, or not, of the bacteria by the innate immune system within the intestine. The intensity of the pro-inflammatory capacity of LPS to interact with the innate immune cells from the intestine would be a first triggering step to the destruction of the bacteria by phagocytosis within the intestine, and therefore reducing the amount of LPS found within the blood (40). Conversely, it could be suggested that LPS of low pro-inflammatory capacity would not be able to engage the full destruction of the bacteria during the phagocytosis process. The second hypothesis is related to our previous observations that a reduced intestinal defense was responsible for the translocation of gut microbes through the intestine and towards tissues (2,14). Notably the intestinal innate immune cells were unable to activate IL17 secreting cells, such at the Th17 lymphocytes which are involved in the intestinal defense against opportunist commensal bacteria (41). The origin of the reduced intestinal defense was due to dysbiosis, since the transfer of the dysbiotic gut microbiota to germ-free mice induced hyperglycemia (42). Therefore, a low pro-inflammatory LPS would not be able to fully engage the activation of Th17 producing cells, and therefore, be responsible for the leaky gut mechanism leading to bacterial translocation. Altogether, from our studies we could expect that a change in the mucosal microbiota would impair the intestinal immune cells by means of specific structures of LPSs.

It was also shown that acyloxyacyl hydrolase (AOAH) mediated partial LPS deacylation and inactivated almost 80% of a subcutaneous dose of LPS before it could travel to draining lymph nodes (43). When LPS enters the blood stream via the gastrointestinal tract, it is transferred to the liver by the portal venous system. There, Kupfer cells which take most of it

are also the major liver AOAH producers (44). The role of LPSs within the adipose tissue for the control of the low grade inflammation can be proposed, as predicted and demonstrated before from translocated bacteria (45).

5. Conclusion

In this paper we aimed at demonstrating the role of LPS in the low grade level of inflammation observed in patients with obesity and diabetes. Some of the *Ralstonia* LPSs, with less FA and less AraN substituents, display structural characteristics known to leading to lower cytokine inducing capacities. We can now explain, element to element and at the molecular level, the state of low-grade inflammation connected to metabolic diseases.

We showed how under-acylated LPS, like the one characterized in *R. pickettii*, among the other species, increased the formation of OMVs particles production from the bacterial membrane (27). It is clear that such structures, rich in low inflammatory LPS molecules, can cross the intestinal membrane much easily than bacteria can do. The observed increased abundance of *Ralstonia* in patients with metabolic diseases thus strongly support our hypothesis on how the underacylated LPS structures increase and participate to the low levels of inflammation observed in metabolic diseases like obesity and type 2 diabetes.

In addition, as already observed, lipid A structures were shown to be a useful tool in phylogenetic bacterial studies (28-31). It applies here again as the three described structures were found to be significantly different from the *Pseudomonas*-type lipid A, to which *Ralstonia* was first related (32).

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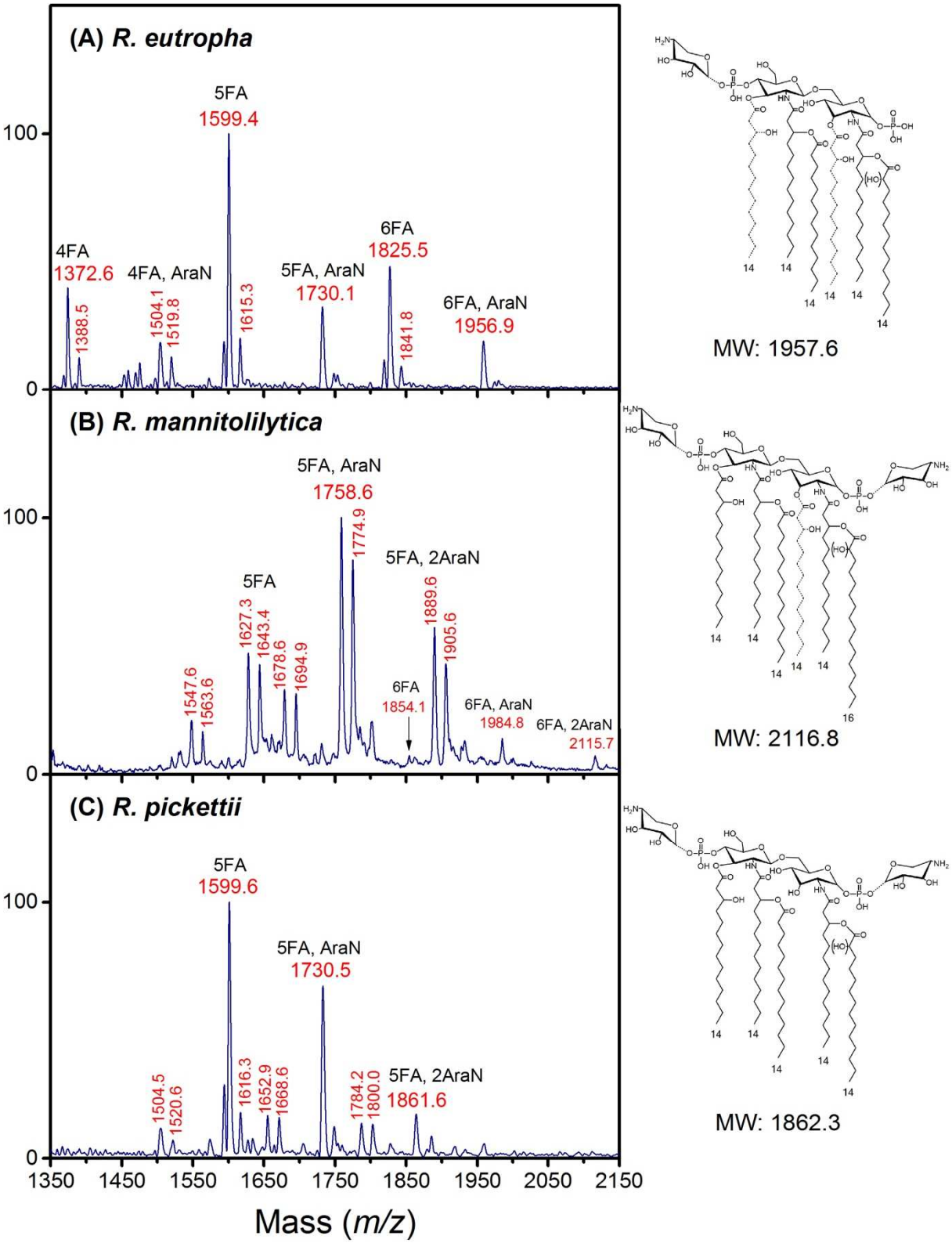
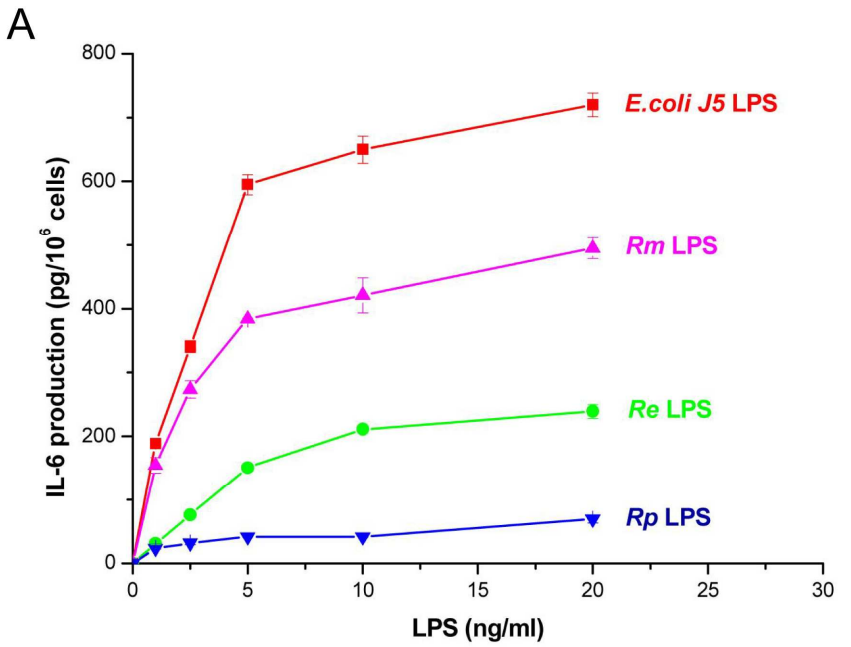
Figure legends

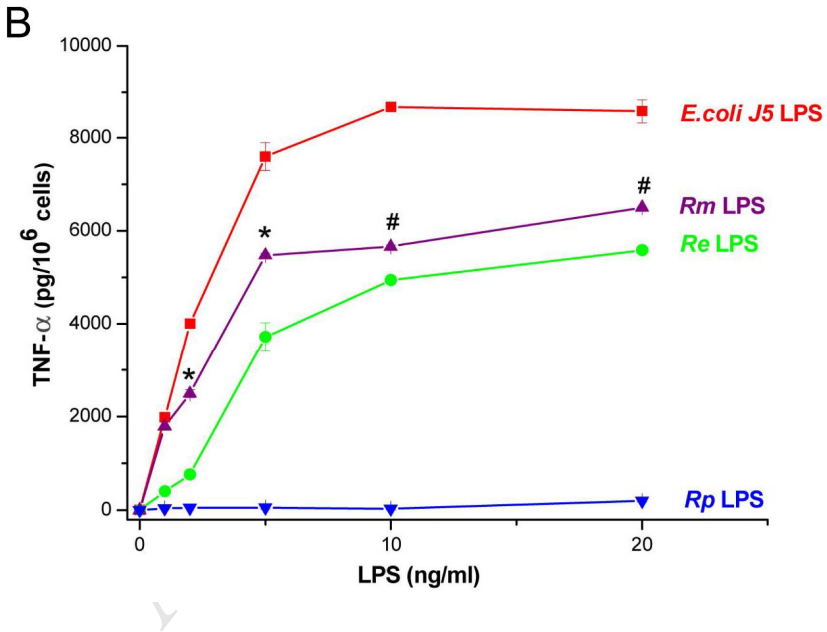
Figure 1. Comparison of lipid A MALDI-MS spectra and corresponding structures of the three *Ralstonia* species. A. *Ralstonia eutropha*, B. *R. mannitolilytica* and C. *R. pickettii*

Figure 2. Comparison of IL-6 (A) and TNF-α (B) production after LPS stimulation of THP-1 cells.

Figure 3. SDS-PAGE displaying the three LPS profiles (A) and electron microscopy (B) of the three *Ralstonia* species. A. *Ralstonia eutropha*, B. *R. mannitolilytica* and C. *R. pickettii*







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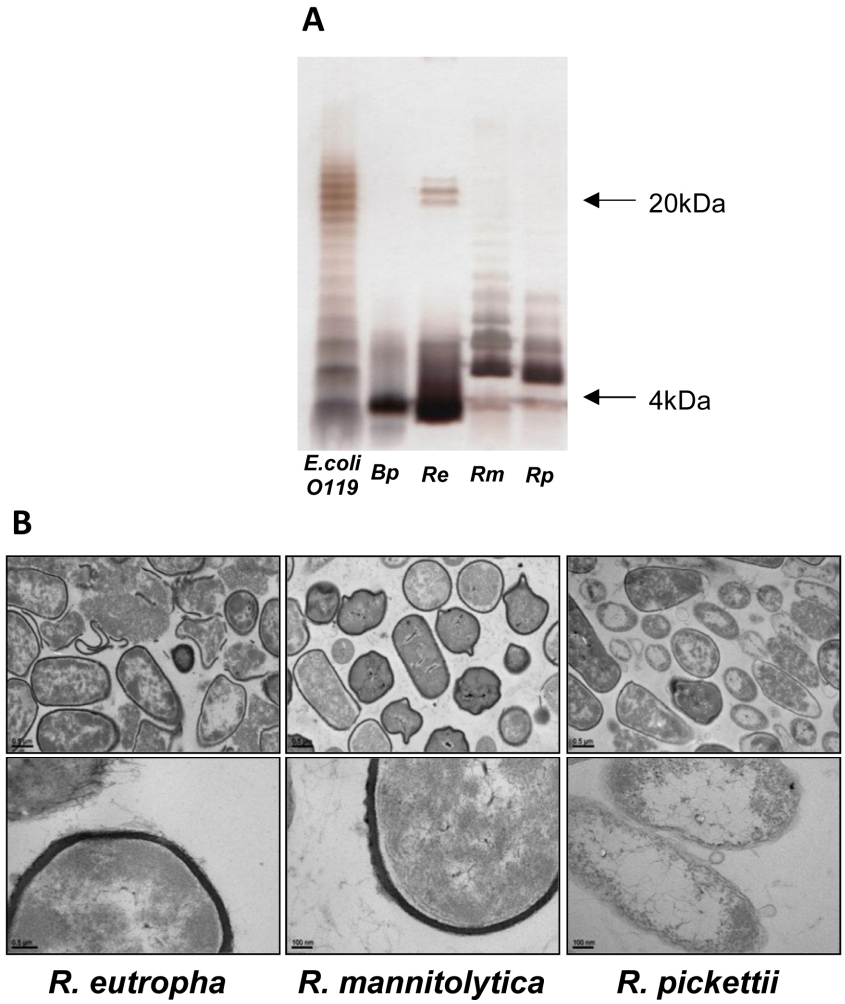

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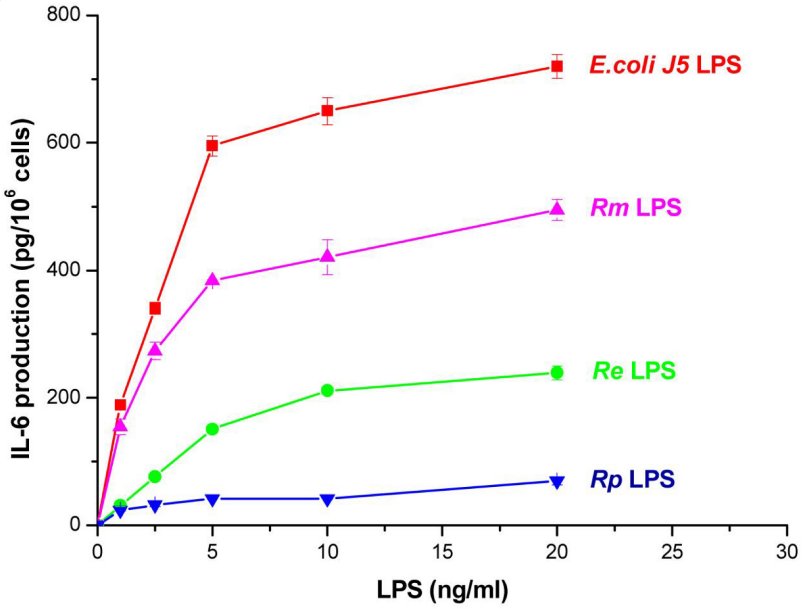

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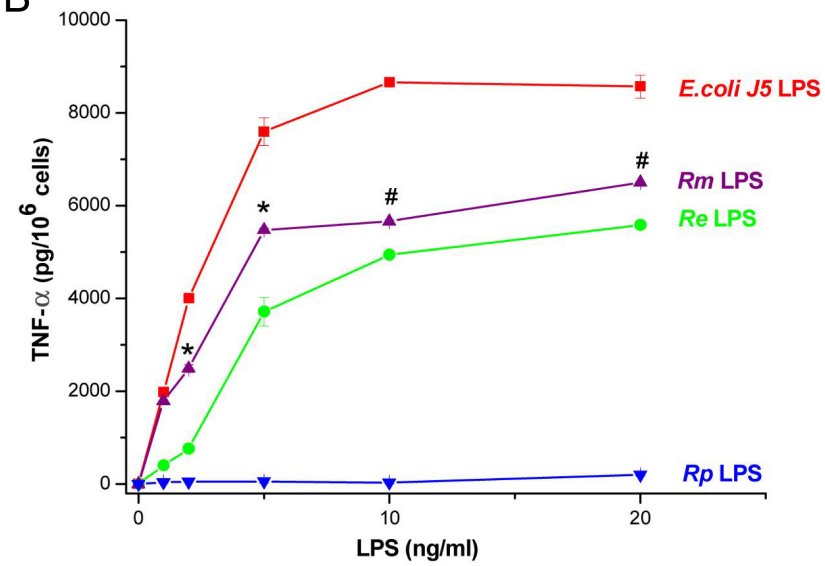

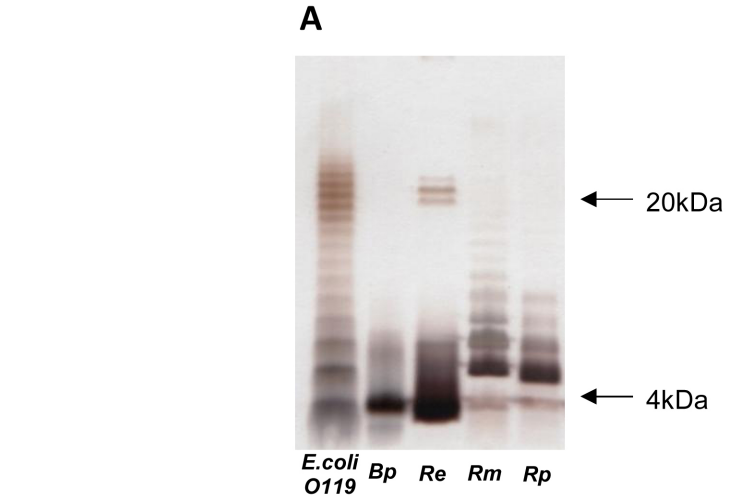

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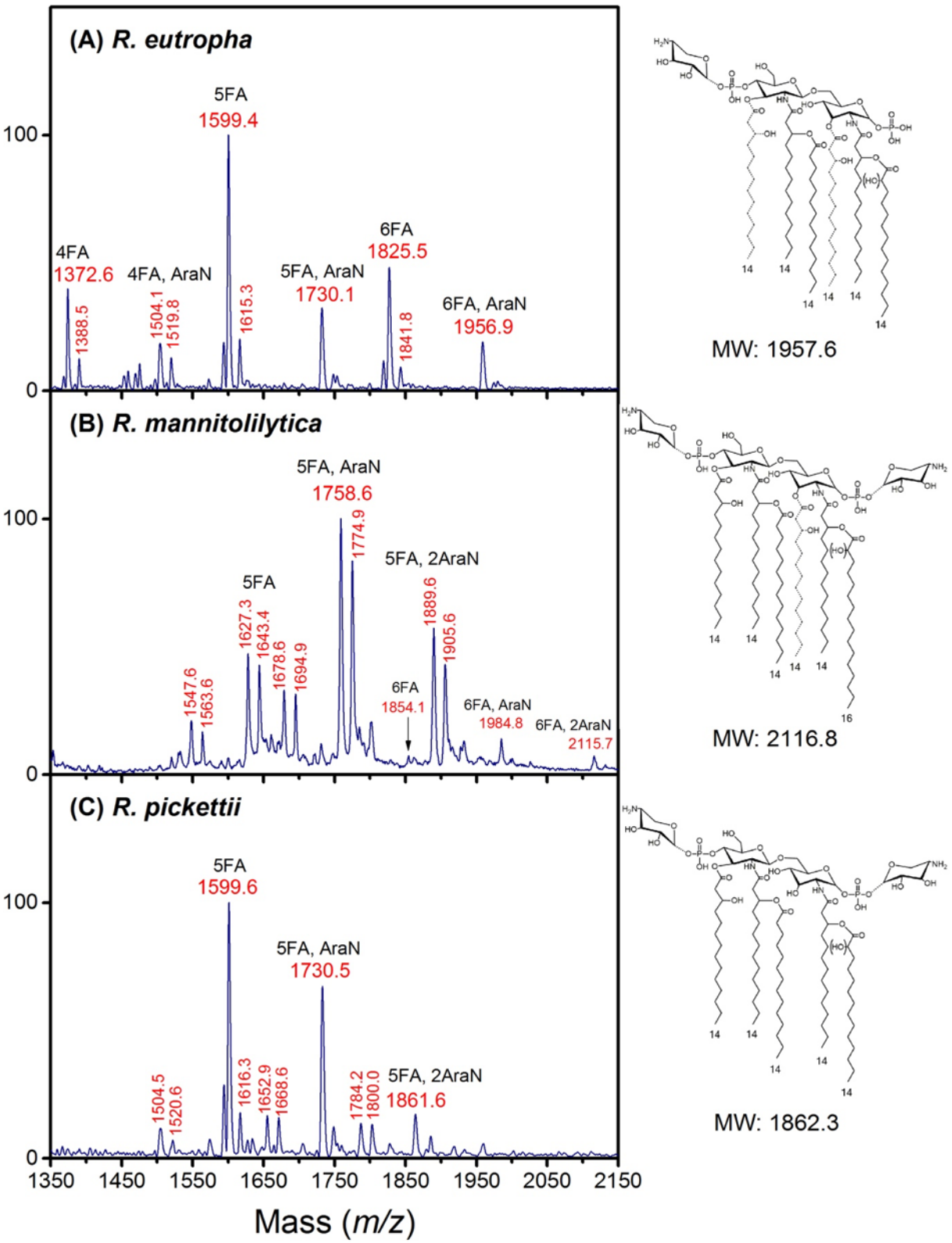
Zhang-Sun, W., Tercé, F., Burcelin, Novikov, A., Sérino, M., Caroff (2019). Structure function relationships in three lipids A from the Ralstonia genus rising in obese patients. Biochimie (159), 72-80. , DOI : 10.1016/j.biochi.2019.01.015



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Highlights

- Characterization of the lipid A structures of *Ralstonia eutropha*, *R. mannitolilytica* and *R. pickettii* was performed in connection to the observed increase of Ralstonia in obese patients.
- The pro-inflammatory activities of the three corresponding *Ralstonia* LPS were tested and compared, explaining the low-grade inflammation level observed in obese patients.
- Transmission electron microscopy performed on the three *Ralstonia* species helps understanding LPS contribution to the bacterial membrane and OMV formation.