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# Microbiome composition of airborne particulate matter from livestock farms and their effect on innate immune receptors and cells

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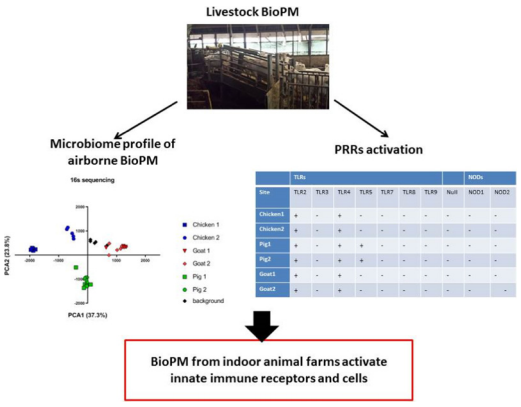
## HIGHLIGHTS

- Airborne farm microbiota profiles grouped according to the animal farm.
- The BioPM contained mainly ligands for TLR4 resulting in a dose-dependent increase of cytokine secreted by MM6 cells.
- Only the pig-derived BioPM induced TLR5 activation
- Coarse BioPM induced similar cytokine production by MM6 cells compared to the fine BioPM fraction

## GRAPHICAL ABSTRACT

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## ABSTRACT

Patients with respiratory diseases in rural areas have been reported to have enhanced responsiveness to ambient particulate matter (PM). In addition to the physical and chemical components, ambient PM can contain microorganisms or parts thereof, referred here as BioPM, that can also contribute to the adverse health effects. This study aimed to characterize the microbial composition of BioPM originating from livestock, and to investigate whether these BioPM can trigger the activation of innate receptors and cells. Coarse ($PM_{2.5–10}$ μm) and fine ($PM_{<2.5}$ μm) BioPM samples were collected from indoor chicken, pig and goat farms using the versatile aerosol concentration enrichment system (VACES) connected to a Biosampler. The fungal and bacterial communities were assessed with an amplicon based approach using Next Generation Sequencing (NGS). In parallel, HEK-Blue cells expressing different pattern recognition receptors (Toll like receptors (TLR) 2, 3, 4, 5, 7, 8, 9 and NOD 1, 2) and a human monocytic cell line (MM6) were exposed to BioPM samples from these sites. Distinct airborne microbiota profiles associated with the corresponding animal farm were observed. Moreover, the various BioPM contained mainly ligands for TLR2 and TLR4 resulting in a concentration-dependent increase of pro-inflammatory cytokine secreted by MM6 cells. In addition, we show for the first time that only the pig-derived BioPM induced TLR5

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activation. These findings suggest that animal farm specific BioPM trigger distinct inflammatory responses, which may contribute to airway diseases in humans.



## 1. Introduction

Epidemiological studies indicate that particulate matter (PM) in ambient air is associated with adverse health effect including respiratory diseases (Cassee et al., 2013; EPA, 2009; EPA, 2004; Holgate, 2008; Pope III et al., 2009), and that the physicochemical properties can affect the potency and type of response (Cassee et al., 2013). Both fine (<2.5 μm, Mass Medium Aerodynamic Diameter (MMAD)) particles and coarse (2.5–10 μm, MMAD) particles play a major role in triggering aggravated asthma or chronic bronchitis (Farina et al., 2013; Santibañez et al., 2013). Vehicular (road traffic) emissions are an important source of PM especially in urban areas. The impact of PM from other sources such as biomass burning and livestock farms are less well understood though it contributes to air quality as well (Steenhof et al., 2011; Stein et al., 2016). Working and residing at close proximity to areas with high density of livestock farms is associated with adverse respiratory health effects (McClendon et al., 2015; Radon et al., 2007) although opposite finding have also been reported (Borlée et al., 2018). Respiratory effects can be caused by inhalation of heterogeneous mixture of PM from livestock that contains pollutants like hydrogen sulphide, ammonium salts, volatile organic compounds, and endotoxins (Cambra-López et al., 2010). PM can contain microorganisms or parts thereof, which comprise of bacterial cells and cellular fragments, fungal spores and fungal hyphae, viruses, and by-products of microbial metabolism (Douglas et al., 2018; Schaechter, 2009), which is here referred to as BioPM. Although there are several studies describing the effect of the physical and chemical components of ambient PM on immune responses (Kroll et al., 2013; Shi et al., 2006) there is limited information on the microbial composition of BioPM, their effect on the immune response and hence the consequences for respiratory diseases.

A better insight in the composition of the air microbiome would therefore substantiate our understanding of the contribution of microorganisms or their components present in ambient air on respiratory diseases. Most of our understanding of airborne microorganisms has been obtained from the study of bacteria and fungi that can be cultured in selective culture media. However, cultivable microorganisms make up only a small fraction of the total airborne microbial composition (Behzad et al., 2015). Molecular typing techniques allow the detection of uncultivable microorganisms in the air, but are often restrict to a few (pathogenic) species. Therefore, recently developed next generation sequencing (NGS) technologies are promising, since it provides the opportunity to profile entire microbial communities from complex air samples, in a cost-effective and reproducible way (Barberán et al., 2015).

Knowledge on which microorganisms are present in the different livestock farms BioPM and their effect on innate immunity is essential towards understanding how specific environmental exposure contribute to the occurrence or worsening of respiratory diseases. Several mechanisms have been proposed to explain the pathogenesis of allergic inflammatory diseases, including the involvement of innate immune responses (Camateros et al., 2006; Feleszko et al., 2006). The innate immune response is of crucial importance for the early containment of infection and the induction of adaptive immunity to pathogens (Pasare and Medzhitov, 2003). However, exposure to external factors such as airborne microbes can influence the type and intensity of innate immune responses, which in turn can contribute to the generation of inflammatory diseases such as asthma (Holgate, 2008).

One of the central components of the innate immune system is the family of pattern recognition receptors (PRRs) which are present either on the cell surface or intracellularly in various (innate) immune cells (Mogensen, 2009). These PRR can recognize the presence of microorganisms by interacting with unique microbial molecular structures, the so-called pathogen-associated molecular pattern (PAMP). Activation of PRR by PAMPs results in a cascade of events leading to activation of transcription factors such as Nuclear Factor-ĸ-gene Binding (NFĸB) and subsequent cytokine production.

Exposure to BioPM derived from livestock farms may predispose workers and residents to respiratory diseases or worsen them. However, little is known about the ambient air microbial composition (external microbiome) and its effect on the immune response. In this study, we aimed to characterize the microbial composition of indoor air of animal farms and to determine the effect that these microorganisms or their components have on innate immune receptors and cells. NGS technology has been applied to profile entire microbial communities from complex BioPM samples and used dedicated cell lines to study immunological consequences.

## 2. Methods

### 2.1. BioPM sampling period and sites

Ambient indoor coarse (2.5–10 μm, MMAD) and fine (<2.5 μm, MMAD) BioPM were collected at seven sites in the Netherlands during July 2016 to July 2017. The sampling sites include six indoor sites (inside or very near the exhaust ventilation pipes of two chicken farms, two pig farms, two goat farms) and a background non-livestock outdoor site (National Institution for Public Health and the Environment (RIVM)). All sites were located in the central region of the Netherlands. Per site, sampling was carried out for 2–6 days and for 6 h per day (between 09:00–16:00 h) in order to collect sufficient material. The daily collected BioPM from each site were pooled in order to carry out in vitro studies. For the background non-livestock site, the maximum concentration of BioPM that could be collected after 6 days sampling was 0.1 μg/ml, which was lower than what was collected for the animal farms (Table 1). Indoor coarse BioPM samples collected at chicken farm 2, on June 2012 were used as reference BioPM for all experiments to correct for possible plate and day variation.

### 2.2. Sampling procedure

The Versatile Aerosol Concentration Enrichment System (VACES) was used to collect BioPM in fine size fraction and coarse collection for the in vitro studies. The BioPM was collected in water at a flow rate of 110 L/min. The procedure to use VACES to sample PM has been described extensively by Kim et al. (Kim et al., 2001). In short, a single-nozzle virtual impactor was used to collect coarse fraction, whereas the fine fraction was collected by drawing air samples through two parallel lines. The fine size fractions go through a saturation-condensation

**Table 1**
Mean value concentration of BioPM collected with the VACES device from different animal farms.

| Site | Date | Coarse ($PM_{2.5-10}$) (μg/ml) | Fine ($PM_{<2.5}$) (μg/ml) |
|---|---|---|---|
| Chicken 1 | 3,8 May | 7850.0 | 3288.0 |
| Chicken 2 | 11–13 July | 17,305.0 | 3782.5 |
| Pig 1 | 3,5,11,19 Jan | 986.0 | 745.0 |
| Pig 2 | 4,5,6,11,13 Apr | 673.0 | 154.0 |
| Goat 1 | 3,9,14–16,28 Feb | 520.0 | 400.0 |
| Goat 2 | 15–17 May,12–14 Jun | 618.0 | 95.0 |

system, which grows particles to 2–3 μm droplets, and then concentrate them by virtual impaction. The concentrated output flow from the virtual impactors is connected to a liquid impinger (BioSampler, SKC West Inc., Fullerton, CA) in order to yield highly concentrated liquid suspensions. The coarse particles do not require to be drawn trough a saturation-condensation system as they are able to penetrate directly into water. Before each use, MilliQ water was used to fill the impinge for the coarse mode.

In parallel, BioPM for bacterial profile studies were collected with Micro-Orifice Deposit Impactors (MOI™; 100-R) on filters (Teflo, R2PJ047/R2PJ037, Pall, Port Washington, NY, USA) as previously described (Marple et al., 1991). These precision cascade impactors are designed for sampling and collecting size-fractionated particle samples. The MOI has a sampling flow rate of 30 l/min and are provided with additional stages to size-fractionate aerosol particle samples. The 8-stage 100-R has a lower cut-size of 0.18 μm. Only four stages (<1, 1–2.5, 2.5–5.62, 5.62–10 μm, MMAD) were used in this study.

The VACES, BioSampler and MOI were cleaned prior to use for each sample collection with ethyl alcohol (70%) and $H_2O_2$ (3%).

### 2.3. DNA isolation

DNA was extracted from environmental air samples that were collected on a filter with the MOI procedure, using magnetic beads. In short, filters were incubated for 1 h in a mixture of NucliSens lysis buffer diluted 2× in PBS. After centrifugation for 15 min at 1000g, the supernatant was collected and spiked with controls for both DNA extraction and qPCR amplification. Subsequent DNA extraction was performed with the NucliSens Magnetic Extraction Kit (bioMérieux, France) according to the manufacturer's protocol.

### 2.4. Library preparation

Amplicon libraries were generated for respectively bacteria and fungi using a 2 step PCR approach. The primer sets for bacteria targeted the V4 (515F-813R) hypervariable region of the bacterial 16S-rRNA gene, while the primer sets for fungi targeted the ITS3-4 (ITS3F- ITS4R) hypervariable Internal transcribed spacer in the ribosomal RNA subunit of fungi (Supplementary table 1). These primers contained the Illumina Nextera Adapters overhang at the 5′ end, which are compatible with the Illumina Nextera XT indices. First PCR amplifications were carried out with the following 25 μl reaction mixture: 1× Phusion HF Buffer, 3% DMSO, 0.25 mM of each dNTP, 1 μM of each primers, 0.5 U Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific), and 10 ng template DNA. The following program was used for amplification: 95 °C for 3 min for initial melting; 20 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min. Amplification products were cleaned using Agencourt AMPure XP PCR purification beads (Beckman Coulter) according to the manufacturer's recommendations. DNA concentration and fragment size were measured on a QIAxcel using a DNA screening kit (Qiagen, Venlo, Netherlands). PCR2 amplification was carried out using the Nextera XT Index Kit according to the manufactures instructions to generate indexed paired-end libraries. In short, this PCR contained 1× Phusion HF Buffer, 3% DMSO, 0.25 mM of each dNTP, 1 μM of each Illumina P5 and P7 index primer, 0.5 U Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific), and 5 μl PCR products from the first PCR. Thermal cycling was carried out at 95 °C for 3 min, followed by 8 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. These PCR reactions were again cleaned via a magnetic bead clean-up and run on a QIAxcel to measure size fragment and DNA concentration as described above.

### 2.5. Sequencing and data analysis

Prior to sequencing, amplicons of the individual samples were equimolar pooled and the PhiX Control v3 (Illumina) was added to the pool at 10% of the final concentration. Paired-end sequencing was performed on a MiSeq (Illumina, San Diego, CA, USA), using MiSeq Reagent Kit v3 (2 × 300 bp Paired-End Reads, 25 Gb output) according to the manufactures' instruction. Raw bcl files were processed using RTA v1.18 (MiSeq). De-multiplexing of the data based on the Illumina index reads was carried out using bcl2fastq v.2.17, which also converted the raw data to FASTQ files.

16S rRNA and ITS amplicon analysis was performed using the mothur software package version 1.35.1 ((Schloss et al., 2009) using the mothur Standard Operating Procedure pipeline for Illumina MiSeq data with minor modifications. Briefly, after forming contigs from the paired-end reads, PCR primers were trimmed off and any sequence with a homopolymer >8 bases, with ambiguous base calls or reads >450 bp were eliminated from further processing. Chimeras were removed with UCHIME (Edgar et al., 2011). Finally, bacterial reads were classified to the species level using SILVA reference database release 128 (Quast et al., 2012). The fungal sequences were assigned to phylum and genus-level phylogeny based on the fungal deposited in the GenBank database (Findley et al., 2013)

### 2.6. HEK-blue cell lines and stimulation

To determine which human PRRs are activated by the BioPM, concentrations of the various farm BioPM samples ranging from 0.39 to 50 μg/ml and a background site BioPM (0.1 μg/ml) were incubated with different Human embryonic kidney (HEK)-Blue cell lines. Each cell line expressed a single PRR (TLR 2, 3, 4, 5, 7, 8, 9 or NOD 1, 2) (InvivoGen, San Diego, California, USA). Parental HEK-Null cells were also used to control for endogenous PRR activation. All cell lines contained a NFκB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. PRR signaling leads to the expression of the reporter SEAP gene, and the activity of the secreted enzyme can be detected in culture supernatants upon addition of the substrate Quanti-Blue (InvivoGen). The levels of SEAP activity are therefore, indicative of PRR activation. The HEK-Blue cells were cultured as previously described (Brummelman et al., 2015). Briefly, HEK-PRR cells were incubated with increasing concentration of farm BioPM o/n in a volume of 200 μl DMEM medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS (Gibco, Maryland, USA)) and 1% penicillin-streptomycin (Gibco, Maryland, USA), in 96-wells plates at 37 °C in humidified air containing 5% $CO_2$. The BioPM derived from the RIVM background site was used only at one concentration. Each HEK-PRR cell lines were also incubated with the indicated concentrations of their respective ligands, as positive controls: Pam3CSK4 (TLR2, 10 ng/ml), polyIC (TLR3, 1000 ng/ml), lipopolysacharide (LPS) (TLR4, 100 ng/ml), flagellum (TLR5, 100 ng/ml), imiquimod (TLR7, 1000 ng/ml), ssPolyU (TLR8, 1000 ng/ml), OND2006 (TLR9, 500 ng/ml), Ie-DAP (NOD1, 1000 ng/ml) and MDP (NOD2, 1000 ng/ml) (all from InvivoGen). PRR activation was expressed as OD value of SEAP activity induced by the BioPM normalized against the SEAP activity of the reference BioPM that was included in every experiment to correct for possible plate and day variation.

### 2.7. MM6 cell line and stimulation

To determine the effect of the different BioPM sources on activation of innate immune cells, a monocytic (MM6) cell line was used. The MM6 cells were cultured as previously described (Bart et al., 2010; Brummelman et al., 2015). Briefly, MM6 were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS (Gibco, Maryland, USA) and 1% penicillin-streptomycin (Gibco). MM6 cells ($5 \times 10^5$ cells/ml) were incubated in the same medium with increasing concentration of farm BioPM collected from different animal farms (two chicken farms, two pig farms, two goat farms) and supernatants were collected after 24 h. Interleukin (IL)-6 was used as a readout for the activation of MM6 cells and measured using an ELISA (eBioscience,

Vienna, Austria) according to the manufacturer's instructions. TLR ligands were added at the concentrations described above. Blocking polyconal antibodies against TLR2 (PAb2, 1 μg/ml), and TLR5 (PAb5, 1 μg/ml) and the TLR4 antagonists LPS from Rhodobacter sphaeroides (LPS-RS, 10 μg/ml) (all from InvivoGen) were added for 2 h prior and during stimulation in order to examine the role of these TLRs on MM6 activation by the different BioPM. All experiments were performed on three separate days. The IL-6 production was expressed as pg/ml induced by the BioPM derived from various farms normalized to the IL-6 production induced by coarse BioPM samples collected at chicken farm 2 on June 2012 as reference BioPM for all experiments, to correct for possible plate/day differences.

### 2.8. Statistical analysis

GraphPad Prism 7.04 (GraphPad Software, Inc.) was used for statistical analysis. Unpaired *t*-test was used to compare SEAP activity or IL-6 induction between medium and positive controls, or when using the TLR blocking antibodies. Difference in the level of SEAP activity and cytokines induced by different BioPM concentration groups were analyzed with One-way Analysis of Variance (ANOVA), followed by post-hoc test (Dunnett's test). All the differences were considered to be statistically significant at $p \leq 0.05$.

## 3. Results

### 3.1. BioPM sampling characteristics

Characteristic features of the collected BioPM for each site and the sampling durations are shown in Table 1. For all BioPM size ranges, the lowest mass concentrations were measured at the RIVM background site (0.1 μg/ml), whereas the highest were measured at chicken farm 2 (17.3 mg/ml). For the in vitro experiments described below on measuring innate receptors and cell activation the BioPM from the different farms were tested at different concentrations.

### 3.2. Airborne microbiome profile in the stable

16 s amplicon sequencing was applied to characterize the bacterial composition in size-resolved BioPM collected in stables of chicken, pig and goat farms as well as a non-farm related location. A total of 257 bacteria at the taxonomic level of genus were detected using the filters based collection system (Supplementary Table 2A). Profiles from both coarse and fine fractions were incorporated into a principal component analysis (PCA), which demonstrated that bacterial profiles from farms grouped according to animal type, irrespective of the size fraction (Fig. 1A). Although the microbiome profile from the chicken farms,

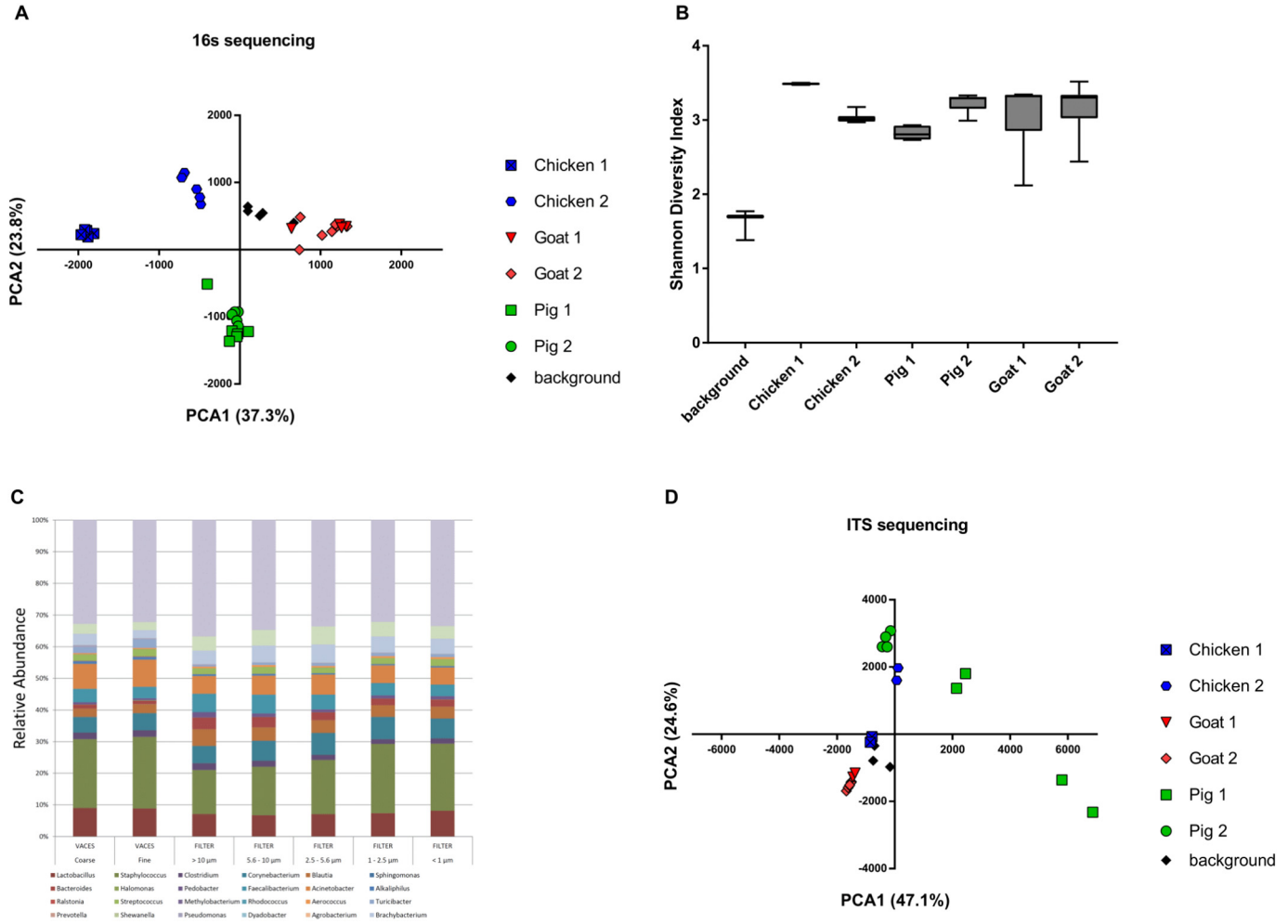


**Fig. 1.** Composition of BioPM bacterial profile in different locations A) Composition of the airborne bacterial profile in two pig, two pig, goat farms and a non-farming reference location. Principal components analysis (PCA) plot characterizes distinct bacterial profiles associated with the type of animals present. Each point represents a sample, each color represents an individual farm. B) Boxplot showing the Shannon-diversity index at the species level for all the sampling locations C) Comparing the relative abundance of bacteria at the genus-level for BioPM collected with the MOI and the VACES. D) PCA analysis on fungal profiles contained by ITS sequencing.

clustered away from the other animal farms, a difference between the two farms was observed. To estimate the relative diversity at each sampling location, we calculated Shannon-Wiener diversity index at the species level classification for all size fractions. Fig. 1B demonstrates a higher diversity in the stable of the different farms compared to a non-farming reference background, but no major differences between the animal farm types. To confirm that there was no bias in the collection method with respect to the observed bacterial communities in the BioPM, we compared the bacterial profile of BioPM collected using the filter based method and the VACES. In Fig. 1C, no difference can be observed between the two collection methods. Since no major difference in bacterial composition was observed between the different fractions, the fine fractions of each farm were used for the comparison of bacterial communities between the farms. Fig. 2 shows the relative abundance for genus-level taxa that represent at least 5% of the bacterial community in one or more samples. The airborne bacteria at the poultry farms are dominated by *Lactobacillus* and *Staphylococcus*, whereas *Lactobacillus* and *Clostridium* are highly abundant in pig farms. *Pedobacter*, *Rhodococcus* and *Sphingomonas* are major constituents of airborne microbiome in goat farms only. Bacterial profiles at the species-level were also generated and are listed in Supplementary Table 3. Among these, several potential pathogenic bacteria were identified including *Streptococcus bovis, Serratia entomophila, Aerococcus viridans,* and *Corynebacterium xerosis.*

In parallel, a similar amplicon based technique was applied to profile the fungal community using Internal Transcribed Spacer (ITS)-profiling. In contrast to 16 s profiling of the v4-variable region, the read length for ITS region was highly variable ranging from 281 to 634 bp. These fungal sequences were assigned to phylum and genus-level phylogeny, 90% of all the phyla belong to the Ascomycota and Basidiomycota. In total, 239 unique genus were identified in at least one of the samples. A complete list of all genus identified can be found in supplemental Table 1B. In line with observations on bacterial composition, PCA analysis of the ITS data at the genus-level indicates that the composition of fungal community is also farm type related (Fig. 1D). The relative abundance for genus-level fungi that represent at least 5% of the community in one or more samples is given in Fig. 2B. Dominant fungal taxa that are consistently present in both sites of a given animal-farm are *Ascochyta* and *Cryptococcus* (goat), *Eurotium* (chicken), and *Emericella* (pig). *Emericella* species that are highly abundant in indoor air of pig farms species are the sexual states of *Aspergillus* species.

### 3.3. Activation of human TLR2, 4 and 5

To determine which PRRs could be activated by the different BioPM, a collection HEK-Blue cell lines expressing individual PRRs were used. All HEK-Blue cells responded as expected when stimulated by their respective PRR ligands (Fig. 3). Furthermore, no endogenous PRR activation on the HEK-Blue cells was observed, as no SEAP activity was measured in the parental untransfected HEK-Blue-Null after stimulating with any of the PRR ligands or BioPM (data not shown).

Our findings indicated that all fine BioPM derived from the livestock farms activated TLR2 and TLR4 on HEK-Blue cells in a dose dependent manner. Only BioPM derived from the pig farms activated TLR5 as well (Fig. 3A–G). When comparing the effect on PRR activation by the two animal farms tested, significant differences in SEAP activity was observed for BioPM derived from all farms. Comparable findings were obtained when using coarse BioPM derived from chicken/pig/goat farms (Supplemental Material, S Fig. 1). When comparing the effect of PRR activation by fine vs coarse BioPM from all animal farms no significance differences was observed except for a stronger activation of TLR2 and TLR4 by the fine compared to coarse BioPM in chicken farm 2 (Fig. 3A, B and Supplemental Material, S Fig. 1 A, B).

### 3.4. IL-6 production by MM6 cells

In addition to using the HEK-Blue cells expressing individual PRRs to screen for innate immune receptors activation by BioPM, the monocytic MM6 cell line expressing simultaneously multiple PRRs (Huang et al., 2009) was also used to measure innate immune cell activation.

Both fine and coarse fraction (Fig. 4A and Supplemental Material S Fig. 2) of the BioPM collected from all animal farms induce IL-6 secretion by MM6 cells in a dose-dependent manner. Significant difference was observed in IL-6 production induced by the fine BioPM derived from the two chicken farms. The level of IL6 induced by the goat farms BioPM was lower compared to that induced by the other animal farms. Four samples (pig and goat farms, 25 and 50 μg/ml) were not included due to too their low BioPM concentration.

### 3.5. Blocking TLRs in MM6 cells

When using the HEK-TLR cell lines, all BioPM samples activated TLR2 and TLR4. In order to investigate the role of these TLRs in MM6 activation by BioPM, MM6 cells were treated with a blocking antibody against

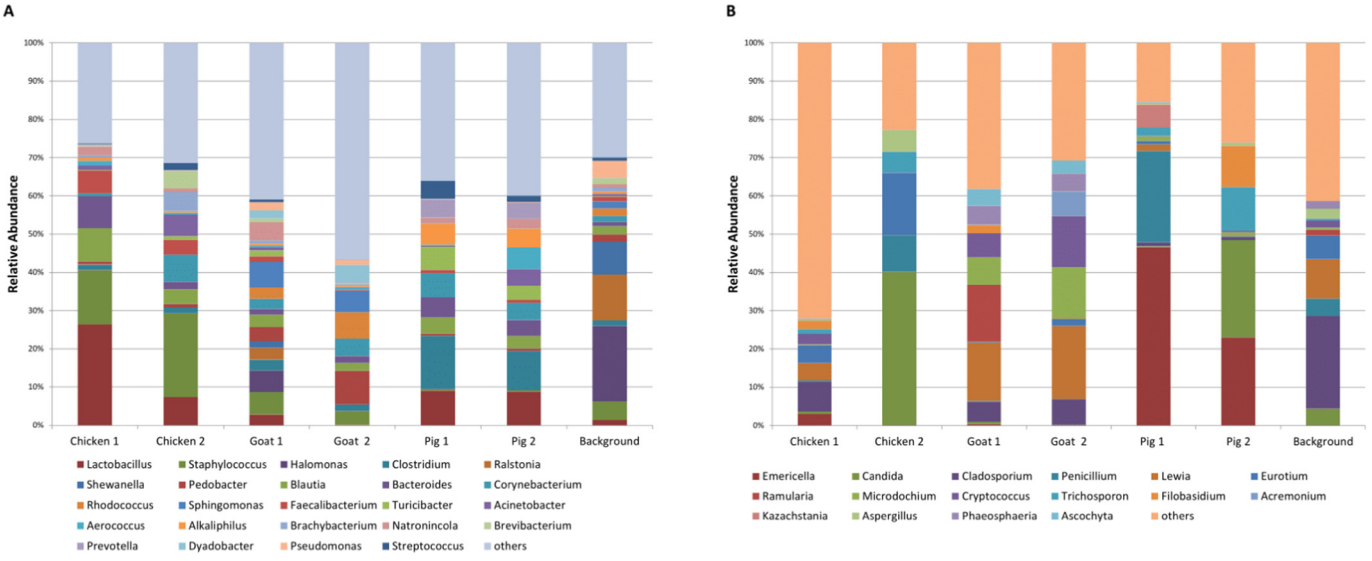


**Fig. 2.** Comparison of the relative abundance of airborne microbiome (A) Bacterial and (B) Fungal taxa at genus-level in the $PM_{2.5-6.2}$ size fraction.

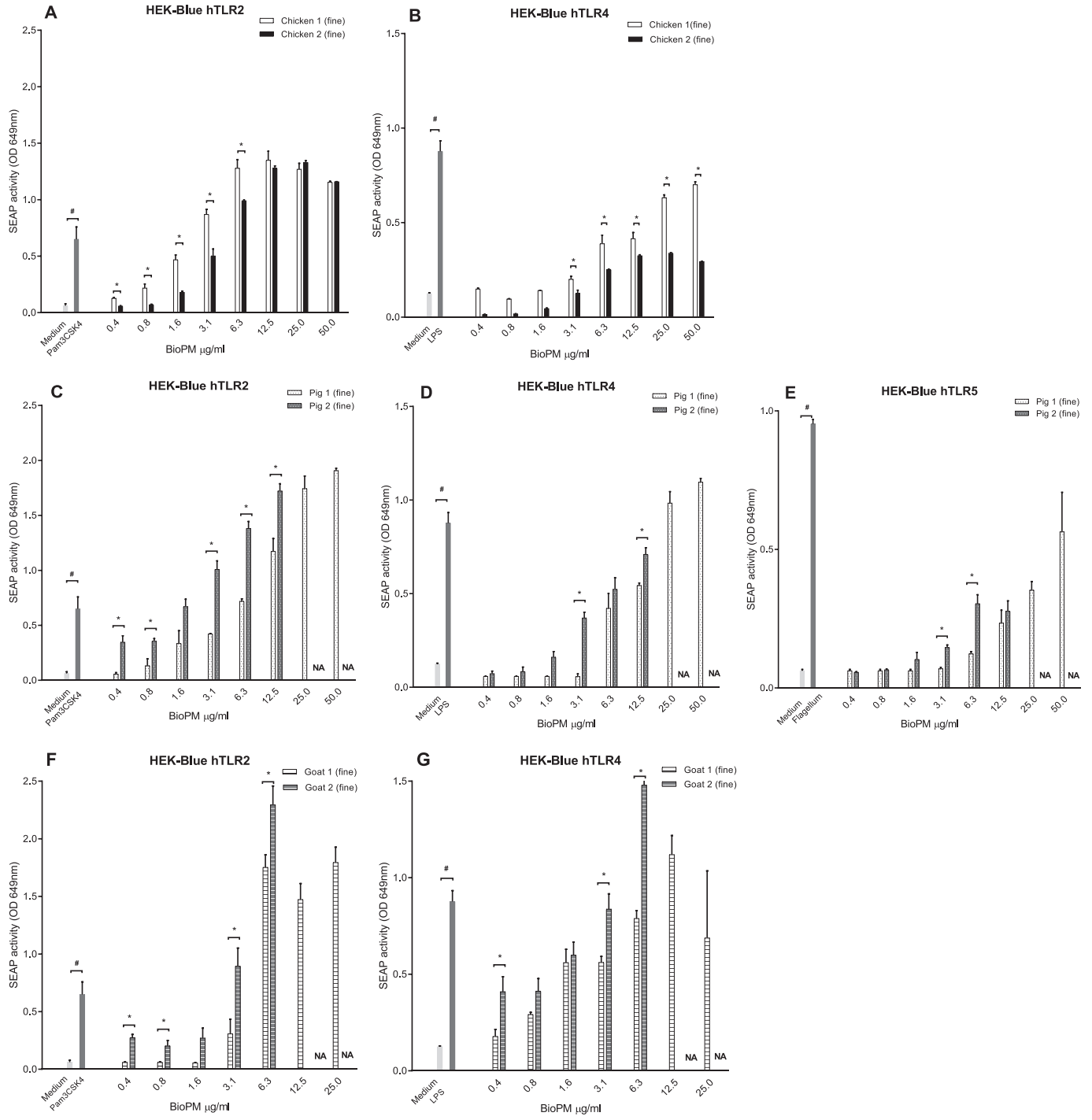


**Fig. 3.** PRR activation by BioPM collected from different farms HEK-Blue cells expressing different PRR were stimulated with fine BioPM collected from different sites: chicken farms with HEK-TLR2 (A) and HEK-TLR4 (B); pig farms with HEK-TLR2 (C), HEK-TLR4 (D) and HEK-TLR5 (E); goat farms HEK-TLR2 (F) and HEK-TLR4 (G). SEAP activity of HEK-Blue cells stimulated with the different BioPM, medium only or with the respective ligands (Pam3CSK4 (10 ng/ml), LPS (100 ng/ml), flagellum (100 ng/ml)) was determined in the supernatant after 24 h. Results are representative of three different experiments, showing mean values of triplicate determinations, with standard deviations represented by vertical bars. # $P \leq 0.05$ (medium vs PRR ligands) * $P \leq 0.05$ (farm1 vs farm2). NA: data not available.

TLR2 or with the TLR4 antagonist prior and during incubation with BioPM derived from these animal farms. Supplemental Material S Fig. 3A shows that MM6 cells can be activated by Pam3SCK4, LPS, and flagellin, ligands for respectively TLR2, −4, and −5 activation. Furthermore, abrogation of IL-6 production by MM6 when using these TLR ligands in the presence of the corresponding blocking antibodies or antagonists is presented in this figure.

Results indicate that only blocking TLR4 interfered with the IL-6 production by MM6 cells upon stimulation with these BioPM (Fig. 5A–C). For the pig farm derived BioPM, in addition to TLR2 and TLR4 we observed that these BioPM also activated TLR5. Therefore, in addition to the TLR4 antagonist and the antibody against TLR2, a blocking antibody against TLR5 was used. Here, we also observed that only blocking TLR4 interferes with activation of the MM6 cells was observed (Fig. 5B). Comparable results were found when using either the coarse or fine fraction of all tested BioPM (Supplemental Material S Fig. 3).

Since antibodies against TLR2 and TLR5 did not significantly interfere with MM6 activation by BioPM and the TLR4 blocking did not

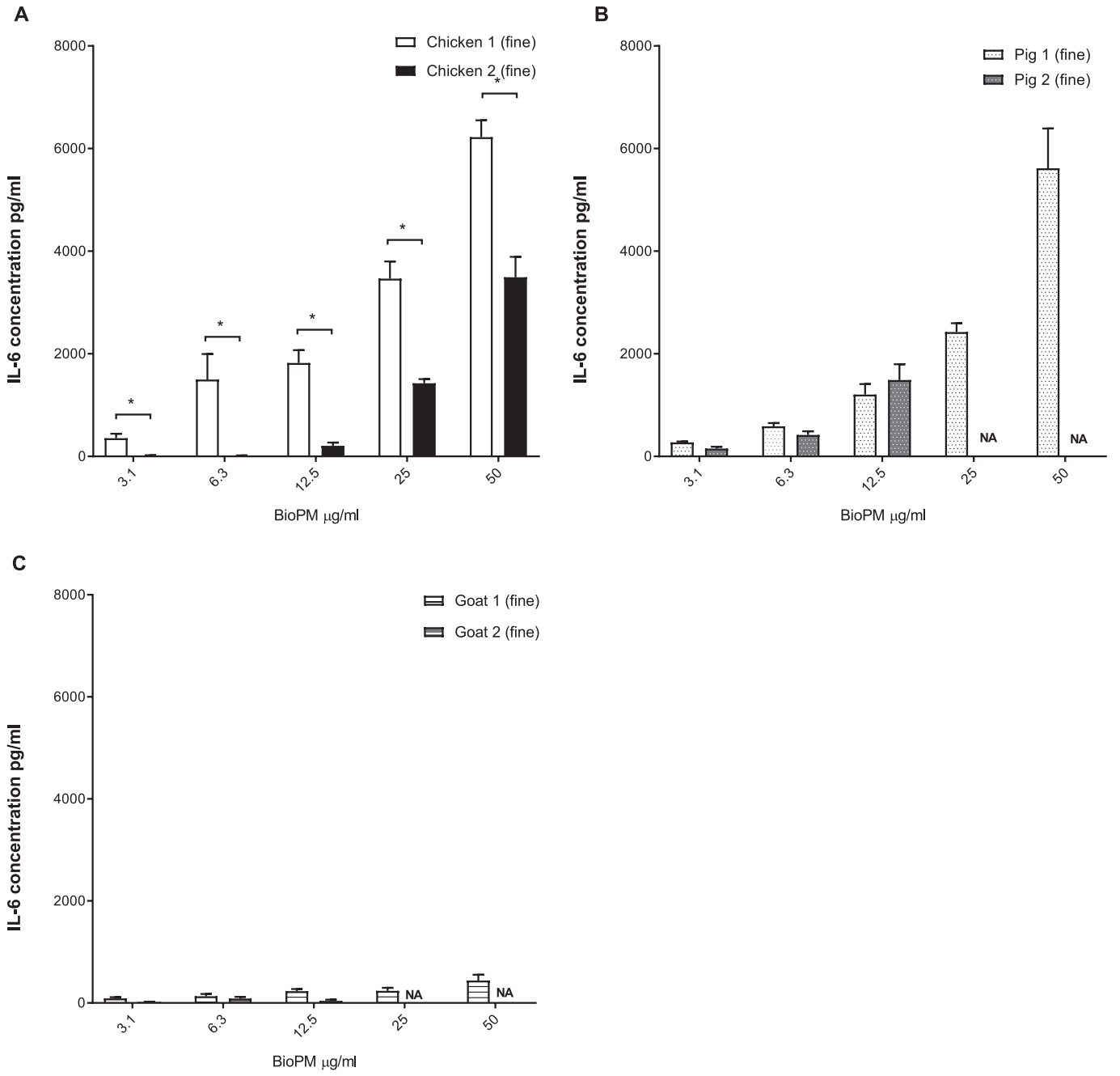


**Fig. 4.** Effect of BioPM collected from different farms on the secretion of IL-6 by MM6 cells The MM6 cells were plated in triplicate and stimulated with fine BioPM collected from different sites, chicken farms (A), pig farms (B), goat farms (C). IL-6 secretion was determined in the supernatant after 24 h stimulation with the different BioPM concentrations. Medium values for IL-6 were below the detection limit for all experiments. Results are representative of three independent experiments, showing mean values of triplicate determinations, with standard deviations represented by vertical bars. # $P \leq 0.05$ (medium vs PRR ligands) * $P \leq 0.05$ (farm1 vs farm2). NA: data not available.

completely abrograte the IL-6 production by these cells, we combined these blocking antibodies with the TLR4 antagonist. No additional decrease in IL6 production by the monocytic cell line MM6 was observed (Supplemental Material S Fig. 3), indicating that engagement of TLR4 on these cells by its ligands present in the tested BioPM, dominates the observed IL6 production.

## 4. Discussion

In this study, we examined the microbial composition of size-resolved BioPM originating from indoor air derived from different livestock in the Netherlands and their effect on innate immune cells. Air microbiome analysis from the different animal farms indicated that the bacterial profiles are associated with the specific animal species, particularly for the pig and goat. The observed association was irrespective of size fraction. BioPM collected from farms contained mainly ligands for TLR2 and TLR4 irrespective the animal source. BioPM from the pig farms contained in addition ligands for TLR5. Despite the fact that BioPM contain multiple TLR ligands, the activation of the monocytic MM6 cell line was mainly driven by TLR4 receptor signaling indicating that LPS from Gram-negative bacteria is likely to be a major contributor in immune activation by BioPM.

In the Netherlands, livestock density is among the highest in the world and the number of intensive livestock farms doubled within the first decade of the 21st century (Bos et al., 2013). Livestock farms are known to emit large amount of PM and thereby contribute to air

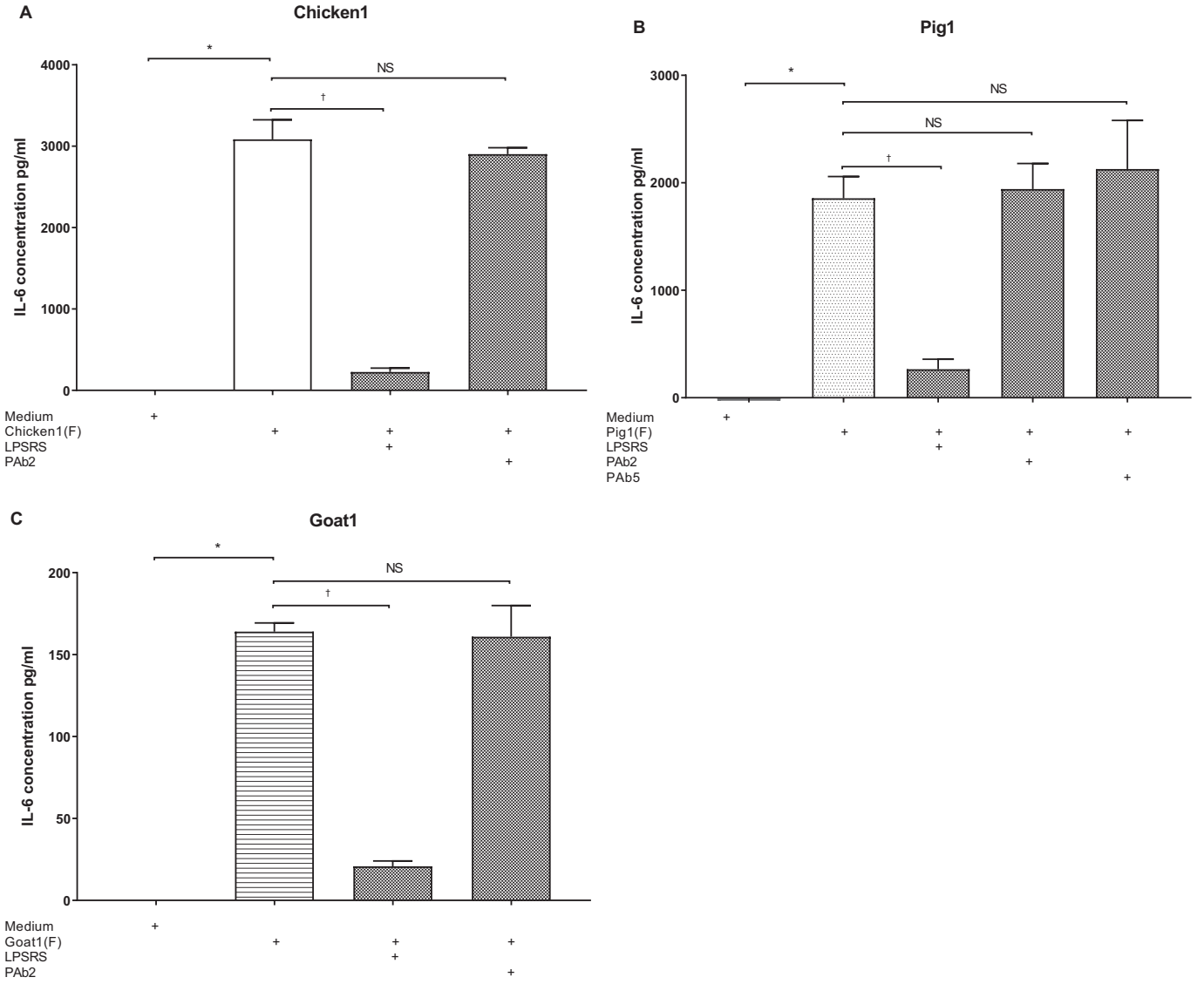


**Fig. 5.** Specific blocking of TLR2, TLR4 or TLR5 receptors on MM6 cells using neutralizing antibodies or antagonist MM6 cells were plated in triplicate treated with neutralizing antibodies against TLR2 and −5 (1 μg/ml) or antagonist LPSRS (10 μg/ml) 2 h prior to stimulation with their fine BioPM collected from chicken farm 1 (A), pig farm 1 (B) and goat farm 1 (C). All BioPM concentration were 10 μg/ml. IL-6 secretion was determined in the supernatant after 24 h. Results are shown as mean values of triplicate determinations, with standard deviations represented by vertical bars. * $P \leq 0.05$ (medium vs BioPM); † $P \leq 0.05$ (addition of blocking agent for receptors vs BioPM).

pollution (Borlée et al., 2017; Lelieveld et al., 2015; Schulze et al., 2006). Indoor PM concentrations in livestock farms have been shown to be up to 5 orders of magnitude higher compared to non-farming background locations. These PMs contain microorganisms and endotoxins which are suggested to induce respiratory infections and airway-related inflammatory responses in both farmers and residents living in close proximity (Cambra-López et al., 2010). However, there is very little information on which microorganisms are present in BioPM emitted from various farm animals and to what extends this affects innate immune receptors and cells.

Airborne fungal and bacterial communities may depend on season, humidity, collection method, size fraction, and environmental source (Barberán et al., 2015; Kumari et al., 2016). In line with previous studies (Schaeffer et al., 2017), our results indicate that the microbial composition does not depend on the size fraction. With respect to the collection method, we found minor effects of the device used for sampling on the bacterial profiles only in goat farms. For this farm Staphylococcus *spp*, *Clostridium spp*, and *Corynebacterium spp* were more abundant in PM collected with sampling in water using the VACES compared to the dry air filter based method. For the other locations, we found comparable microbiome profiles between samples that were collected via filter and the liquid impinger methods. This implies that BioPM collected with the VACES is representative for the actual microbial community present in indoor air.

Interestingly, the composition of microbial communities in the BioPM collected in the present study is mainly reflecting the animal species. This is consistent with a previous publication (Hong et al., 2012) in which it was reported that different livestock (poultry and pig) were associated with a distinct airborne microbial community. In our study, the association between the bacterial profiles and the specific animal species was most evident for the goat and pig farms. Although the profile from chicken farms grouped away from the other animal farms, a difference between the two chicken farms was observed. These findings are in line with the significant differences in TLR2, TLR4 and MM6 activation observed when using these two chicken farm BioPM. A possible explanation for these findings is the two types of chicken farms used in this study. We collected indoor BioPM from chicken farm 1, which is a breeding farm whereas farm 2 is an egg-laying farm. Remarkably,

although both pig farm shows a highly comparable microbiome profile (Fig. 1A), much more variation with respect to their mycobiome profile was observed (Fig. 1D). Future studies determining the microbial composition of indoor BioPM should include larger number of the same type of animal farms to pinpoint drivers of these differences. Among the predominant bacterial taxa, we observed *Lactobacillus*, *Clostridium*, and *Prevotella* as in pig farms, and *Lactobacillus* in chicken farms. Although tracing exact source of the airborne bacteria was beyond the scope of this study, airborne bacteria in a dairy farm have reported to be predominantly derived from animal feces (Schaeffer et al., 2017). To the best of our knowledge, there is only one study describing the airborne fungal communities from an animal farm type, performed in an unbiased way using ITS sequencing (Kumari et al., 2016). In line with this study, we also found *Ascomycota* and *Basidiomycota* as dominant phyla in our pig farm BioPM samples, representing >90% of the fungal community. We also observed high variability of fungal communities at the genus level related to the animals present in the farms. Except for *Emericalla*, high variability between the two pig farms were observed, and this probably also explains the poor similarity of fungal taxa at the genus level described by Kumari et al. (2016).

Among the different bacterial and fungal components, we observed that all BioPM samples contained potential zoonotic pathogens. Although we cannot exclude that the small size fractions only contain fragmented bacteria (or their DNA), it is tempting to suggest that airborne microbes in BioPM can infect the human respiratory tract. The potential pathogenic *Steptococcus spp, Corynebacterium spp, Clostridium spp* and *Aerocossus spp* were present in the BioPM samples in low abundance. The complete list of potential pathogens at the species levels (Supplemental Material Fig. 2) shows 24 taxa that comprise at least 1% of the community. These are identified as infectious agents in human lung, skin or the urinary tract infections of (susceptible) individuals. This list should be taken with the precaution, since the taxonomy of an organism does not necessarily provide the information about the pathogenic level the organism (Kumari et al., 2016).

Innate immunity relies on signaling by PRR, including NLRs and TLRs to alert the immune system of the presence of invading bacteria, viruses and fungi (Gerold et al., 2007). In this study, we aimed to screen for the microbial ligands in BioPM that bind to these receptors. Our results indicated that all BioPM derived from the livestock farms contains mainly bacterial and fungal ligands, evident from TLR2, TLR4 and TLR5 activation. In only a few studies the involvement of TLR2 and 4 in recognition of BioPM collected from various sources has been investigated. For example, TLR2 and TLR4 were activated by BioPM collected from urban air and cytokine productions induced by PM were inhibited by TLR2 and TLR4 neutralizer in human airway macrophages (Becker et al., 2002). TLR2 recognizes lipoprotein or peptidoglycan which is one of the main components of the cell wall of gram-positive bacteria. TLR4 is one of the well-characterized TLRs that recognizes LPS which is a common constituent of the cell wall of gram-negative bacteria (Hopkins and Sriskandan, 2005). Recently, it has been shown that PM2.5 may exacerbate allergic inflammation in the murine lung via a TLR2, TLR4 and Myeloid Differentiation factor 88 (MyD88) coupled signaling (He et al., 2017). Previous in vivo findings have indicated that low level of LPS could cause TLR4-dependent Th2 response to OVA (Eisenbarth et al., 2002). This suggests that the BioPM collected in our study could be an important risk factor for inflammatory and allergic lung diseases.

To our knowledge, we are the first to show that BioPM derived from pig farms activate TLR5. The most commonly known ligand for TLR5 is flagellin, which is a major component of flagellum found in certain bacteria (Hopkins and Sriskandan, 2005). Although blocking TLR5 did not alter the levels of IL-6 produced by the MM6 cell upon stimulation with the pig BioPM, we cannot discard and effect of TLR5 engagement on cell activation. Other cytokines produced by these cells or peripheral blood monocytes as well as their expression profile upon stimulation with the pig BioPM should also be investigated. In one study, the authors show that TLR5 agonists reduced development of airway hyperreactivity, eosinophilic airway inflammation and the production of Th2 type cytokines (Shim et al., 2012). According to these findings TLR5 activation would have a protective role against airway inflammation. However, this finding remains controversial as other studies demonstrated that flagellin drives MyD88-dependent Th2 immune responses to indoor allergens in mice (Didierlaurent et al., 2004; Wilson et al., 2012). Future studies on the role of TLR5 activation by BioPMs in respiratory diseases should be performed. When analyzing the airborne microbial composition, we could not find distinct bacterial populations in the pig farms that could explain the TLR5 activation by these samples. TLR5 expression is elevated in peripheral blood mononuclear cells (PBMCs) of children that live at pig farms, but not in children that live in other types of farms (Ege et al., 2007). Whether this response is due to the abundance of airborne TLR5 agonists in the environment of pig farms remains to be investigated.

Our findings indicate that the BioPM size fraction did not influence IL-6 production by MM6. Other studies however showed that the coarse fraction of PM has similar or higher potency to induce release of the pro-inflammatory cytokines compared to the smaller fractions (Becker et al., 2005; Hetland et al., 2005). This difference may be due to the source of the PM as well as the sampling technique, i.e. filters versus liquid based sampling. It is important to consider that coarse and fine fraction PM deposit in different regions of the respiratory tract. The coarse PM commonly deposit in the upper airway where asthma pathology occurs (Kim et al., 1996). The use of monocytic cells derived from the upper and lower respiratory tract would be an alternative approach to study whether the different BioPM size fractions influence the cytokine secretion profile of these cells.

To conclude on the effect of BioPM derived from specific animal farms on innate immune responses, future studies should include a larger number of livestock farms. In this study, the BioPM samples used were limited to 2 for each animal farm, which may not be representative for the entire type of livestock emissions. Moreover, the BioPM were collected only indoors whereas sampling in the surroundings of the farms can provide further information on the effect of BioPM on people living nearby. In the present study, we have used cell lines to study the innate immune response. It would be interesting to substantiate these observations using primary human PBMCs and airway macrophages. Finally, future in vivo studies should test the effect of fine and coarse PM on the airways. Although in vitro studies indicate that the different BioPM fractions induce comparable immune activation, in vivo responses may be different due to differences in regional deposition across the airway.

## 5. Conclusions

In summary, the results from this study indicate that distinct airborne microbiota profiles are associated with the corresponding animal farms and LPS is the most critical component of BioPM responsible for activation of innate immune cells. Whether the induced immune response has consequences for inflammatory responses upon inhalation of the different BioPM affecting (ongoing) respiratory diseases requires further investigation. The role of other ligands, such as lipoprotein and flagellum emitted from different animal farms on the airway should also be investigated. The knowledge generated is important for the development and implementation of measures to control microbiome air pollution from livestock farms.

## Disclaimer

All authors declare they have no actual or potential competing financial interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.06.217.

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