Rhamnolipids, Microbial Virulence Factors, in Alzheimer’s Disease

Eleni Andreadou, Anastasia A. Pantazaki, Makrina Daniilidou and Magda Tsolaki

Department of Chemistry, Laboratory of Biochemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece

3rd Department of Neurology, “G. Papanikolaou” General Hospital of Thessaloniki, Aristotle University of Thessaloniki, Greece

Accepted 27 April 2017

Abstract. Alzheimer’s disease (AD) has been attributed to chronic bacterial infections. The recognition of human microbiota as a substantial contributor to health and disease is relatively recent and growing. During evolution, mammals live in a symbiotic state with myriads of microorganisms that survive at a diversity of tissue micro-surroundings. Microbes produce a plethora of secretory products [amyloids, lipopolysaccharides, virulence factors rhamnolipids (RLs), toxins, and a great number of neuroactive compounds]. The contribution of infectious microbial components to the pathophysiology of the human central nervous system including AD is considered potentially substantial, but the involvement of the RLs has never been reported. Here, RLs were isolated from serum and identified through various conventional methods including the colorimetric orcinol method, thin-layer chromatography, attenuated total reflection Fourier transform infrared (ATR-FTIR), and dot blot using antibodies against RLs. Dot blot demonstrated elevated RL levels in sera of AD patients compared to controls (p = 0.014). Moreover, ELISA showed similarly elevated RL levels in cerebrospinal fluid of both AD (0.188 versus 0.080) (p = 0.04) and mild cognitive impairment (0.188 versus 0.129) (p = 0.088) patients compared to healthy, and are well-correlated with the AD stages severity assessed using the Mini-Mental State Examination. These results provide conclusive evidence for the newly-reported implication of RLs in AD, adding it to the list of bacterial components, opening new avenues for AD investigation. Moreover, they strengthen and vindicate the divergence of research toward the exploration of bacterial involvement in AD generation and progression.

Keywords: Alzheimer’s disease, bacteria, blood serum, cerebrospinal fluid, rhamnolipids

INTRODUCTION

Alzheimer’s disease (AD) is a human neurodegenerative disorder that displays several pathological features, including the bundles of amyloid-β plaques in the brain that occur alongside tau neurofibrillary tangles, and exhibits misfolded structure and deteriorated clearance. Some of these characteristics have been causally attributed to microorganisms, which contribute to protracted periods of persistent bacterial infections as already proposed a century ago by Alois Alzheimer and colleagues. Microbes could originate either from the gut microbiota or the oral cavity. The result of this symbiosis is the genesis of the collective genomes of these co-residing microorganisms, named microbiome, which in turn are determined by several factors of the host like hereditary and nutritional ones that possibly affecting host behavior in both healthy and diseased conditions [1]. Furthermore, microbes can survive on blood tissues or relocate in unusual places, a case well described by the Greek terms “dysbiosis: bad-bios (=bad+life)” or “atopobiosis: bios (=life) in other topos (=place+life)”, respectively [1]. This situation may influence the evolution and dynamics of a variety of inflammatory diseases. Microbiome genes within
the human gut microbiota, which significantly outnumber human genes in the body, can produce a plethora of neuroactive proteins/peptides [2].

Microbes may arrange host nutrition and metabolism crucially affecting the evolution and functionality of the immune system [1]. When the composition of the bacterial population is disturbed or modified, or when the host homeostasis becomes unbalanced under certain circumstances, these conditions may constitute causes for the host transition from a healthy situation to a pathogenic one. Studies have demonstrated the inextricable correlation between the diverse forms of neuroimmune and neuropsychiatric disorders with the microbiome variations and the microbiota-derived products [3, 4]. Even local microflora (such as this of the gut) could affect both neighboring and remote locations within the host organism [3].

Recent reports emphasize that the intestinal microflora can affect the outcome of some neurological circumstances, enhancing the occurrence, progression, or deterioration of certain central nervous system (CNS) disorders [5], as well as the extent and the ways in which microbes influence brain physiology, functions, and behavior [4, 6]. A bi-directional relationship linking the gut microbiota and the brain along the so-called the microbiota-gut-brain axis, contributes to the pathogenesis of certain disorders via the involvement of brain inflammation [7].

Microbes under unbalanced nutrient conditions produce an excessive amount of secondary metabolites, which are localized on the outer membrane surface of the Gram-negative bacteria, like amyloids and lipopolysaccharides (LPSs) or secreted components, i.e., exotoxins such as rhamnolipids (RLs), pyocyanin, alginate, proteases, etc. [8–12]. Research highlights the contribution of amyloids and other bacterial components, ligands, and/or products like LPSs, to the appearance or progression of AD [11, 12], even within the developing intestine, which plays a key role in the early programming of the CNS and the regulation of the intestinal innate immunity [13]. Amyloids, despite their sufficient solubility as proteinaceous monomers, can be assembled by auto-aggregation resulting in fibrils, which are often associated with AD pathogenicity [14]. The concentration of endotoxin LPSs in plasma from AD patients increased compared to controls. This increase was reflected in a proportionally-dependent degree of blood monocytes activation, which was positively associated with the genesis of AD [15]. Bacterial cells in the blood of AD patients in dormancy were visualized with ultrastructural microscopy: when iron deficiency has been overcome, it leads to their revivification [16]. The surviving cells are able to expel strongly inflammatory components including LPSs [16]. The imputed role of pathogens in AD has been extensively reported for about over 200 different helical bacterial spirochete species [17], which may co-infect with other Gram-negative phylotypes [18–29].

Among the extensively-studied bacterial infectious components and their inductive role in immune and inflammatory responses, the involvement of RLs as microbiota-derived metabolites in AD has never been accounted for. RLs are glycolipids, initially reported to be produced by P. aeruginosa, but later several other bacteria were added to the list [30–33]. RLs are composed of a gluconic and a lipidic segment, commonly comprising one or two rhamnoses and hydroxyl-substituted fatty acids [33]. RLs production is regulated by the quorum sensing system depending on the bacterial density in the culture medium [33]. They are released from the periplasm toward the extracellular environment and have been characterized as bacterial virulence factors [32]. Several biological activities of RLs emanating from and being attributed to their detergent-like properties have been identified [30, 34], including immune system modulation [34]; promotion of early infiltration of primary human airway epithelia by P. aeruginosa; morphological alterations in the membrane provoking alterations to its integrity especially by causing selective tight-junction changes to the respiratory epithelium [35]; cytotoxicity induced on the fibroblastic cell line [36]; hemolytic activity of erythrocytes [37]; and induction of cyotogenetic damage in human lymphocytes and bind DNA in vitro [38].

Therefore, based on the concept that AD could be attributed to bacterial infections, and given that RLs are bacterial virulence factors secreted by a wide range of pathogens residing in humans, the objectives of this study were: a) to investigate the possible and temporary presence of RLs in blood serum using methods such as thin-layer chromatography (TLC), attenuated total reflection Fourier transform infrared (ATR-FTIR), and immune-detection methods [dot blot and enzyme-linked immunosorbent assay (ELISA)] and antibodies against bacterial RLs; b) furthermore, we endeavored to elucidate whether there are visible differentiations in the quantities of RLs present in serum and cerebrospinal fluid (CSF).
of small cohorts of both mild cognitive impairment (MCI) and AD patients in comparison with normal individuals; and finally c) to evaluate whether the increased amounts of plasma RLs would be reflected proportionally in the clinical AD progressive stages.

MATERIALS AND METHODS

Materials

Antibodies against RLs (anti-RLs) were prepared after immunizing rabbits subcutaneously with RLs, using standard techniques. The rabbits were titrated and evaluated, whereas the utilized RLs were produced, purified from *T. thermophilus* HB8, and validated as described above [37]. The goat anti-rabbit IgG-alkaline phosphatase conjugate was used as secondary antibodies, purchased from Sigma-Aldrich (St. Louis, USA). Nitrocellulose membranes were of the WhatmanTM Company (Protran BA 85, Germany), polystyrene 96 wells plates were of the Greiner BioOne Company (Germany).

Participants

Fifty-nine AD patients, recruited from the Memory and Dementia outpatient clinic of the “G. Papanicolaou” General Hospital of Thessaloniki, Greece, provided serum and CSF samples, following written informed consent. Probable AD was diagnosed according to the NINCDS–ADRDA criteria [39] with the use of neurological, neuropsychological, blood, CSF, and neuroimaging examination. Cognitively normal and not normal individuals were assessed by means of clinical and neuropsychological tests. Serum samples were collected from 18 participants (9 AD and 9 controls) and immediately frozen at –80°C. The two groups were well matched for gender (*p* = 0.637), education (*p* = 0.106), and age (*p* = 0.595) according to the *χ²* and Mann–Whitney test respectively (Table 1).

CSF samples were taken from 41 individuals (21 AD patients, 13 MCI patients, and 7 healthy volunteers, not paired with the sera samples) during a lumbar intervention, which was carried out with a standard protocol, and kept at –80°C. The statistical data concerning CSF samples are given in Table 2.

**Cultivation conditions for RL production**

In this study, bacteria *T. thermophilus* HB8 (or *E. coli*) were first cultivated overnight at 37°C in basal-rich medium [40, 41]. For RL production, the bacteria were cultivated in batch cultivation in mineral salt medium supplemented with 1.5% (w/v) sodium gluconate as sole carbon source [40, 41]. Then, 10% (v/v) of the inoculums of the initially fully-grown cultures of *T. thermophilus* HB8 (or *E. coli*) in basal-rich medium were added in 2-l Erlenmeyer flasks, containing 700 mL medium and subsequently incubated on a rotary shaker at 37°C for 48 h.

**RL extraction from *T. thermophilus* or *E. coli* cultures**

RLs were extracted either from the supernatant *T. thermophilus* HB8 (or *E. coli*) cultures according to our previous report [41] or from blood serum following the same protocol. Specifically, 2 mL of

---

Table 1

Demographic and clinical characteristics of Alzheimer’s disease (AD) and normal groups in serum samples

<table>
<thead>
<tr>
<th>Demographics</th>
<th>AD</th>
<th>Normal</th>
<th><em>p</em>-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>74.7 (7.2)</td>
<td>70.1 (4.1)</td>
<td>0.595</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>4/5</td>
<td>5/4</td>
<td>0.637</td>
</tr>
<tr>
<td>Education, years</td>
<td>6.6 (4.5)</td>
<td>11.7 (3.5)</td>
<td>0.106</td>
</tr>
</tbody>
</table>

The depicted values shown represent mean values, with standard deviations appearing in parentheses. The statistical significance of the differences obtained, was analyzed with the Mann-Whitney (for age and education) and *χ²* (for gender) tests. The *p*-values of less than 0.05 were considered as statistically significant.

Table 2

Demographic and clinical characteristics of Alzheimer’s disease (AD) and normal groups in cerebrospinal fluid samples

<table>
<thead>
<tr>
<th>Demographics</th>
<th>AD</th>
<th>Normal</th>
<th>MCI</th>
<th><em>p</em>-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants</td>
<td>21</td>
<td>7</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>74.3 (7.4)</td>
<td>75.8 (7.9)</td>
<td>69.9 (11.0)</td>
<td>0.586 (AD versus MCI)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>11/10</td>
<td>4/3</td>
<td>4/9</td>
<td>0.217</td>
</tr>
</tbody>
</table>

The depicted values shown represent mean values, with standard deviations appearing in parentheses. The statistical significance of the differences obtained was analyzed with the Mann-Whitney (for age) and *χ²* (for gender) tests. The *p*-values lower than 0.05 were considered as statistically significant. No data were available concerning the educational level of the participants. MCI, mild cognitive impairment.
concentrated culture supernatant or blood serum were adjusted to pH 2 with 5 N H_2SO_4. After adding an equivalent volume of chloroform to methanol ratio (2:1), the liquid was then mixed well with a vortex for 1 min. Thereafter, the mixture was subjected to centrifugation at 10,000 × g for 15 min in order to separate the aqueous from the organic phase and retrieve the latter. This extraction operation protocol was repeated once more. RLs were dissolved in the organic phases, which were pooled and concentrated by rotor evaporation. The quantification of the RLs content was performed indirectly by the determination of rhamnose in the samples according to the spectrophotometric orcinol method and the use of a standard rhamnose curve [42]. Finally, to calculate the amount of RLs, the obtained rhamnose values were multiplied by 3.4 which originated from the RLs/rhamnose ratio (since it was found that 1.0 mg of rhamnose is approximately equivalent to 3.4 mg of RLs) [43].

**Thin layer chromatography of RLs**

Samples containing RLs were analyzed by TLC on silica gel plates (Kieselgel 60/Kieselgur F254; Merck, Darmstadt, Germany). Prior to separation, TLC plates were dried for 30 min at 110°C. The samples were spotted on the plates and separated, employing a mobile phase composed of 80% (v/v) chloroform, 18% (v/v) methanol and 2% (v/v) acetic acid. The TLC plates were sprinkled with a solution in atomizer containing 0.5 g thymol in EtOH-concentrated H_2SO_4 (95:5, v/v). After spraying, the plates were heated for 20 min at 100 to 120°C, and the RLs appeared as pink spots [44].

**Attenuated total reflection Fourier transform infrared spectroscopy of RLs**

In order to identify and confirm the presence of RLs, the sample of RLs isolated from the blood serum was subjected to ATR-FTIR spectroscopy, a kind of FTIR which allows the characterization of compounds in solution, as described previously [45, 46 and references therein]. FTIR analysis of RLs was performed on a Nicolet 6700 FTIR spectrometer (Thermo Scientific, Massachusetts, USA) with an Orbit Diamond Crystal W (SPA-2 Tower for Nexus) in an ATR-Dura test chamber. The sample was dissolved in CHCl_3:CH_3OH 2:1 (v/v); a small quantity (1 drop) was deposited on the ATR diamond, followed by solvent evaporation before taking the spectra width from 600 to 4,000 cm⁻¹ and applying 32 scans as well as a resolution of 4 cm⁻¹.

**Production of anti-RL specific antibodies**

To prepare polyclonal rabbit antiserum against RLs (anti-RLs), rabbits were immunized subcutaneously with purified RLs from *T. thermophiles* HBS, using standard techniques [37], whereas, in our previous work, RLs were produced, purified, and identified from *T. thermophilus* HB8 [36]. All animal procedures were carried out by the Animal Resources Unit of the Aristotle University of Thessaloniki, with the approval of the institutional Animal Ethical Committee.

**Detection of RLs in serum by dot blot immunodetection**

To compare the levels of RLs in serum between AD patients and age-matched controls, 10 µL of 22 sera in total (11 pathological and 11 healthy) were spotted on the nitrocellulose membrane. When the required time elapsed for the binding of proteins onto the nitrocellulose membrane (approximately 1 h), the latter was immersed for 1.5 h in a solution of 5% (w/v) skimmed milk in PBS 1 × (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, and 0.2 g/L KH_2PO_4) for blocking. Each membrane was probed by incubating it with anti-RLs antisera diluted 1000-fold in 5% v/v skimmed milk in PBS overnight at 4°C under shaking. The non-specific-binding primary antibody was removed after washing it three times with PBST (phosphate buffer saline +0.05% w/v Tween-20) for 5 min. The nitrocellulose film was then immersed for 2 h in a solution containing 5% v/v skimmed milk in PBS and an anti-rabbit polyclonal secondary antibody-attached to alkaline phosphatase (1:2,500, v/v). The non-specific-binding antibody was removed with the same washing procedure. The membrane was also washed three times with PBS 1 × for 5 min. The membrane staining was developed using the typical procedure with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) in alkaline phosphatase buffer containing 100 mM Tris pH 9.5, 100 mM NaCl, and 5 mM MgCl_2. The quantification of the spots was done with the Total Lamp Quantification software.
Detection of RLs in CSF by ELISA

To determine the content of RLs in the CSF of AD, MCI patients, and healthy individuals, the ELISA method was performed with the use of antibodies against RLs [37]. Optimization of various parameters of the protocol was achieved by including the concentration of CSF used for coating, dilutions of the primary and secondary antibody as well as assay conditions before the CSF samples determination. Polystyrene 96 wells plates (Greiner BioOne, Germany) were coated overnight at room temperature with 100 μL CSF diluted 1:100 in PBS 1x. After washing three times with PBS 1x, the plates blocked by adding 1% w/v egg albumin in PBS 1x (blocking buffer) for 2 h, at ambient temperature and rinsed carefully with PBS 1x. Each well was then incubated with 100 μL of primary serum (anti-RLs), diluted 1000-fold in blocking buffer, for 2 h at 37°C in agitation, washed 4-times with PBST and treated with 100 μL of alkaline phosphatase conjugated goat anti-rabbit IgG diluted 1000-fold for 2 h at 37°C in agitation. After four washes with PBST, the alkaline phosphatase activity was developed by immersing in 100 μL of p-nitrophenyl phosphate (1 mg/mL, Sigma Co., USA) in 10 mM di-ethanolamine buffer pH 9.8 and 1 mM MgCl₂ for 3 h at room temperature. The optical density (OD) of each reaction was measured at 405 nm using a micro Elisa Reader (BioTek Instruments Inc., USA). Each CSF sample was assayed in duplicate. The immunoreactivity was calculated after subtracting the mean OD values obtained from the human albumin from the mean OD values obtained from the CSF samples.

Statistical analyses

All statistical analyses of the results were carried out using the SPSS 17 software (SPSS Inc., Chicago, IL).

RESULTS

Detection of RLs in blood serum using various methods

The orcinol method

RLs were extracted from blood serum and in parallel they were produced and purified from the supernatants T. thermophilus HB8 (or E. coli) bacterial cultures. They grew in the presence of sodium gluconate, according to the same well-established chloroform extraction protocol [36, 45, 46]. Subsequently, the concentration of RLs was quantified in all samples with the colorimetric orcinol method using a standard rhamnose curve. Surprisingly, the concentration of the blood serum-derived RLs after the chloroform extraction reached as high as 57.8 μg/mL. This finding corroborates another experiment [47], in which RLs were also produced and isolated at an equally high concentration, i.e., 63 ± 10 μg/mL, after blood cultivation for 24 h. The presence of RLs was attributed to the clinical isolates of Pseudomonas strain PA227 [47].

Thin layer chromatography

TLC is usually employed for qualitative or quantitative analysis of RL types [33, 46, 48, 49]. Thus, the presence of RLs in the blood serum isolated sample was further confirmed by TLC, using as positive standards the purified RLs which were isolated from the T. thermophilus HB8 (or E. coli) bacterial cultures grown in sodium gluconate, i.e., the commercial white saponin and rhamnose. RLs originating from blood serum isolation through chloroform extraction gave an RL-specific positive thymol staining, which was concentration-dependent for the RLs, as expected (Fig. 1). Additionally, serum RLs migrated with the same mobility as RLs isolated from T. thermophilus HB8 culture [46] and as saponin white, confirming again the presence of RLs in serum. A similar pattern was observed with E. coli RLs, giving support to the presence of RLs in serum.

Statistical analyses

All statistical analyses of the results were carried out using the SPSS 17 software (SPSS Inc., Chicago, IL).

RESULTS

Detection of RLs in blood serum using various methods

The orcinol method

RLs were extracted from blood serum and in parallel they were produced and purified from the supernatants T. thermophilus HB8 (or E. coli) bacterial cultures. They grew in the presence of sodium gluconate, according to the same well-established chloroform extraction protocol [36, 45, 46]. Subsequently, the concentration of RLs was quantified in all samples with the colorimetric orcinol method using a standard rhamnose curve. Surprisingly, the concentration of the blood serum-derived RLs after the chloroform extraction reached as high as 57.8 μg/mL. This finding corroborates another experiment [47], in which RLs were also produced and isolated at an equally high concentration, i.e., 63 ± 10 μg/mL, after blood cultivation for 24 h. The presence of RLs was attributed to the clinical isolates of Pseudomonas strain PA227 [47].

Thin layer chromatography

TLC is usually employed for qualitative or quantitative analysis of RL types [33, 46, 48, 49]. Thus, the presence of RLs in the blood serum isolated sample was further confirmed by TLC, using as positive standards the purified RLs which were isolated from the T. thermophilus HB8 (or E. coli) bacterial cultures grown in sodium gluconate, i.e., the commercial white saponin and rhamnose. RLs originating from blood serum isolation through chloroform extraction gave an RL-specific positive thymol staining, which was concentration-dependent for the RLs, as expected (Fig. 1). Additionally, serum RLs migrated with the same mobility as RLs isolated from T. thermophilus HB8 culture [46] and as saponin white, confirming again the presence of RLs in serum. A similar pattern was observed with E. coli RLs, giving support to the presence of RLs in serum.

Statistical analyses

All statistical analyses of the results were carried out using the SPSS 17 software (SPSS Inc., Chicago, IL).

RESULTS

Detection of RLs in blood serum using various methods

The orcinol method

RLs were extracted from blood serum and in parallel they were produced and purified from the supernatants T. thermophilus HB8 (or E. coli) bacterial cultures. They grew in the presence of sodium gluconate, according to the same well-established chloroform extraction protocol [36, 45, 46]. Subsequently, the concentration of RLs was quantified in all samples with the colorimetric orcinol method using a standard rhamnose curve. Surprisingly, the concentration of the blood serum-derived RLs after the chloroform extraction reached as high as 57.8 μg/mL. This finding corroborates another experiment [47], in which RLs were also produced and isolated at an equally high concentration, i.e., 63 ± 10 μg/mL, after blood cultivation for 24 h. The presence of RLs was attributed to the clinical isolates of Pseudomonas strain PA227 [47].

Thin layer chromatography

TLC is usually employed for qualitative or quantitative analysis of RL types [33, 46, 48, 49]. Thus, the presence of RLs in the blood serum isolated sample was further confirmed by TLC, using as positive standards the purified RLs which were isolated from the T. thermophilus HB8 (or E. coli) bacterial cultures grown in sodium gluconate, i.e., the commercial white saponin and rhamnose. RLs originating from blood serum isolation through chloroform extraction gave an RL-specific positive thymol staining, which was concentration-dependent for the RLs, as expected (Fig. 1). Additionally, serum RLs migrated with the same mobility as RLs isolated from T. thermophilus HB8 culture [46] and as saponin white, confirming again the presence of RLs in serum. A similar pattern was observed with E. coli RLs, giving support to the presence of RLs in serum.

Statistical analyses

All statistical analyses of the results were carried out using the SPSS 17 software (SPSS Inc., Chicago, IL).

RESULTS

Detection of RLs in blood serum using various methods

The orcinol method

RLs were extracted from blood serum and in parallel they were produced and purified from the supernatants T. thermophilus HB8 (or E. coli) bacterial cultures. They grew in the presence of sodium gluconate, according to the same well-established chloroform extraction protocol [36, 45, 46]. Subsequently, the concentration of RLs was quantified in all samples with the colorimetric orcinol method using a standard rhamnose curve. Surprisingly, the concentration of the blood serum-derived RLs after the chloroform extraction reached as high as 57.8 μg/mL. This finding corroborates another experiment [47], in which RLs were also produced and isolated at an equally high concentration, i.e., 63 ± 10 μg/mL, after blood cultivation for 24 h. The presence of RLs was attributed to the clinical isolates of Pseudomonas strain PA227 [47].

Thin layer chromatography

TLC is usually employed for qualitative or quantitative analysis of RL types [33, 46, 48, 49]. Thus, the presence of RLs in the blood serum isolated sample was further confirmed by TLC, using as positive standards the purified RLs which were isolated from the T. thermophilus HB8 (or E. coli) bacterial cultures grown in sodium gluconate, i.e., the commercial white saponin and rhamnose. RLs originating from blood serum isolation through chloroform extraction gave an RL-specific positive thymol staining, which was concentration-dependent for the RLs, as expected (Fig. 1). Additionally, serum RLs migrated with the same mobility as RLs isolated from T. thermophilus HB8 culture [46] and as saponin white, confirming again the presence of RLs in serum. A similar pattern was observed with E. coli RLs, giving support to the presence of RLs in serum.

Statistical analyses

All statistical analyses of the results were carried out using the SPSS 17 software (SPSS Inc., Chicago, IL).

RESULTS

Detection of RLs in blood serum using various methods

The orcinol method

RLs were extracted from blood serum and in parallel they were produced and purified from the supernatants T. thermophilus HB8 (or E. coli) bacterial cultures. They grew in the presence of sodium gluconate, according to the same well-established chloroform extraction protocol [36, 45, 46]. Subsequently, the concentration of RLs was quantified in all samples with the colorimetric orcinol method using a standard rhamnose curve. Surprisingly, the concentration of the blood serum-derived RLs after the chloroform extraction reached as high as 57.8 μg/mL. This finding corroborates another experiment [47], in which RLs were also produced and isolated at an equally high concentration, i.e., 63 ± 10 μg/mL, after blood cultivation for 24 h. The presence of RLs was attributed to the clinical isolates of Pseudomonas strain PA227 [47].

Thin layer chromatography

TLC is usually employed for qualitative or quantitative analysis of RL types [33, 46, 48, 49]. Thus, the presence of RLs in the blood serum isolated sample was further confirmed by TLC, using as positive standards the purified RLs which were isolated from the T. thermophilus HB8 (or E. coli) bacterial cultures grown in sodium gluconate, i.e., the commercial white saponin and rhamnose. RLs originating from blood serum isolation through chloroform extraction gave an RL-specific positive thymol staining, which was concentration-dependent for the RLs, as expected (Fig. 1). Additionally, serum RLs migrated with the same mobility as RLs isolated from T. thermophilus HB8 culture [46] and as saponin white, confirming again the presence of RLs in serum. A similar pattern was observed with E. coli RLs, giving support to the presence of RLs in serum.
Characterization by ATR-FTIR spectroscopy and interpretation of the absorbance spectrum of the blood serum extracted RLs

The blood serum extracted RLs were also characterized and evaluated by the representative ATR-FTIR spectra (Fig. 2). A strong, characteristic, and wide band of the free hydroxyl-group (–OH) stretch due to hydrogen bonding was observed at 3473 cm⁻¹.

Similarly, the characteristic aliphatic bonds CH₃, CH₂, and C–H stretching as well as the carbonyl (C = O) stretching with strong intensities bands were shown in regions (2925–2856 cm⁻¹) and 1736 cm⁻¹ respectively, which also appeared in characteristic bacterial RL spectra [41, 45, 46]. Moreover, feeble bands were noticed in the positions of pyranyl I and α-pyranyl II adsorption bands in regions at 918–940 cm⁻¹ and 838–844 cm⁻¹, respectively, denoting the presence of di-RLs in the mixture.

These characteristic adsorption peak bands prove that these RLs hold a chemical structure identical to RLs, with rhamnose rings and long hydrocarbon chains. Thus, according to the ATR-FTIR spectrum results, the serum isolated RLs belong to the glycolipid group, which is made up of aliphatic acid and ester. The characteristic peaks marked in the spectrum are attributed to the presence of RLs and are similar to previously reported peaks [41, 45, 46].

Detection of RLs in sera probed with polyclonal antibodies against RLs by dot blot assay

The presence of RLs in the blood serum isolated sample was also confirmed by the immune detection method of dot blot immunoassay, using our prepared polyclonal primary antibody against RLs. Rhamnose and saponin were used as positive reference samples for the isolated RLs from the supernatant of T. thermophilus HB8, which were cultivated in the presence of sodium gluconate as a carbon source (Fig. 3). Both serum RLs and bacterial T. thermophilus HB8 RLs as well as saponin (glycolipid) resulted in positive immune-staining. Rhamnose (sugar) gave no staining with the anti-RLs antibody, which is consistent with the already known specific recognition of the antibody on the lipidic part of glycolipid and not the gluconic part, i.e., rhamnose [37]. Moreover, previous studies provide evidence that among the constituents of RLs, the lipidic part (but not the gluconic like rhamnose) triggers some tremendous biological activities on cells, such as cytotoxicity and hemolytic activity [36, 37].
Detection of RLs in sera from AD patients probed with polyclonal antibodies against RLs by dot blot assay

Based on the detection of RLs in blood, the question raised was whether their levels may vary in the sera of AD patients and whether they could be associated with AD as bacterial metabolites. Therefore, the variation of RLs serum levels between AD patients and healthy participants was compared, by performing a dot blot immune-assay using the antibody prepared against RLs; subsequently, the intensity of immune-staining of the dot blot analysis was quantified. Surprisingly, it was discovered that the levels of RLs in AD patients were elevated as compared to the control group (Fig. 4).

The median value of the RLs immuno-signal was 37,374 versus 25,380. The standard deviation value for AD patients was 14,696.87, while the control group one was 5,902.7. As shown in the Mann–Whitney test (Tables 1 and 3), the increase of RLs levels observed in patients with AD as compared to controls was statistically significant ($p = 0.014$).

This result indicates that the increased concentration of RLs, as bacterial metabolites, in AD patients, revealed a possible abundant microbiota residing temporarily or nearly permanently in these patients, as it was reflected in the increased RLs secretion. Another hypothesis might be the progressively temporary accumulation of RLs without the necessary permanent survival of microbes. Measuring the concentration of RLs may be considered as a potential predictive factor for AD, associated with the bacterial potential.

**Table 3**

Statistical analysis of the immune reactivity of Alzheimer’s disease (AD) and normal groups against rhamnolipids (RLs) in serum samples with dot blot method

<table>
<thead>
<tr>
<th></th>
<th>AD</th>
<th>Normal</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLs</td>
<td>37374.2 (14696.9)</td>
<td>25380.5 (5902.7)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

The depicted values shown represent mean values, with standard deviations appearing in parentheses. The $p$-value lower than 0.05 was considered as statistically significant.
Fig. 5. Immune signal intensity of rhamnolipids (RLs) obtained from cerebrospinal fluid (CSF) samples analysis by ELISA method results were performed using the antibody against rhamnolipids, among Alzheimer’s disease (AD) patients, mild cognitive impairment (MCI) patients, and healthy individuals belonging to the same age group. A volume of 100 μL of CSF samples was diluted with PBS (dilution factor 1:100) and placed in a 96-wells plate.

indirect ELISA to measure the RL levels in CSF. The CSF samples of AD patients (21), MCI patients (13), and controls (7) were used. The CSF samples were taken from different individuals than those who provided the serum samples. The average age of AD patients was 75 years (56–85), of MCI patients 70 years (48–81), and of controls 73.3 years (68–81). Each sample was assayed in duplicate. According to the results depicted in Fig. 5, the RLs levels in the CSF of AD patients were elevated compared to healthy individuals (0.188 versus 0.080). The difference in RL levels between MCI and healthy controls was smaller compared to healthy individuals (0.188 versus 0.129). The p-values of the AD and MCI patients against the control group were 0.04 and 0.088, respectively, while the p-value of the AD patients against the MCI group was 0.023 (Table 4).

The CSF donors of the experiment were assessed based on the Mini-Mental State Examination (MMSE) and were classified in three groups according to the obtained MMSE values: <10, 10–19, and >20, which correlate with the severe, moderate, and mild stages of the disease, respectively. The median values of the RLs levels, which have been determined, were found increased in the patients of the first cohort suffering from severe stage of AD, while gradually were decreased in the two other cohorts of patients suffering from moderate and mild stages of the disease, respectively, but in all cases, they remained higher than those of the control cohort (Fig. 6). It is noteworthy that statistical analysis of these results showed that the MMSE values obtained for the three cohorts of the severe, moderate, and mild stages of AD ranged from <10, 10–19, >20, respectively, were found statistically significant versus normal group (AD MMSE 0–10 versus Normal p = 0.01, AD MMSE 10–19 versus Normal p = 0.016, AD MMSE 20–30 versus Normal, p = 0.038). On the contrary, MCI MMSE values versus normal group values were not statistically significant (MCI MMSE 0–10 versus Normal p = 0.097). No other comparisons between the values gave statistically significant differentiations.

These results are consistent with old data reporting that microorganisms of helical shape were found in the CSF, blood, and cerebral cortex of AD patients while they were absent in the control samples [50]. RLs, characterized as small bacterial metabolites, are expected to circulate in the blood and pass rather easily in the CSF. The increased levels of RLs in AD patients may be well explained by the hypothesis suggesting a microbial population diversity residing within each individual and secreting RLs.

**DISCUSSION**

This article revolves around the idea of the possible microbial embroilment in AD pathology via infection by pathogens, an issue that constitutes a long-standing debate [3, 11, 12, 18–29, 50, 51].

<table>
<thead>
<tr>
<th>ELISA</th>
<th>AD (0.188 (0.0801))</th>
<th>MCI (0.129 (0.0462))</th>
<th>Normal (0.080 (0.071))</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLs</td>
<td>AD versus MCI</td>
<td>AD versus Normal</td>
<td>MCI versus Normal</td>
</tr>
<tr>
<td></td>
<td>0.023</td>
<td>0.04</td>
<td>0.088</td>
</tr>
</tbody>
</table>

The depicted values shown represent mean values, with standard deviations appearing in parentheses. The p-values lower than 0.05 were considered as statistically significant. MCI, mild cognitive impairment.
Fig. 6. Median values of the levels of rhamnolipids (RLs) obtained in the cerebrospinal fluid (CSF) of donor participant groups, which were classified according to their Mini-Mental State Examination (MMSE) score. RL levels in Alzheimer’s disease (AD) and mild cognitive impairment (MCI) groups were compared to those in the normal group. AD groups with MMSE <10, 10–19 and >20 displayed statistically significant RL levels compared to the Normal group (AD MMSE 0–10 versus Normal, p = 0.01, AD MMSE 10–19 versus Normal, p = 0.016, AD MMSE 20–30 versus Normal, p = 0.038). On the contrary, when comparing MCI versus Normal group RLs values, no statistical significance was observed (MCI versus Normal, p = 0.097). Statistical analysis was also performed between the different AD groups and MCI group but no significant correlation was found.

The first question raised is: what is the possible origin of the detected RLs? Except for the numerous strains of Pseudomonas, the most frequent human colonizers, various other bacteria have been reported to produce RLs, including Acinetobacter, Bacilli, Betaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria (Acinetobacter, Enterobacter, Pantoea, Burkholderia) [32]. Numerous non-Pseudomonas URPB, “uncommon”-RLs producing bacteria have also been reported, including both Gram (–), like Pseudoxanthomonas sp. PKN–04 [52], Acinetobacter calcoaceticus, Enterobacter hormaechei, Pantoea stewartii, and E. asburiae [53], and Gram (+) bacteria [54–56].

Notably, some of the RLs producing bacteria are potential pathogens, such as E. hormaechei [57] and E. asburiae [58]. Several Burkholderia species, such as B. thailandensis, B. plantarii, B. pseudomallei, and B. mallei may also produce RLs [59–62]. Among the Burkholderia species, the last two are pathogenic for both humans and animals, and known for their role in the infectious process [61, 62]. Enterobacter, a genus of common Gram (–) gammaproteobacteria of the Enterobacteriaceae family, and a member of the coliform group of bacteria (https://en.wikipedia.org/wiki/Enterobacter) has been reported as an RL producer [32]. Several strains of these bacteria are pathogenic and cause opportunistic infections in immuno-compromised (usually hospitalized) hosts, and in those who are on mechanical ventilation. The urinary and respiratory tracts are the most common sites of infection.

Serratia spp. is an opportunistic pathogen and one of the ten most common causative factors of bacteremia in North America [63]. They are responsible for a variety of infections, including bacteremia, pneumonia, intravenous catheter-associated infections, osteomyelitis, endocarditis, and, rarely, endogenous and exogenous endophthalmitis [64, 65]. The mortality rate from bacteremia due to Serratia spp., 6 months after infection, is 37% [66]. S. rubidaea has also been accounted for as an RLs producer among the Serratia spp. pathogens (http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/serratia-spp-eng.php).

Moreover, Acinetobacter has been reported among the RLs producers. Acinetobacter baumannii is a pleomorphic aerobic gram-negative bacillus (similar in appearance to Haemophilus influenzae) commonly isolated from the hospital environment and hospitalized patients. A baumannii is often cultured from the sputum or respiratory secretions, wounds, and urine of hospitalized patients. In a hospital setting, Acinetobacter commonly colonizes irrigating and intravenous solutions. Acinetobacter species have low virulence but are capable of causing infection in organ transplants and febrile neutropenia. Most Acinetobacter isolates recovered from hospitalized patients, particularly those recovered from respiratory secretions and urine, represent colonization rather than infection (http://emedicine.medscape.com/article/236891-overview). Interestingly, the low frequency phenomenon of RLs production among natural isolates, besides P. aeruginosa or Burkholderia, is concomitant with the lack of orthologues of the genes participating in the RL synthesis (rhl genes), among the plethora of the sequenced bacterial genomes. Probably, the scarcely mentioned cases of RLs-producing bacteria have acquired this feature through horizontal gene transfer either from the pre-mentioned bacteria or from not yet studied ones [67].

The possible contribution of the virulent RLs that derive from microbiota co-residing in humans, in
chronic infections, was first reported for cystic fibrosis. The presence of RLs (up to 8 μg/mL) in saliva specimens, yielded from cystic fibrosis patients, had been attributed to infection by *P. aeruginosa*, and denoted a direct relationship between the increased level of RLs and the clinical deterioration of the patient [68]. The RL amounts found in saliva specimens were much higher (as high as 65 μg/mL) than these detected in secretions of a lung obtained from a cystic fibrosis patient [69].

It is apparent that the RL differences observed in AD patients might be attributed either to the different composition of the microbiota population which displays significant fluctuations, or to alterations in the equilibrium and the content of ordinary microbes. These fluctuations depend on the homeostasis state of the host, which affects the production of fermentation products, including secreted secondary metabolites, virulence factors, toxins, or neurotransmitter molecules. The homeostasis balance may depend on a plethora of factors like nutritional deficiencies, environmental toxins, tenacious bacterial and viral infections, various autoimmune immunological responses, vascular diseases, head injury, and accumulation of fluid in the brain. It has been demonstrated that RLs secretion by bacteria is promoted under unbalanced conditions of homeostasis such as nutrient limitation especially phosphate [41] and nitrogen. This is significant especially in humans, when the host organism is temporarily under specific circumstances of homeostatic imbalance, like nutrients limitation, e.g., phosphate (in hypophosphatemia), nitrogen, etc. Various environmental and alimentary factors are crucial parameters influencing the disease manifestation [53].

Overall, many diseases of chronic inflammation may be attributed to the presence of microbial components such as LPSs [1, 15, 70]. The continuous generation of bacterial components, considered as potent inflammatory agents, as well as their feedback from the pool of the dormant bacteria, may well justify the persistent, but mild-manifested inflammation, followed by inflammatory cytokine production, which is characteristic of many diseases [20]. Moreover, it is alleged that the presence of bacterial antigens, derived from the bacterium *Borrelia burgdorferi* belonging in the spirochete phylum, in concomitance with genes in the brain of AD patients, demonstrate that these patients had undergone chronic Lyme neuroborreliosis [71]. Bacterial antigens and AD-like pathology have been detected in the CNS of BALB/c mice models resulting from intranasal infection due to a strain of *Chlamydia pneumoniae* [20]. An excessive amount of virulence markers encoded virulence-related genes which have been recorded to originate from *Staphylococcus aureus* strains isolated from hemodialysis catheters of Mexican patients; they were held responsible for nosocomial bacterial infections due to biofilm formation [72].

Several bacterial determinants termed ‘pathogen-associated molecular pattern’ (PAMP) of the representative opportunistic pathogen *P. aeruginosa* have been considered responsible for the innate immune system activation in epithelial cells. For instance, the presence of bacterial DNA and RNA leads to an increased expression of pro-inflammatory molecules, which further activates the innate and adaptive immune systems, leading to persistent infection, chronic inflammation, neuronal destruction, and amyloid-β deposition [21]; it is toward this direction that RLs have been implicated in DNA transfer phenomena [38].

Polar lipids of *Burkholderia pseudomallei* like ornithine lipids and RLs induce antibodies production and the most polar stimulate cellular immune response [73]. Moreover, bacterial amyloid proteins after their recognition as PAMP activate the toll-like receptor-2, an intermediate significant factor of the inflammation pathway, further leading to the activation of the nuclear factor kappa B, a main regulator of inflammation, chronic inflammation, neuronal destruction, and amyloid-β deposition [21]; it is toward this direction that RLs have been implicated in DNA transfer phenomena [38].

Polar lipids of *Burkholderia pseudomallei* like ornithine lipids and RLs induce antibodies production and the most polar stimulate cellular immune response [73]. Moreover, bacterial amyloid proteins after their recognition as PAMP activate the toll-like receptor-2, an intermediate significant factor of the inflammation pathway, further leading to the activation of the nuclear factor kappa B, a main regulator of inflammation, chronic inflammation, neuronal destruction, and amyloid-β deposition [21]; it is toward this direction that RLs have been implicated in DNA transfer phenomena [38].
detected and identified in samples from patients with AD. This finding suggests that bacterial sequences represented the 2.3% even in the control CSF sample from an encephalitis-uninfected patient. The bacterial sequences corresponded mainly to *Psychrobacter, Acinetobacter* and *Corynebacterium* genera [82]. A metagenomic survey in the CSF of multiple sclerosis (MS) patients (a common cause of non-traumatic neurologic disability with high incidence in many developed countries) and patients suffering from other type of neurologic conditions, was performed. Bacterial reads were detected in 8 out of the 15 non-MS patients and in a single MS patient, at an abundance >1% of the total classified reads. Two patients were of special interest: one non-MS patient harbored ~73% bacterial reads, while an MS patient had ~83% bacterial reads. In the former case, *Veillonella parvula*, a bacterium occasionally associated with meningitis was the predominant species, whilst *Kocuria flava*, apparently an environmental bacterium, predominated in the latter case. Thirty-four out of 43 samples contained <1% bacterial reads, which was regarded as a cross- or environmental contamination [83]. The involvement of bacteria, the relevance and the mechanisms of infection/inflammation to AD pathogenesis has been recently reviewed [84].

The obtained results provide strong evidence for the association of RLs with AD. The presence of RLs may be well attributed to chronic bacterial infections, which constitute bacterial virulence factors secreted by a wide range of pathogens. In conclusion, these results substantiate the involvement of RLs as bacterial virulence factors in AD, adding them in the list of bacterial constituents after amyloid, LPSs, and other current treatment strategies for Alzheimer’s disease (AD). Moreover, they strengthen and vindicate the turn of research toward the exploration of bacterial involvement in AD generation or progression. However, further studies might be needed to support the suggestion that measuring RL concentration could be considered as a potential and critical predictive factor for AD.

**ACKNOWLEDGMENTS**

We thank the Memory and Dementia Outpatient Clinic of the “G. Papanicolaou” General Hospital of Thessaloniki as well as the Greek Alzheimer’s Association and Greek Federation of Alzheimer’s Disease created by M.T. for accessing and sampling AD patients. We thank D. Giagkas for the preparation of antibodies against RLs in a previous proposal. We also thank Prof. T. Choli-Papadopoulou for her valuable suggestions.

Authors’ disclosures available online (http://j-alz.com/manuscript-disclosures/16-1020r3).

**REFERENCES**


[16] Pretorius E, Bester J, Kell DB (2016) A bacterial component to Alzheimer’s-type dementia seen via a systems biology approach that links iron dysregulation and


