Dietary Pectin–Derived Acidic Oligosaccharides Improve the Pulmonary Bacterial Clearance of *Pseudomonas aeruginosa* Lung Infection in Mice by Modulating Intestinal Microbiota and Immunity

Henry Bernard, Jean-Luc Desseyn, Nana Bartke, Lennart Kleinjans, Bernd Stahl, Clara Belzer, Jan Knol, Frédéric Gottrand, and Marie-Odile Husson

1Unité INSERM 995, Faculté de médecine, Université Lille 2, France; 2Nutricia Research, Utrecht, and 3Laboratory of Microbiology, Wageningen University, The Netherlands

**Background.** A predominantly T-helper type 2 (Th2) immune response is critical in the prognosis of pulmonary *Pseudomonas aeruginosa* infection. But the mucosal and systemic immune responses can be influenced by the intestinal microbiota.

**Methods.** We assessed the effect of microbiota compositional changes induced by a diet enriched in 5% acidic oligosaccharides derived from pectin (pAOS) on the immune response and outcome of chronic pulmonary *P. aeruginosa* infection in mice.

**Results.** pAOS promoted Th1 polarization by increasing interferon γ release, upregulating *t-bet* gene expression, decreasing interleukin 4 secretion, and downregulating *gata3* gene expression. pAOS also sustained the release of keratinocyte chemoattractant, recruited polynuclear leukocytes and macrophages, stimulated M1 macrophage activation and interleukin 10 release, and decreased tumor necrosis factor α release in the lung. These effects led to increased bacterial clearance after the first and second *P. aeruginosa* infections. pAOS modified the intestinal microbiota by stimulating the growth of species involved in immunity development, such as *Bifidobacterium* species, *Sutturella wadsworthia*, and *Clostridium* cluster XIVa organisms, and at the same time increased the production of butyrate and propionate.

**Conclusion.** These results suggest that pAOS may have beneficial effects by limiting the number and severity of pulmonary exacerbations in patients chronically infected with *P. aeruginosa*, such as individuals with cystic fibrosis.

**Keywords.** prebiotics; intestinal microbiota; Th1 and M1 polarization; *P. aeruginosa* lung infection.
preliminary clinical data have suggested that M2 activation indicates declining pulmonary function [6]. These observations suggest that skewing of the Th1/Th2 balance toward a predominantly Th1 response might be beneficial for patients with cystic fibrosis infected by *P. aeruginosa*.

Studies have demonstrated that immune-modulating functions of nondigestible carbohydrates such as short-chain galactooligosaccharides (scGOS) and long-chain fructooligosaccharides (lcFOS) simulate the function of human milk oligosaccharides (HMOS). Defined as prebiotics [7], these carbohydrates stimulate the growth of bifidobacteria and lactobacilli [8], which induce Th1 polarization [9] and regulatory T cell (Treg) responses [10]. In addition, they are degraded by intestinal microbiota in beneficial short-chain fatty acids (SCFAs), such as butyric and propionic acid, which exert immune effects by interfering with T-cell differentiation [11] and Treg functions [12].

We hypothesized that, by modulating the intestinal microbiota, acidic oligosaccharides derived from pectin (pAOS) could shift the Th1/Th2 balance toward a predominantly Th1 response and would improve the outcome of *P. aeruginosa* pulmonary infection. To test this hypothesis, we fed BALB/c mice with a control diet or a diet enriched with 5% pAOS for 5 weeks and we compared the intestinal microbiota and fecal SCFA concentrations. We then induced *P. aeruginosa* chronic pulmonary infection and compared inflammatory markers, Th1 markers, M1 markers, survival, and bacterial clearance during the first 4 days of infection. Surviving mice were reinfected 2 weeks later before comparing specific immunoglobulin G (IgG) levels and pulmonary bacterial clearance. Our results showed that the pAOS diet, by modulating gut microbiota, induced a shift of the Th2/Th1 balance to a Th1 response and induced M1 activation that improved the pulmonary bacterial clearance after a first and a second infection.

**MATERIALS AND METHODS**

**Mice and Nutrition**

Male BALB/c mice (Harlan, Gannat, France) were randomized at 3 weeks of age into 2 groups fed for 35 days. The diet was a AIN-93G rodent diet [13] into which either 5% pAOS extracted from citrus or 5% cellulose were incorporated (Nutricia Reformulate, Utrecht, The Netherlands). The 2 diets were identical in energy, protein, mineral, micronutrient, and vitamin content. All experiments were conducted following the French Guide for the Care and Use of Laboratory Animals and the Guidelines of the European Union.

**Intestinal Microbiota Analysis and Fecal SCFA Concentration**

Fecal samples from 3 mice of each group housed in separate cages were collected at the beginning of the study and each week thereafter for 5 weeks and stored immediately at −80°C. The microbiota were analyzed using the Mouse Intestinal Tract Chip (MITChip) as described previously [14, 15]. The data were analyzed using a set of R-based scripts (https://github.com/microbiome) in combination with a custom-designed database run under the MySQL database management system (http://www.mysql.com) as described previously [16, 17].

SCFA concentrations were measured concomitantly by solid-phase microextraction headspace gas chromatography with flame ionization detection. Frozen samples (30–50 mg), glass beads, and 960 µL of demineralized water were placed in the headspace tube, which was crimped with a polyfluorotetraethylene/silicone-lined aluminum cap. Twenty microliters of an aqueous standard solution (2-ethylbutyric acid, 100 µg/1 mL) and 20 µL of 5% HCl were added, and solid-phase microextraction was performed in a TriPlus Autosampler (Agilent Technologies, Waldbronn, Germany) under magnetic stirring at 80°C for 20 minutes. Fiber desorption was performed at 220°C for 3 minutes in an Agilent 6890N gas chromatograph. For gas chromatography with flame ionization detection, the conditions were as follows: a fused silica capillary column coated with DB-FFAP (30 m, 0.25 mm, 0.25 µm, Agilent J&W Scientific); an oven temperature of 50°C for 1 minute, which was increased at 10°C/min until reaching 180°C and then decreased at 40°C/minute until reaching 220°C, holding the final temperature for 2 minutes; hydrogen at 40 cm/minute as the carrier gas; and flame ionization detection at 260°C. After each sample run, the solid-phase microextraction fiber was conditioned for 20 minutes at 270°C. SCFAs were identified by comparing the peak retention times between the sample and external standards: acetic acid (C2:0), propionic acid (C3:0), butyric acid (C4:0), isobutyric acid (C4:0 iso), valeric acid (C5:0), and isovaleric acid (C5:0 iso; all from Sigma-Aldrich, Germany). Data acquisition and analysis of the results were performed using Agilent ChemStation software. All analyses were performed in triplicate.

**Lung Infection and Lung, Spleen, and Bronchoalveolar Lavage (BAL) Fluid Collection**

*P. aeruginosa* strain PAO1 was grown at 37°C in tryptic soy broth (bioMérieux, France) for 14 hours and entrapped in agar beads as described previously [18]. Mice were anesthetized mildly by sevoﬂurane inhalation (Abbott, UK) and placed in dorsal recumbency, and 50 µL of agar beads containing either 5 × 10^5 *P. aeruginosa* or 0.9% saline solution was administered by endotracheal insertion of a 24-G animal feeding needle. Groups of 5 animals per time point were euthanized on days 1–4 after the inoculation, and spleens and lungs were excised aseptically. Spleens were frozen immediately at −80°C for quantitive real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis. Lungs were homogenized in 1 mL of RNase-free water. Part of each homogenized lung sample was stored at −20°C for measurement of cytokine concentrations, another part was frozen at −80°C for qRT-PCR, and a third part...
was used for bacterial counts. BAL fluid was collected as described previously [18]. Neutrophils and macrophages were counted on cytospin slide preparations stained with Wright–Giemsa coloration, and the supernatant was collected and immediately frozen at −20°C for measurement of cytokine concentrations. Surviving mice were reinfected according to the same protocol 2 weeks after the first infection. As described above, the mice were euthanized on days 1–4. The lungs were collected for bacterial counts, and blood was collected via cardiac puncture for measurement of IgG concentration.

qRT-PCR
Total RNA from frozen tissues was extracted using an InviTrap Spin Tissue RNA kit (Eurobio, France). RT was performed using a Thermoscript Reverse Transcriptase kit (Invitrogen, France), and assays were performed using the ABI Prism 7000 (Applied Biosystems, France) sequence detection system, with SYBR green as the fluorescent intercalant. Sequence primers were as follows: for \( t\)-bet, 5′-GCAAGGACGGGGAATGTTC CCA-3′ and 5′-TCCACACTGCACCTGGCC-3′; for gata3, 5′-AAGGCTGTCGGCAGAAGGA-3′, and 5′-AGACCGGGT CCCCATAGCGT-3′; for nos2, 5′-TTGAGGCCCTTGTGC AGCCCT-3′ and 5′-AAGGCCAGGCGACATGCAA-3′; for arg1, 5′-GTGGAGACCACAGTCTGGCAGT-3′ and 5′-GGAC ACAGGTGTCCCATGCAG-3′; and for \( \beta\)-actin, 5′-GCCAC AGTTCGCCCATGGA-3′ and 5′-AATACAGCCCGGGAG CATCG-3′. The reaction mixture contained 5 µL of complementary DNA, 12.5 µL of SYBR green PCR master mix (Applied Biosystems, France), and 300 mmol/L of either primer. qRT-PCR analyses were performed in duplicate, and results were normalized against the expression of \( \beta\)-actin as an endogenous control. Data are expressed as fold-changes in expression for infected mice at each day, compared with values for the uninfected controls.

Measurement of Cytokine Concentrations
Concentrations of keratinocyte chemoattractant (KC), tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)), and interleukin 10 (IL-10) were measured in filtered BAL fluid, and concentrations of interleukin 4 (IL-4) and interferon \( \gamma \) (IFN-\( \gamma \)) were measured in homogenized lung samples, using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems and CliniSciences, France).

Serology
Concentrations of \( P. \) aeruginosa–specific IgG1, IgG2a, and IgG2b were measured in serum by using ELISAs in microplates (Maxisorp Nunc, France) coated with 100 µL of \( P. \) aeruginosa antigens (Statens Serum Institute, Denmark). Serum samples were applied in serial dilutions (1:100 to 1:3200) and incubated for 2 hours at room temperature. IgG concentrations were measured with specific horseradish peroxidase-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b. The reaction was developed with \( o\)-phenylenediamine and stopped with \( H_2SO_4 \). Absorbance was read at 450 nm on a microplate reader (Mithras, LB940, Bertold Technologies).

Statistical Analyses
To assess the diversity in the total microbiota and subgroups, we calculated the Simpson reciprocal index of diversity and Shannon diversity index. The difference in diversity was compared over time within the pAOS and control groups and between the 2 groups, using the Wilcoxon rank sum test for the Shannon diversity index. Multivariate analysis was performed using Canoco5 software (http://www.canoco5.com/). Significant differences between bacteria at the genus level were assessed with the Wilcoxon rank sum test, with adjustment of \( P \) values for multiple comparisons. SCFA concentrations are expressed as means and were compared between groups at various times, using the Mann–Whitney \( U \) test. The inflammatory markers, immune parameters, and bacterial load are expressed as medians and were compared over time within the pAOS and control groups, to assess their evolution, and between the 2 groups at the different times, using the Mann–Whitney \( U \) test. The statistical software GraphPad Prism 5.0 was used. A \( P \) value of <.05 was considered statistically significant. Cumulative survival rates were compared using the log-rank test.

RESULTS

Intestinal Flora Analysis

Microbiota Analysis
To assess the extent to which the pAOS could modify the intestinal microbiota, we used the MITChip to profile the microbial composition. Hierarchical cluster analysis of the microbial profiles based on Pearson product–moment correlation coefficients (\( r \)) indicated separation of the control and pAOS-treated mice. On day 0, the pAOS-treated mice clustered with the control samples. Within the pAOS-treated mice, separation occurred between early (days 7, 14, and 21) and later (days 21, 28, and 35) time points. This pattern was not seen in the control samples and shows the microbiota adjustment period in the pAOS group. Several bacterial species were significantly affected by diet and the duration of intervention and are plotted in a heat map (Figure 2).

Supervised clustering of samples indicated a clear partitioning between control and pAOS-treated groups and explained 29.2% of the total data variance (Figure 2). This analysis also showed the adjustment period and the separation of early and late time points within the pAOS-treated mice. The separation was associated with SCFA production and certain bacterial groups. pAOS promoted a significant increase in \( E. \) coli, \( A. \) Allobaculum species, \( S. \) Sutterella wadsworthia, \( B. \) Bacteroides vulgatus, \( B. \) Bifidobacterium species, \( C. \) Clostridium difficile, \( C. \) Clostridium ramosum, \( C. \) Clostridium sphenoides, and unclassified \( C. \) Clostridium.
Figure 1. Hierarchical clustering shown in a heat map of the Mouse Intestinal Tract Chip profiles of samples from mice fed the control diet or the 5% acidic oligosaccharides derived from pectin (pAOS) diet after 7, 14, 21, 28, and 35 days of dietary intervention. Red spots represent upregulated and blue spots downregulated bacterial species found in mice fed either of the diets. The darkness of the spots corresponds to the bacterial abundance in the sample. Abbreviation: et rel., and related.

Figure 2. Correlation between microbiota composition, diet, and fecal concentrations of short-chain fatty acids (SCFA). In the redundancy analysis, the SCFA concentrations and mice fed either the control diet or the 5% acidic oligosaccharides derived from pectin (pAOS) diet were used as variables. Black circles represent individual mice in the pAOS group, and gray circles represent individual mice in the control group. The size of each circle increases with the duration of dietary intervention. The responding bacterial subgroups differed significantly between groups. Abbreviation: et rel., and related.
XIVa organisms similar to *Anaerostipes caccae*. Another major change that could be observed after pAOS treatment was a significant decrease in microbial diversity.

**SCFA Concentrations**
The concentrations of butyric acid, propionic acid, and other SCFAs except acetic acid were significantly higher in the feces of mice fed the pAOS diet, compared with control mice, after 5 weeks (Supplementary Figure 1). This higher production could result from bifidobacteria abundance, since bifidobacteria produce butyric and propionic acid, and is supported by the correlation we observed (Figure 2).

**Inflammatory Response**
The inflammatory response was affected by the diet only after *P. aeruginosa* infection (Figure 3). The neutrophil count was
significantly lower in the pAOS-infected mice, compared with the control mice, on day 1 ($P < .01$; Figure 3A); neutrophil recruitment remained stable during the first 3 days and then decreased significantly on day 4 in the pAOS group ($P < .01$). By contrast, the neutrophil count and recruitment decreased from days 1 to 4 in the control group ($P < .01$). The kinetics of macrophage recruitment was similar in the 2 groups (Figure 3B), but the number of macrophages was significantly higher in the pAOS group, compared with the control group, for the first 3 days of infection ($P < .01$). The KC concentration remained stable during the first 2 days in pAOS-treated mice but decreased significantly after day 1 in control mice ($P < .01$); the KC concentration was higher on day 2 in mice fed the pAOS diet, compared with the control group. The peak of IL-10 release was 3-fold higher in the pAOS group than in the control group ($P < .05$; Figure 3E).

Immune Response

**T-Helper Immune Response**

The T-helper response was first assessed by measuring the kinetics of IFN-γ (Th1) and IL-4 (Th2) release and the ratio of IFN-γ/IL-4 release in infected lung tissues (Figure 4A). It was not affected by the diet alone (Figure 4). In pAOS-treated mice, a significant increase in the IFN-γ concentration ($P < .01$) on day 2 and a decrease in the IL-4 concentration on days 2 ($P < .05$) and 4 ($P < .01$) led to an increase in the IFN-γ/IL-4 ratio ($P < .01$), indicating Th1 polarization. In comparison, the decrease in the IFN-γ concentration from days 2 to 4 ($P < .01$) and the stable IL-4 concentration led to a decrease in the IFN-γ/IL-4 ratio ($P < .05$) during the course of infection in mice fed the control diet, indicating Th2 polarization. However, the IL-4 concentration increased on days 1 and 3 in the pAOS group. This increase might have been caused by the presence of *P. aeruginosa*, which can stimulate the Th2 immune response through the secretion of the N-3(oxododecanoyl)-l-homoserine lactones [19, 20]. Thus, the T-helper response was also assessed by comparing the t-bet (Th1) and gata3 (Th2) messenger RNA (mRNA) levels and the t-bet/gata3 ratio in the spleen, where there were no bacteria on or after day 2 (Figure 4B). The higher mRNA level of t-bet ($P < .05$) and the lower mRNA level of gata3 ($P < .05$) led to a higher t-bet/gata3 ratio in the pAOS group, compared with the control group ($P < .01$), thus confirming the Th1 polarization.

**Macrophage Activation**

Macrophage activation was assessed by measuring the mRNA levels of nos2 (M1) and arg1 (M2) and the nos2/arg1 ratio in the lungs (Figure 5A) and comparing these levels with those
in the spleen (Figure 5B). In the lungs of the pAOS group, the nos2 mRNA level increased significantly during the first 2 days (P < .01) and the arg1 mRNA level remained stable until day 3. This led to an increased nos2/arg1 ratio on day 2 (P < .01), indicating M1 activation. By contrast, in the lungs of the control group, nos2 mRNA and arg1 mRNA levels increased until day 3, and the nos2/arg1 ratio remained stable, suggesting no significant M1 or M2 activation. Similar conclusions could be drawn when spleen samples were analyzed. A higher nos2 mRNA level was observed in the pAOS group on days 2 and 3 (P < .01, vs the control group), and a higher nos2/arg1 ratio was observed in the pAOS group and on days 2 (P < .01 vs the control group) and 3 (P < .05, vs the control group).

**First and Second P. aeruginosa Infection**

The survival rate and pulmonary bacterial clearance were measured after the first infection and 2 weeks later, after reinfection of the mice that survived the first infection. After the first infection, animals died between days 2 and 4, but the survival rate did not differ between the 2 groups (Supplementary Figure 2). No animal died after the second infection.

Bacterial clearance was more efficient and rapid in the pAOS group, as demonstrated by the absence of bacteria for 3, 2, and 2 mice on days 2, 3, and 4, respectively, in the pAOS group. By contrast, all mice in the control group remained infected until day 3. The pAOS group had a lower bacterial load after day 2 during the first infection (P < .05; Figure 6A). The number of bacteria decreased more rapidly during the second infection in the 2 groups but was significantly lower in the pAOS group, compared with the control group (P < .01; Figure 6B).

The T-helper immune response was assessed during the second infection by measuring P. aeruginosa–specific IgG1 level (Th2) and IgG2a and IgG2b levels (Th1). pAOS did not affect the P. aeruginosa–specific IgG1 level, but the IgG2a and IgG2b levels increased on day 2 (P < .05), confirming Th1 polarization after the infection (Supplementary Figure 3).

**DISCUSSION**

Oral administration of pAOS for 5 weeks in BALB/c mice modified the intestinal microbiota composition and fecal SCFA production. In turn, this lead to modification of the immune response to chronic P. aeruginosa lung infection by inducing a shift in the Th2/Th1 balance toward a Th1 response and M1 activation. Th1 polarization suggests a greater abundance of T cytotoxic lymphocytes active against infected cells, and the M1 activation suggests an increase in macrophage phagocytic activity. pAOS administration also influenced the inflammatory response, as shown by sustained KC release, neutrophil and macrophage recruitment, reduced TNF-α release, and increased IL-10 release. The Th1 polarization and M1 activation associated with the sustained recruitment of phagocytes led to a reduction in the bacterial load in the lungs after both the first
and second infections. The increase in IL-10 concentration in the airways is important because IL-10 counteracts the effects of inflammatory cytokines on disease progression and is probably involved in the reduction in TNF-α release.

The strong modulating effect of pAOS on gut microbiota probably explains the observed their immune-modulating function. pAOS strongly stimulated the growth of bacteria with immune properties, inducing the growth of bifidobacteria, which can shift the Th2 response toward the Th1 response [21] and promote Treg activity [22], and the growth of S. wadsworthia and unclassified Clostridiales XIVa organisms, which also have immune functions. S. wadsworthia is a sulfite-reducing gram-negative bacterium that is usually stimulated by bile and a milk-fat-based diet that increases the production of bile acids as a fat emulsifier [23]. Recent studies have reported that pure bacterial lysates of S. wadsworthia can stimulate immune cells to produce Th1 cytokines in vivo and in vitro [24]. Clostridiales XIVa organisms stimulate the Treg population and induce IL-10 release, and oral administration of Clostridium XIVa organisms attenuates the symptoms of disease in models of colitis and allergic diarrhea [25]. There is no clear evidence that B. vulgatus has direct immune activity, but lipopolysaccharide from B. vulgatus has immune activity similar to that of B. fragilis [26], and lipopolysaccharide from B. fragilis induces the production of IL-10 by both Tregs and lamina propria macrophages [27, 28]. pAOS also increased the amount of butyric acid, propionic acid, and other SCFAs, such as isovaleric acid, in feces. These SCFAs are the products of carbohydrate fermentation by intestinal species. A. caceae and bifidobacteria can produce butyric and propionic acid [29, 30], and pAOS also stimulate the growth of Allobaculum species, which also produce butyrate [31]. The increase in the content of these 2 SCFAs is important because after crossing the intestinal barrier, they can interfere with T-cell differentiation [11], induce IL-10 production by Tregs [12], and increase the antimicrobial activity of macrophages [32, 33]. Thus, pAOS appear to act as a prebiotic, and their properties may reflect their effects on the intestinal growth of bacteria that can interact directly with immune cells in the intestine or produce butyrate and propionate.

We cannot exclude the possibility that the beneficial effects of pAOS occur independently of their effects on intestinal microbiota. A previous study showed that pAOS derived from different pectins can act directly on immune cells and promote Th1 polarization [34]. To act directly on immune cells, pAOS must cross the intestinal barrier. This hypothesis is supported by a recent report showing the in vitro transfer of pAOS and scGOS/lcFOS through a Caco-2 monolayer [35]. Human-milk-derived oligosaccharides were reported in the urine of infants fed with human milk at a concentration compatible with their effect on T cells [36]. pAOS may also act directly on β-galactoside-binding proteins or galectins present at the surface of intestinal epithelial or dendritic cells [37].

Our results add to previous findings demonstrating the influence of nondigestible oligosaccharides such as lcFOS, scGOS, and HMOS on the systemic immune response. These compounds upregulate the growth of probiotic Lactobacilli species and bifidobacteria known to Shift the Th1/Th2 balance toward a Th1 response, stimulate the Treg population [38, 39], and increase the amount of butyrate and propionate in feces [31]. Their oral administration can correct immune disorders associated with the upregulation of the Th2 response, which appears as allergic symptoms [40, 41], increase the virus-induced delayed-type hypersensitivity response by increasing the Th1-dependent response [42, 43], and protect mice against virus and bacterial infections [44–46].

In conclusion, we are the first to demonstrate that oral administration of pAOS strongly influences the susceptibility to P. aeruginosa pulmonary infection by acting on systemic immunity and that the effects of pAOS on the host are mediated by gut microbiota. The immune properties of pAOS include a shift...
in the Th1/Th2 balance toward the Th1 response, increased recruitment and activation of phagocytic cells, and control of the inflammatory response. Pretreatment with P AoS improves the outcome of P. aeruginosa pulmonary infection by increasing bacterial clearance in mice after both primary infection and reinfection. Our results support the oral administration of P AoS as a promising treatment that may help reduce the number and severity of P. aeruginosa exacerbations, and they suggest the need for human trials to confirm the beneficial properties of P AoS in patients with cystic fibrosis and chronic P. aeruginosa infection.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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F. G. and M.-O. H. designed the experiments. H. B. conducted the research on mice. C. B. and L. K. analyzed the intestinal microbiota. N. B. analyzed the SCFAs. H. B. and all authors analyzed the data. H. B. and M.-O. H. wrote the manuscript. M.-O. H. had primary responsibility for the final content. H. B. and J.-L. D. checked the statistical data. F. G. and N. B. had primary responsibility for scientific advice and reading. All authors read and approved the final manuscript.

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