

Acellular filtrate of a microbial-based cleaning product potentiates house dust mite allergic lung inflammation

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ARTICLE INFO

Keywords:

Allergic inflammation
Bacillus
Consumer product
House dust mite
Lung

ABSTRACT

Microbial-based cleaning products (MBCPs) contain bacteria and chemical constituents. They are used in consumer applications such as odor reduction, unclogging drains, and surface cleaning. To determine the capacity of a model MBCP to contribute to acute allergic lung inflammation, a two-week repeated exposure regimen was used. Mice were exposed by endotracheal instillation to saline alone, MBCP alone, house dust mites (HDM) alone, or sequentially (i.e., MBCP followed by HDM, HDM followed by MBCP, or HDM + MBCP followed by HDM). Both whole MBCP and acellular MBCP filtrate were investigated, and showed minimal differences in the endpoints examined. HDM exposure caused pulmonary perivascular inflammation, bronchiolar mucous cell metaplasia, elevated bronchoalveolar lavage fluid (BALF) eosinophils, and HDM-specific IgG1. For MBCP, notable changes were associated with sequential exposures. MBCP/HDM caused elevated T_H2 cytokines in BALF, and elevated neutrophils, eosinophils and IL-5 in peripheral blood. Co-administration of MBCP and HDM followed by HDM resulted in elevated blood and BALF eosinophils and HDM-specific IgE and IgG1. These results demonstrated that acellular MBCP filtrate, and not bacteria within MBCPs, potentiated the acute allergic inflammation to HDM. This methodology could be extended to investigate chronic allergic inflammation and inflammatory potential of other MBCPs and biotechnology products with complex compositions.

1. Introduction

Spray-type microbial-based cleaning products (MBCPs) are marketed internationally for use by consumers and smaller industries such as restaurants and recreational facilities. They have a wide range of applications, including cleaning a variety of surfaces, deodorizing pet or sports equipment, and removing oil and pet residue stains (Vandini et al., 2014; SpÖk and Klade, 2015). An important distinction between these and non-microbial products is that the living microorganisms and proprietary enzymes, surfactants and detergents collectively contribute to the efficacy of the product. This complex composition also creates uncertainties for regulators responsible for assessing the human health risk during manufacture or use by the consumer.

Spray MBCPs could pose a hazard from accidental inhalation exposures during product use, or after surface treatment where chemicals adsorbed to dust or other particles are aerosolized and subsequently inhaled (Poulsen et al., 2000). Furthermore, the prevalence of occupational allergy resulting from industrial enzyme exposure is an ongoing concern (Lindstedt et al., 2005). Currently, there are no studies

investigating the respiratory hazard of MBCPs, but the potential for acute symptoms such as local airway inflammation or exacerbation of pre-existing respiratory conditions is possible.

Either the filterable formulation constituents or microbial components of MBCPs could contribute to the allergic response. Chemical and enzymatic cleaning agents are known to be occupational and domestic allergens. For example, disinfectants, detergents, and bacterial enzymes can act as sensitizers, and are capable of elevating the risk of asthma and rhinitis (Lindstedt et al., 2005; Clausen et al., 2000; Folletti et al., 2014; Rosenman, 2006). Alternatively, the microbes within the MBCPs may function as inducers of allergies. Although exposure to bacteria or substances derived from them most often result in a T_H1 type response (North et al., 2016), biomonitoring data and mouse exposure studies have demonstrated that bacteria or substances derived from them (e.g., cell wall constituents) can contribute to allergic asthma by functioning as sensitizers or adjuvants (Bernstein et al., 1999; Berstad et al., 2000; Huang et al., 2011; Kawai et al., 2002; Schröder et al., 2008). Moreover, *Bacillus* spores have been reported to induce hypersensitivity pneumonitis, which is also known as detergent workers disease or humidifier

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<https://doi.org/10.1016/j.fct.2018.02.030>

Received 19 September 2017; Received in revised form 6 February 2018; Accepted 14 February 2018
0278-6915/ © 2018 Published by Elsevier Ltd.

lung (Reynolds, 1988).

Thus, there is sufficient evidence to suggest that cleaning agents and/or microbial constituents have the capacity to elicit or contribute to the allergic response. The aim of this study was to determine if whole MBCP or its acellular formulation filtrate could induce or potentiate the early allergic response. To address this, an acute (two week) mouse exposure model of allergic airway inflammation was used, which has been well-characterized using a known respiratory allergen, the house dust mite (HDM; *Dermatophagoides farina*) (Cates et al., 2004; Piyadasa et al., 2016; Bazett et al., 2016), which is a relevant allergen when considering residential MBCP usage. MBCP exposures with and without HDM were investigated by measuring indicators of inflammation, airway remodeling, and induction of HDM-specific immunoglobulins. The data presented here demonstrate the capacity of MBCP components to contribute to the allergic inflammatory response caused by HDM.

2. Materials and methods

2.1. MBCP characterization and preparation of MBCP filtrate

A liquid MBCP was selected based on its application in spray format and our initial analysis of contents. The product is used for pet odor reduction and could potentially result in a substantial respiratory exposure during application. It was described by the manufacturer to contain non-pathogenic bacteria, natural enzymes and mild cleansers. The microbial content was assessed by spread plating serial dilutions and incubating at 28 °C for 24 h on Trypticase Soy Agar (TSA) with or without 5% sheep blood (37 °C). Colonies were enumerated and processed for identification as outlined by the MIDI-Sherlock® fatty acid methyl ester (FAME) microbial identification system (MIDI Inc., Newark, DE). FAME profiles from isolated colonies were compared to both the RTCLIN6 clinical library and a previously developed custom in-house library consisting of FAME profiles from domestic microbial strains approved for biotechnology applications in Canada. Acellular product filtrates were prepared by passing the contents through a 0.22 µm pore size hydrophilic polyethersulfone (PES) membrane. Lack of microbial growth was confirmed by streaking the filtrate onto TSA.

2.2. Animals used in this study

All procedures involving animals were approved by the Health Canada Animal Care Committee and overseen by a board certified veterinarian. Female BALB/c mice between 18 and 20 g (8 weeks old) were purchased from Charles River Laboratories Inc. (Saint-Constant, QC), and acclimated for at least 1 week prior to experimentation. Animals were housed in cages under a 12-h light/dark cycle at a constant room temperature (20 ± 2 °C) and relative humidity of 50–70%. The commercial pellet diet and reverse osmosis (RO) water were available *ad libitum*.

2.3. Experimental design and animal exposure

The inflammatory effects of exposure to either whole MBCP or its filtrate were investigated using a modification of a procedure used for HDM-induced inflammation in allergic airway disease (Cates et al., 2004; Piyadasa et al., 2016; Bazett et al., 2016). Animal exposure schedules are shown in Fig. 1. Six mice were used for each two-week treatment with the following: (1) saline alone (2) HDM alone (3) MBCP alone (4) MBCP (week 1) followed by HDM (week 2) referred to as MBCP/HDM (5) HDM (week 1) followed by MBCP (week 2) referred to as HDM/MBCP, and (6) HDM mixed with MBCP (week 1) followed by HDM (week 2) referred to as HDM + MBCP/HDM.

For endotracheal instillation, mice were anesthetized with isoflurane (4–5% for induction, and 1–3% for maintenance). The animals were treated with a final volume of 25 µL of saline with either *Dermatophagoides pteronyssinus* (HDM; 25 µg as used by (Bazett et al.,

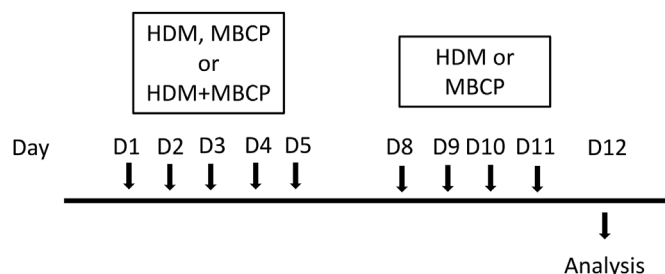


Fig. 1. Exposure schedule for administration of MBCP and HDM. The schedule followed was for 2 weeks as shown for days (D) 1–5 in the first week followed by D8–11 in the second week, and sample collection and analysis on D12.

2016)) (Greer Laboratories, Lenoir, NC) or MBCP (10 µg). The 10 µg dose was chosen since it corresponds to 2×10^4 cfu, which did not cause notable toxicity in a previous mouse exposure study with *Bacillus* species (Tayabali et al., 2011), which are used in the formulation of most MBCPs (Spök and Klade, 2015). Control animals were treated with vehicle alone (0.9% saline). The doses were administered by instillation using a syringe-style nebulizer (Microsprayer™, Penn Century, Philadelphia, PA) through the trachea into the lungs. Treated mice typically recovered from anaesthesia and dosing within 2 min.

2.4. Blood analysis

On Day 12, treated mice were anesthetized with isoflurane as described above, and euthanized by exsanguination. For this, 0.5–1.0 mL of blood was collected by cardiac puncture and transferred to tubes containing ethylenediaminetetraacetic acid (EDTA). Hematological analysis was performed using an automated haematology analyzer (Sysmex XT-2000iV-1, Kobe, Japan), and the remaining plasma was stored at -80 °C for pro-inflammatory cytokine analyses and immunoglobulin IgG1 and IgE measurements. Cytokines were measured according to the manufacturer's instructions (Bio-Plex, Biorad, Hercules, CA). A panel of 10 cytokines were investigated: IL-4, IL-5, IL-10, IL-13, Eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon (IFN)-γ, keratinocyte chemoattractant (KC), regulated on activation normal T cell expressed and secreted (RANTES), and tumor necrosis factor (TNF)-α. Data were analyzed by Bio-Plex Manager 6.0 software. For measurement of HDM-specific IgG1 and IgE, reagents from ELISA Ready-SET-Go! IgG1 and IgE kits (eBioscience, San Diego, CA) were used and a modification of the procedure by Bazett and colleagues was followed (Bazett et al., 2016). Briefly, HDM (2.5 µg/well) was coated into wells of a 96-well plate. Following blocking with 5% fetal bovine serum in PBS, undiluted plasma was incubated in the wells overnight at 4 °C. Following PBS washes, wells were incubated with horse-radish peroxidase (HRP) conjugated anti-mouse IgG1 or IgE polyclonal antibody for 1 h at 37 °C. Colorimetric detection of antibody conjugation was done with tetramethylbenzidine (TMB) substrate solution and measurement with a multi-well spectrophotometer (Molecular Devices, CA) at optical densities of 450 nm and 570 nm. Standard curves were generated by coating wells with known concentrations of IgG1 or IgE, binding with HRP-conjugated anti-mouse IgG1 or IgE, and detection with TMB.

2.5. Bronchoalveolar fluid (BALF) collection and analysis

Lung lavage fluid was collected by securing a 22-gauge plastic cannula (Instech Laboratories, Inc., Plymouth Meeting, PA) in the trachea using surgical thread. A 3-way stopcock (Harvard Apparatus, Holliston, MA) with two syringes was secured on the cannula. One syringe was used to inject sterile saline into the bronchoalveolar compartment, while the other syringe was used for BALF recovery.

For the cell population analysis, cells were retrieved by

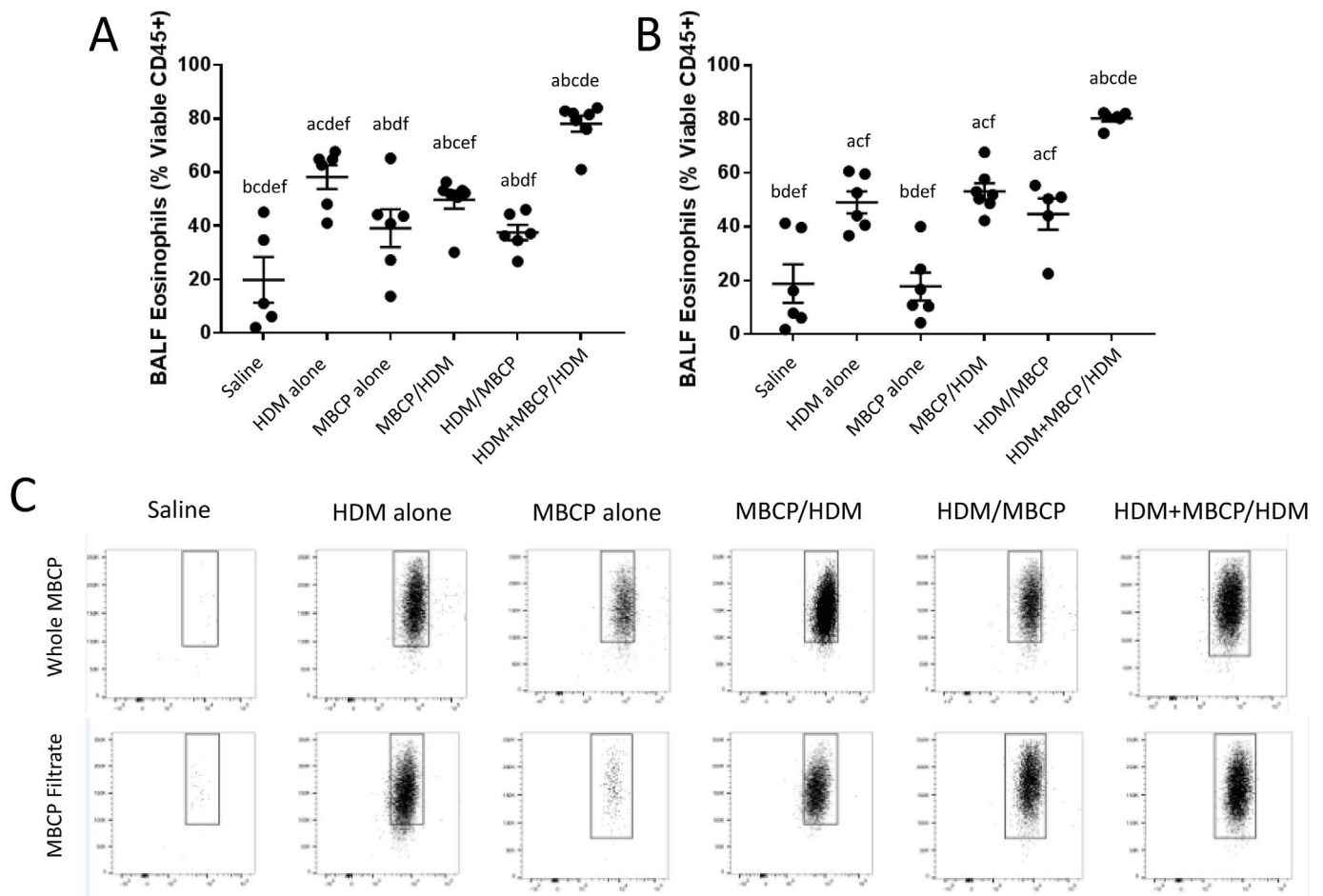


Fig. 2. Alterations in eosinophil levels in BALF following MBP and HDM exposures. BALF fluid was collected on day 12 following two week exposures with the indicated treatments. Cells were stained with leukocyte-specific fluorescent antibodies and numbers of leukocyte populations were enumerated by flow cytometry after gating for viable cells. Percent viable eosinophils are shown following whole MBP exposure (A) MBP filtrate exposure (B). Representative scatter plots gated for eosinophils are shown in (C), with y-axis representing side scatter and x-axis representing level of Siglec-F. Eosinophils were gated as viable CD45⁺, Gr-1^{lo}, CD11b⁺, F4/80⁺, Siglec-F⁺, and SSC^{hi}. Data in (A) and (B) were compared using 1-way ANOVA followed by Tukey's multiple comparison test with a value of $p < 0.05$ indicating statistical difference. The 'a' denotes a difference from control, 'b' denotes a difference from HDM alone, 'c' denotes a difference from MBP alone, 'd' denotes a difference from MBP/HDM, 'e' denotes a difference from HDM/MBCP, and 'f' denotes a difference from HDM + MBP/HDM.

centrifugation and resuspended in 0.5 mL of Hanks Balanced Salt Solution (HBSS). The cell-free BALF was saved for cytokine measurements. The cell fraction was used for flow cytometric analysis. Briefly, cells were stained with fixable viability dye eFluor 506 for 30 min on ice in the dark (eBioscience, ThermoFisher Scientific, Waltham, MA), incubated with BD Fc Block (BD Biosciences, San Jose, CA) for 10 min on ice in the dark followed by a single panel of fluorochrome-conjugated antibodies for 25 min on ice in the dark. Panel: F4/80 PE, CD11b PE-Cy7, SiglecF APC-R700, CD45 BV786, CD11c APC-Cy7, I-A/E Alexa 488, CD24 BV421, CD3 PE-CF594, CD117 BV605 (BD Biosciences, San Jose, CA) and Gr-1 PerCp-e710, FcεRI APC (eBioscience). BD Horizon Brilliant Stain Buffer was added to the panel prior to addition to the cells (BD Biosciences, San Jose, CA). Staining and washes were performed with Flow Buffer consisting of HBSS with no calcium, no magnesium, no phenol red, and supplemented with 0.5% bovine serum albumin and 0.09% sodium azide. Compensation controls were prepared with OneComp Beads or Ultracomp eBeads (eBioscience). Cells were analyzed on a BD LSR Fortessa flow cytometer using FACS Diva software and analyzed using FlowJo software (TreeStar, Inc., Ashland, OR). All plots were gated for viable leukocytes, as determined by live-cell gating by forward scatter, side scatter, a lack of viability dye uptake, and positive staining for CD45.

Cell-free BALF was also used for measurement of IL-4, IL-5, IL-10, IL-13, Eotaxin, GM-CSF, IFN- γ , KC, RANTES and TNF- α using Bio-Plex

Pro™ Assays, a Bioplex 100 instrument (BioRad), and analysis was done using Bio-Plex Manager 6.0 software and GraphPad Prism™ (version 7.00).

2.6. Lung histopathology

After BALF collection, 1 mL of 10% (v/v) formalin was injected into the lung through the cannula connection. The inflated lung tissues were collected immediately by excising and immersing them into 10% formalin. Tissues were embedded into paraffin, sectioned to a thickness of 4 μ m, and mounted on a glass microscope slide using standard histopathological techniques. The sections were stained with hematoxylin-eosin (HE) or Periodic Acid-Schiff (PAS) and examined by light microscopy. Tissue pathology was evaluated by a veterinary pathologist who was blind to the origin of the samples. Scoring of lung and blood vessel lesions was done using the INHAND (International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice) system (Berridge et al., 2016; Renne et al., 2009).

2.7. Statistical analysis

Results were analyzed in GraphPad Prism™ (version 7.00). Data were compared by 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All data were expressed as mean $\pm$

standard error. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. MBCP characterization

The MBCP product was analyzed by spread plating on TSA to contain $2.1 \times 10^6 \pm 30.7\%$ cfu/mL ($n = 8$). When spread plated on sheep blood agar, some colonies caused haemolysis; however when MBCP filtrates were deposited onto the sheep blood plates, no haemolysis was observed. MIDI-FAME phenotyping from colonies grown on either TSB or 5% sheep blood demonstrated that the product consisted of *Bacillus subtilis* and *Bacillus licheniformis* with similarity indices between 0.636 and 0.889. Aside from the contents description on the product label (i.e., natural enzymes and mild cleansers), the chemical formulation constituents were unknown.

3.2. Murine exposure experiments

3.2.1. Alterations in BALF

Flow cytometry was used to identify and quantify the proportions of leukocyte populations present in the BALF. Several cell types were measured (neutrophils, alveolar macrophage, mast cells, basophils, CD11b + dendritic cells, interstitial macrophage, Ly6c⁺ monocytes, eosinophils and T lymphocytes). Data presented in Fig. 2 show changes in BALF eosinophil levels resulting from whole MBCP (Fig. 2A) or MBCP filtrate (Fig. 2B) exposures, and representative side-scatter versus eosinophil-specific Siglec-F plots (Fig. 2C). Eosinophils were gated as viable CD45⁺, Gr-1^{lo}, CD11b⁺, F4-80⁺, Siglec-F⁺, and SSC^h. Data demonstrate that saline-treated animals had eosinophil levels of ~20% of viable CD45⁺ leukocytes. In treatments with either whole MBCP (Fig. 2A) or its filtrate (Fig. 2B), eosinophils were significantly elevated to ~50% viable CD45⁺ cells in all exposures that included HDM (HDM alone, MBCP/HDM, HDM/MBCP). The HDM + MBCP/HDM exposure caused the largest elevation of eosinophils (~80% of viable CD45⁺ cells).

Given that lung eosinophils were elevated following exposure, levels of BALF cytokines and chemokines were measured. Fig. 3A and B shows that when either whole MBCP or its filtrate was used, MBCP/HDM caused statistically increased levels of Th2 cytokines IL-4, IL-5, and IL-13, as well as the anti-inflammatory cytokine IL-10. In contrast to elevated proportion of eosinophils with HDM + MBCP/HDM, no elevation of these cytokines was observed with this treatment.

3.2.2. Pulmonary pathology following MBCP and HDM exposure

Following exposures and BALF collection, the lungs of mice were inflated and examined for pathological effects. Table 1 summarizes lung lesions scores that were conducted by a pathologist that was blind to the animal exposures. Since BALF fluid was already collected from these lungs, airway infiltration could not be assessed by histopathology; however evidence for airway remodeling was examined. Saline-treated lungs did not demonstrate any damage resulting from the lavage procedure with the indices examined. The predominant lesions occurred in HDM, MBCP/HDM, and HDM + MBCP/HDM treated groups, which demonstrated perivascular cuffing and blood vessel mural inflammation. Perivascular cuffing was characterized by a mixed population of leukocytes, including eosinophils, mononuclear cells, lymphoid cells, and plasma cells, with few neutrophils, which occurred in most mouse lungs (Fig. 4A-F). Blood vessel inflammatory changes, or mural inflammation, were characterized by monocytes and eosinophils occupying the vessel walls; these leukocytes could be responding to vessel injury and/or transmigrating into the lung. Airway lesions in these mice consisted of mucous cell metaplasia of subterminal and larger bronchioles, accompanied by mild changes in terminal bronchiolar epithelium (Fig. 5A-F). This was especially apparent with HDM, MBCP/HDM and

HDM + MBCP/HDM treatments (Fig. 5D, E and 5F, respectively). Further, there was only mild alveolar septal thickening due to congestion, minimal Type 2 pneumocyte hypertrophy and hyperplasia, and the presence of fibroblast or spindle cells with suspect minimal focal fibrosis. No differences were apparent between exposures to whole MBCP or its filtrate.

3.2.3. Alterations in peripheral blood

Since significant pulmonary changes were observed in animals exposed to MBCP and HDM, the systemic response to exposure was assessed. Fig. 6 shows the blood leukocytes that were significantly altered. When either whole MBCP or its filtrate was used for treatments, HDM + MBCP/HDM caused an approximately 4-fold elevation in eosinophils (Fig. 6A and C). With MBCP/HDM treatment, an approximate 2-fold elevation of neutrophils (Fig. 6B and D) was apparent. There was no significant difference between whole MBCP (Fig. 6A and B) and MBCP filtrate (Fig. 6C and D) exposures.

Since a significant increase in granulocytes was observed within the blood and elevation of Th2 cytokines were elevated in the BALF, expression of inflammation-related cytokines and chemokines was investigated in the plasma. Fig. 7 shows that only MBCP/HDM caused elevated levels of IL-5 and KC when whole MBCP was used (Fig. 7A and B), and IL-5 when MBCP filtrate was used (Fig. 7C). Although KC was not statistically significantly different from control with MBCP filtrate exposure, there were two mice that showed elevated levels (Fig. 7D).

3.2.4. Levels of HDM-specific IgE and IgG1 following exposures

As a further assessment of immune response, the levels of HDM-specific IgE and IgG1 were measured following exposures. HDM + MBCP/HDM treatments of either whole MBCP (Fig. 8A) or MBCP filtrate (Fig. 8B) induced significantly elevated levels of HDM-specific IgE. HDM alone did not induce significant elevation of HDM-specific IgE. For HDM-specific IgG1, data was limited to saline, HDM and HDM + MBCP/HDM, since sufficient plasma was not available for all treatments. HDM exposure resulted in significantly elevated HDM-specific IgG1 (saline = 185.6 ± 83 ng/mL ($n = 9$ pooled replicates from whole MBCP and its filtrate) versus HDM = 2050.3 ± 70 ng/mL ($n = 3$)). HDM + MBCP/HDM exposure with whole MBCP also resulted in significantly elevated HDM-specific IgG1 (2049.2 ± 46.9 ng/mL ($n = 4$)). However, HDM + MBCP/HDM with filtrate was elevated without being statistically different from control (1857.4 ± 111.6 ng/mL ($n = 2$)).

4. Discussion

The primary goal of this study was to determine if MBCPs could contribute to the allergic response using early indicators of allergic inflammation. Further, the study was designed to differentiate between the effects of whole MBCP and its acellular filtrate. Results demonstrated that although MBCP itself did not induce allergic inflammation, it could potentiate acute allergic inflammation to HDM, which is relevant when considering allergies in the residential context where MBCPs are used. This heightened allergic inflammation was similar after exposures from whole MBCP or its filtrate, which demonstrated that the filterable formulation constituents and not the bacteria contributed to this activity.

The model MBCP investigated here and several others have been characterized in greater detail as part of a separate publication (Subasinghe et al., 2018). This study also identified bacterial isolates using 16S rDNA sequencing from cultivable bacteria and metagenomic sequencing for bacterial identification. These data confirmed the presence of substantial numbers of bacteria belonging to the *Bacillus* species. In addition to *B. subtilis* and *B. licheniformis* observed with MIDI-FAME analysis presented here, 16S rDNA sequencing of cultivable bacteria also identified *B. amyloliquefaciens*. Metagenomic sequencing identified *B. flexus* and *B. licheniformis* as the predominant species.

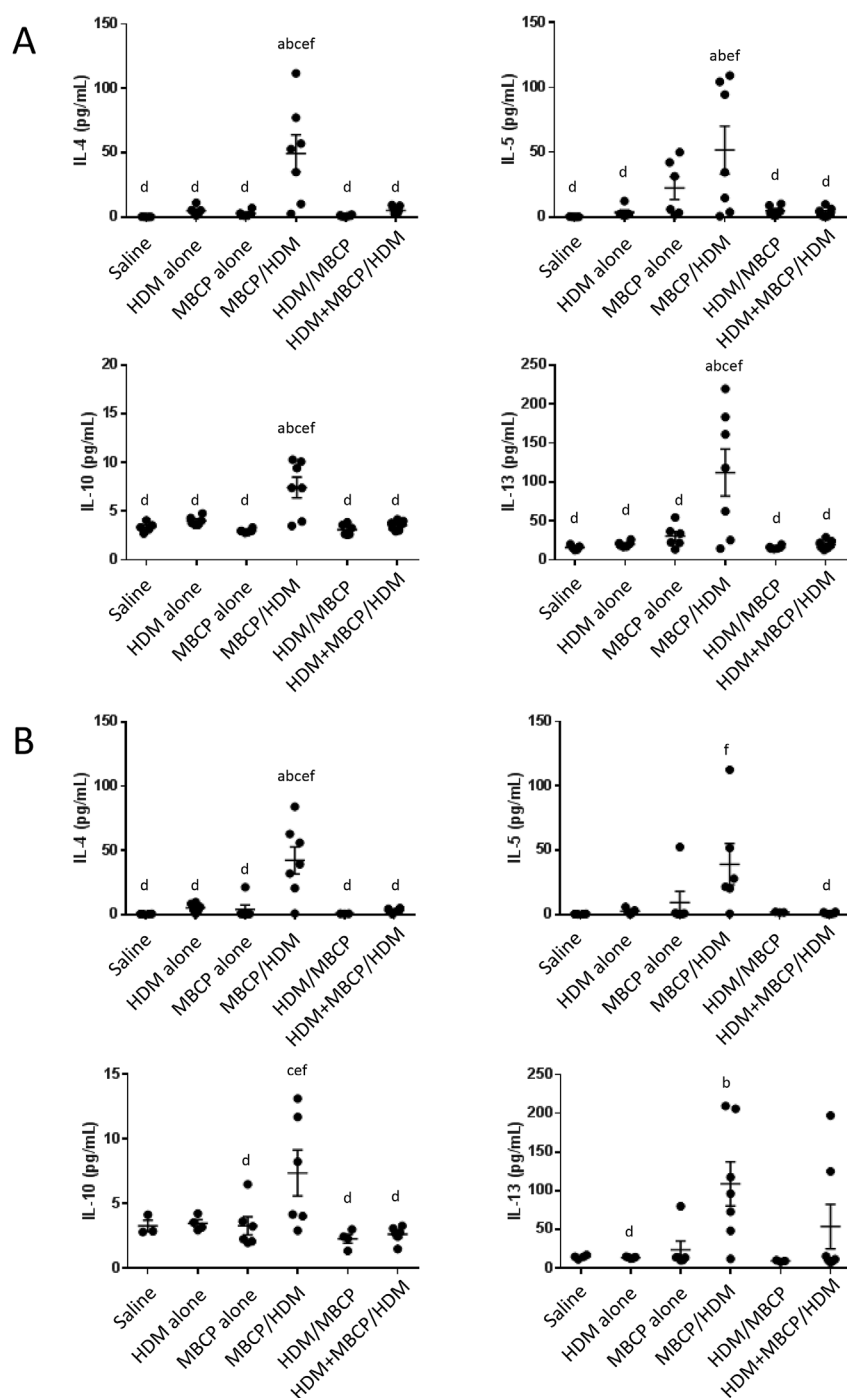


Fig. 3. Levels of BALF cytokines/chemokines following MBCP and HDM exposures. BALF fluid was collected on day 12 following sensitization/challenge with the indicated treatments. BALF was filtered to remove cells and subjected to a 10-plex Bioplex™ bead array cytokine measurement system. Only cytokines that demonstrated statistical differences to other exposures were shown here. (A) Whole MBCP was used in the exposures (B) MBCP acellular filtrate was used in the exposures. Data were compared using 1-way ANOVA followed by Tukey's multiple comparison test with a value of $p < 0.05$ indicating statistical difference. The 'a' denotes a difference from control, 'b' denotes a difference from HDM alone, 'c' denotes a difference from MBCP alone, 'd' denotes a difference from MBCP/HDM, 'e' denotes a difference from HDM/MBCP, and 'f' denotes a difference from HDM + MBCP/HDM.

Although these methods identified different species, most (i.e., aside from *B. flexus*) were members of the 'B. subtilis group', which includes *B. amyloquifaciens*, *B. atrophaeus*, *B. licheniformis* and *B. subtilis* (Dunlap et al., 2015). *Bacillus* species are known to be primary constituents of MBCPs (Spök and Klade, 2015), and produce a number of lytic enzymes that could contribute to MBCP efficacy (Fujinami and Fujisawa, 2010). Quantification by colony counting showed large numbers of these bacteria with high variability between eight separate samplings of the product. This high variability in cfu has been observed with *Bacillus* species that demonstrate even small amounts of clumping (Hazan et al., 2012). Nevertheless, spores of several strains within the *B. subtilis* Group, including *B. subtilis sensu stricto* and *B. licheniformis* have been tested by us using single endotracheal exposures with BALB/c mice, but did not result in changes in lung or blood cytokine or

leukocyte levels (unpublished data).

The acute HDM challenge model that was adapted here (Cates et al., 2004; Piyadasa et al., 2016; Bazett et al., 2016) is relatively short in duration (2 weeks) and is marked by neutrophilic and eosinophilic airway inflammation and induction of pro-inflammatory and T_H2 cytokines. In contrast, chronic HDM challenge of 5–8 weeks results in airway remodeling and fibrosis (Piyadasa et al., 2016). Our methodology differed from the typical acute HDM challenge model in that doses were administered by endotracheal instillation and not the intranasal route, which from our experience, has demonstrated consistent and efficient *Bacillus* spore dose delivery to the lungs (Tayabali et al., 2011). Furthermore, we introduced some variations in the dosing regimen; some treatments consisted of serial exposures of either: MBCP followed by HDM, HDM followed by MBCP, or a mixture of HDM and

Table 1

Mean Scores ($\pm$ SEM) of lung lesions associated with MBCP & HDM exposures. Mean scores were calculated from individual lesion scores from 6 mice for each treatment.

Treatment	Perivascular Inflammation	Bronchiole Epithelial Hyperplasia	Bronchiole Epithelial Regeneration	Mucous Cell Metaplasia
Control	1.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
HDM Alone	3.0 $\pm$ 0.6	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	3.0 $\pm$ 0.0
MBCP Alone	2.2 $\pm$ 0.4	0.8 $\pm$ 1.3	0.3 $\pm$ 0.5	1.0 $\pm$ 1.0
MBCP/HDM	3.7 $\pm$ 0.8	0.0 $\pm$ 0.0	0.3 $\pm$ 0.8	2.7 $\pm$ 0.8
HDM/MBCP	1.8 $\pm$ 0.4	1.0 $\pm$ 1.0	1.0 $\pm$ 1.0	2.0 $\pm$ 0.7
HDM-MBCP/HDM	3.0 $\pm$ 0.6	0.3 $\pm$ 0.8	0.1 $\pm$ 0.4	3.0 $\pm$ 0.0
Control (Filtrate)	0.5 $\pm$ 0.5	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
HDM Alone (Filtrate)	3.0 $\pm$ 0.6	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	3.0 $\pm$ 0.0
MBCP Alone (Filtrate)	1.2 $\pm$ 1.0	0.0 $\pm$ 0.0	1.2 $\pm$ 0.8	0.3 $\pm$ 0.8
MBCP/HDM (Filtrate)	3.9 $\pm$ 0.4	1.6 $\pm$ 0.8	1.3 $\pm$ 0.8	2.9 $\pm$ 0.4
HDM/MBCP (Filtrate)	2.0 $\pm$ 0.0	0.5 $\pm$ 0.8	0.7 $\pm$ 0.8	2.3 $\pm$ 0.8
HDM-MBCP/HDM (Filtrate)	2.8 $\pm$ 0.4	0.3 $\pm$ 0.8	0.7 $\pm$ 0.8	3.0 $\pm$ 0.0

MBCP followed by HDM. This was done to determine if MBCP could potentiate the allergic inflammation response of HDM.

Whenever HDM was used in challenges with or without MBCP, BALF eosinophil levels were elevated. The BALF was only examined at 24 h post-exposure; however, the eosinophilic response in acute HDM challenge has been observed to be maximal at 48 h (De Alba et al., 2010). An unexpected observation was that HDM + MBCP/HDM exposure induced higher levels of eosinophils than HDM alone or MBCP/HDM, which supports the notion that MBCP can potentiate the allergic inflammatory response to HDM. Eosinophilic lung infiltration is a hallmark of allergic asthma (Walsh et al., 2010). Upon induction and chemoattraction by IL-5, eosinophil progenitors differentiate and mediate effects in the lung that contribute towards the early allergic response. Some of these include antigen presentation, and secretion of a number of cytokines, chemokines, metalloproteases, and leukotrienes.

To further investigate the potentiating effect of MBCP, BALF cytokine levels were measured. Only MBCP/HDM exposures demonstrated elevated levels of T_H2 cytokines IL-4, IL-5, IL-13 that are typically observed in allergy-affected airways (Williams et al., 2012). The cytokine IL-4 is pleiotropic during the early allergic response, mediating the isotype switch for IgE production, up-regulating expression of IgE

receptors on the surface of leukocytes, activating mucin production from goblet cells, increasing vascular cell adhesion molecule expression in endothelium, and promoting eosinophil survival, and differentiation of T_H0 to T_H2 lymphocytes for secretion of other T_H2 cytokines (Steinke and Borish, 2001). One of these cytokines is IL-5, which drives differentiation, release, and survival of eosinophils (Greenfeder et al., 2001). Our data also indicated significantly elevated levels of blood and BALF IL-5, which is consistent with the high levels of eosinophils observed. Another T_H2 cytokine, IL-13, contributes towards IgE synthesis and induces mucous secretion and airway remodeling (Rael and Lockey, 2011). The elevation of the immunosuppressive cytokine, IL-10, suggests that the allergy induction that was observed is regulated, since IL-10 has been implicated in suppression of IgE production and effector cells of allergic inflammation (eosinophils, basophils, mast cells) (Cezmi and Mübeccel, 2014).

Examination of HDM-exposed lungs demonstrated perivascular inflammation (or cuffing) and bronchiolar mucous cell metaplasia, which was corroborated by elevated levels of BALF IL-4 and IL-5. These data are indicative of allergy-associated airway inflammation (Voynow et al., 2004) and support the acute and chronic HDM induced airway inflammation model previously described (Piyadasa et al., 2016).

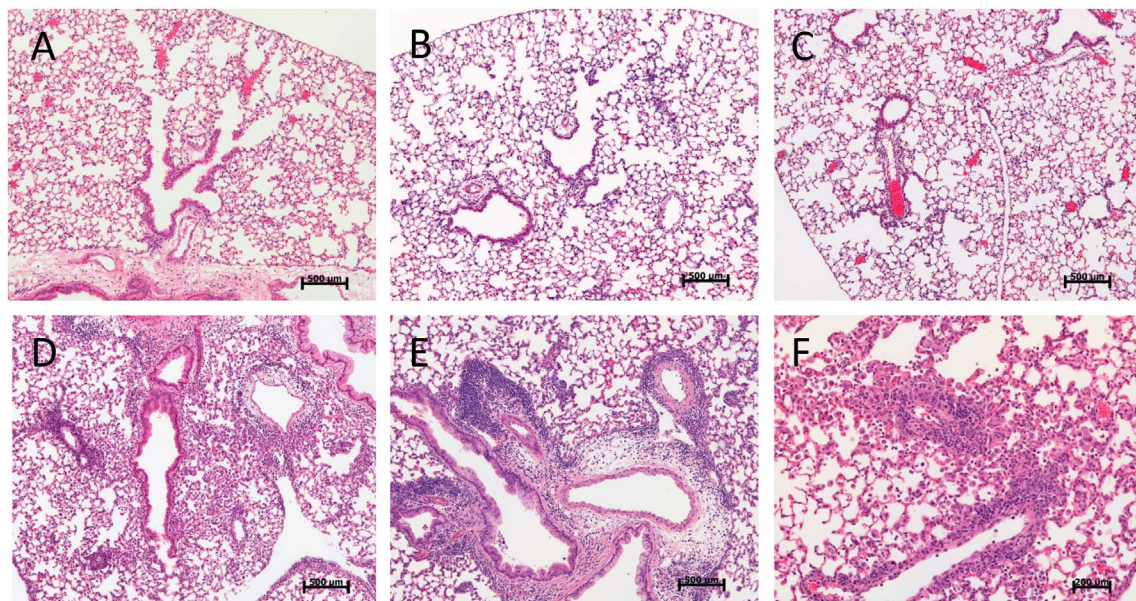


Fig. 4. H&E stained histological lung sections demonstrating scoring for perivascular cuffing. **A.** Normal lung after saline exposure. Category 0 perivascular cuffing. **B.** Lung from HDM/MBCP exposure showing Category 1 perivascular cuffing. The cuffs average one leukocyte in thickness. There is mild alveolitis in adjacent alveoli. **C.** MBCP only exposure showing Category 2 perivascular cuffing. The cuffs average 2-3 cells in thickness. **D.** HDM alone exposure showing Category 3 perivascular cuffing. The leukocytes surrounding blood vessels are about 4-5 cells thick. There is mild alveolitis with macrophages and eosinophils occupying alveoli. The large pale airway epithelial cells represent mucus metaplasia. **E.** MBCP/HDM exposure showing Category 4 perivascular cuffing. These cuffs average 6-15 cells in thickness. There is mucous cell metaplasia of airway epithelium and mild alveolitis. **F.** MBCP filtrate/HDM exposure. These 2 blood vessels have mural inflammation with mononuclear cells and eosinophils infiltrating tunica intima and tunica media. There is accompanying alveolitis.

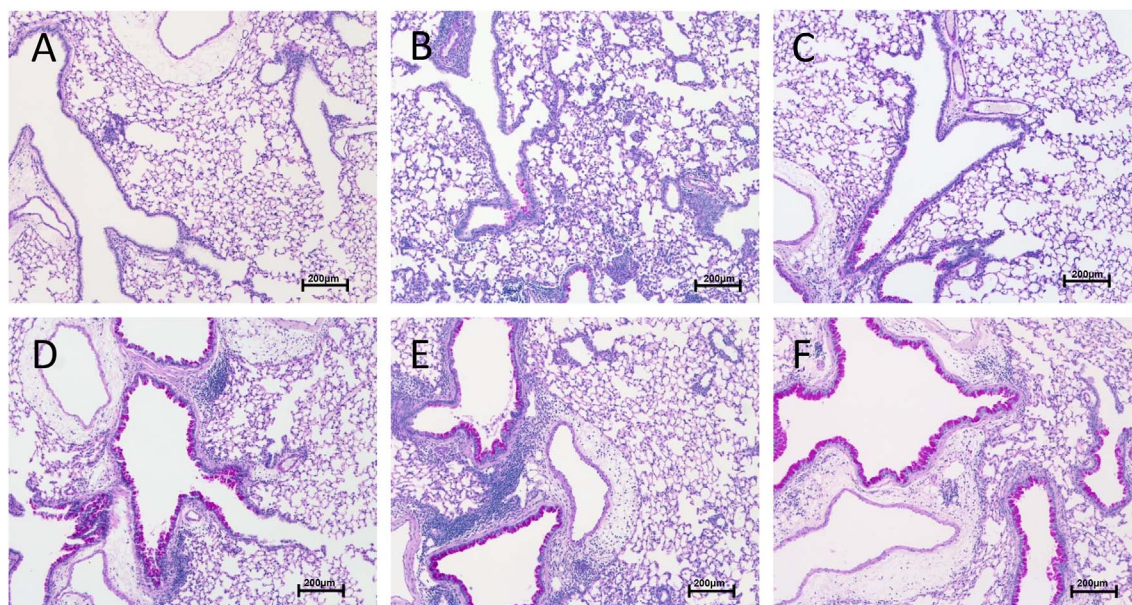


Fig. 5. PAS stained histological lung sections demonstrating scoring for mucous cell metaplasia of bronchiolar epithelium. **A.** Normal lung after saline exposure showing Category 0 mucous cell metaplasia. There are no PAS-positive stained cells. **B.** Lung from MBCP alone exposure showing Category 1 mucous cell metaplasia. Only a few PAS-positive stained cells are apparent. **C.** Lung from HDM/MBCP exposure showing Category 2 mucous cell metaplasia. Frequent groups of PAS stained cells are apparent. **D.** Lung from HDM alone exposure showing Category 3 mucous cell metaplasia. Many cells have PAS staining. **E.** Lung from MBCP/HDM exposure showing Category 3 mucous cell metaplasia. Many cells have PAS staining. **F.** Lung from HDM + MBCP/HDM exposure showing Category 3 mucous cell metaplasia. Many cells have PAS staining.

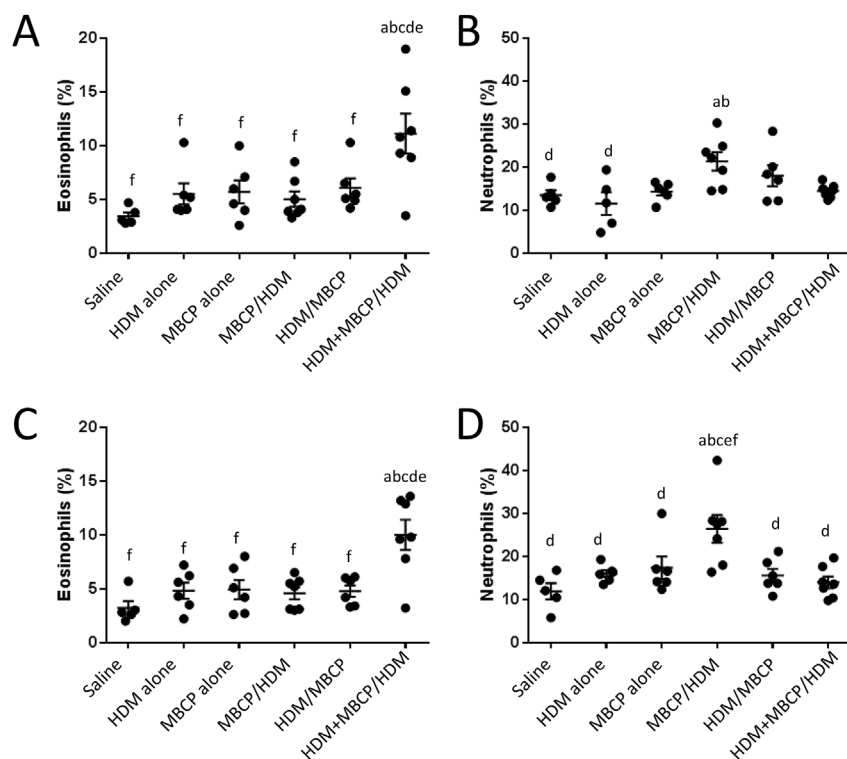


Fig. 6. Alterations in leukocyte levels in blood following MBCP and HDM exposures. Blood was collected by cardiac puncture into EDTA tubes on day 12 following exposure with the indicated treatments. Blood cells were analyzed with a haematology analyzer. Only those leukocytes that demonstrated significant changes compared to other exposures are shown here. (A, B) Whole MBCP was used in the exposures (C, D) MBCP acellular filtrate was used in the exposures. Data were compared using 1-way ANOVA followed by Tukey's multiple comparison test with a value of $p < 0.05$ indicating statistical difference. The 'a' denotes a difference from control, 'b' denotes a difference from HDM alone, 'c' denotes a difference from MBCP alone, 'd' denotes a difference from MBCP/HDM, 'e' denotes a difference from HDM/MBCP, and 'f' denotes a difference from HDM + MBCP/HDM.

During inflammation, the capillaries are the main sites of cellular recruitment, so the perivascular cuffing was consistent with the elevated levels of BALF eosinophils. Furthermore, increased airway mucous producing goblet cells are characteristic of acute allergic inflammation caused by HDM (Bazett et al., 2016) and are indicative of respiratory epithelium that is repeatedly replaced and transformed into a more resistant cell type (Renne et al., 2009).

A hallmark of allergy induction is the production of allergen-specific immunoglobulins, IgE and IgG1 (Warrington et al., 2011). Induction of

IgE was observed when whole MBCP or its filtrate was used for HDM + MBCP/HDM exposures. Yet, neither HDM alone nor MBCP/HDM exposures were able to generate the same level of IgE response. This supports the notion that MBCP is capable of potentiating allergy induction of HDM. HDM alone caused elevation of IgG1 levels, but as mentioned, not IgE. This discrepancy may be because IgG1 production precedes that of IgE during class switching in the early allergic response (Erazo et al., 2007), and because the IgE response is more transient compared to that of IgG1 in mice (Wu and Scheerens, 2014). IgE levels

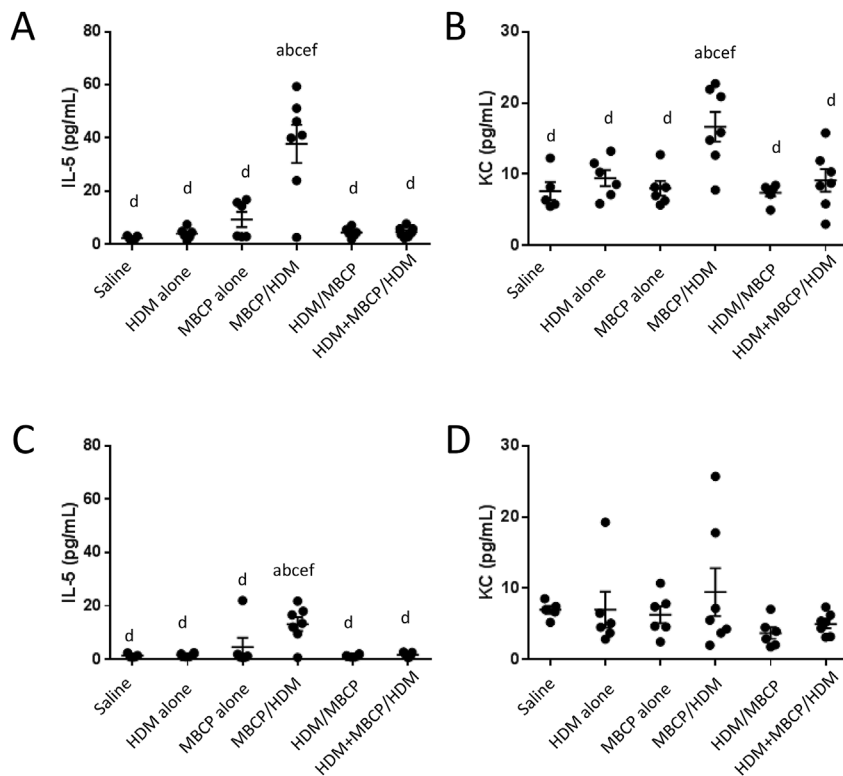


Fig. 7. Levels of plasma cytokines/chemokines following MBP and HDM exposures. Blood was collected by cardiac puncture into EDTA tubes on day 12 following exposure with the indicated treatments. After removal of cells, the plasma was subjected to a 10-plex Bioplex™ bead array cytokine measurement system. Only cytokines that demonstrated statistical differences to other exposures are shown here. (A) Whole MBP was used in the exposures (B) MBP acellular filtrate was used in the exposures. Data were compared using 1-way ANOVA followed by Tukey's multiple comparison test with a value of $p < 0.05$ indicating statistical difference. The 'a' denotes a difference from control, 'b' denotes a difference from HDM alone, 'c' denotes a difference from MBP alone, 'd' denotes a difference from MBP/HDM, 'e' denotes a difference from HDM/MBP, and 'f' denotes a difference from HDM + MBP/HDM.

have been observed to be low with 12 days of HDM treatment compared to longer exposures (Bracken et al., 2015). So, the early expression of IgG1 versus IgE, and high turnover and/or low levels of IgE may have resulted in the lack of HDM-specific IgE detected here.

These results demonstrated that the filterable constituents within MBCPs were responsible for the allergic inflammation potentiating activity observed. Although the exact chemical constituents of this MBCP are not known, several chemicals in cleaning products such as disinfectants, detergents, and bacterial enzymes have been documented to contribute to allergic alveolitis, asthma and rhinitis (Folletti et al., 2014; Rosenman, 2006; Tripathi and Grammer, 2001). For example, mice were exposed to an enzyme used in laundry detergent, Subtilisin Carlesberg (Alcalase), with and without a detergent matrix containing anionic and non-ionic surfactants, silicate builders and perborate bleach (Kawabata et al., 1996). Results demonstrated that endotracheal exposure to the enzyme could elevate levels of specific IgG1 and IgE and that the detergent matrix further enhanced Alcalase-specific antibody levels. Another microbial-derived enzyme, lipase, is also known to be allergenic (Lindstedt et al., 2005). In another study, the anionic surfactant sodium dodecyl sulphate (SDS) and other anionic and non-ionic surfactants were studied for their adjuvant activity and capacity to elicit an IgE response in BALB/c mice using ovalbumin (OVA) as an allergen (Clausen et al., 2000). The SDS, but not other detergents, could

act as an adjuvant after a single booster.

Overall, we were able to demonstrate several interesting observations. However, some limitations with the exposure model should be clarified. This study does not rule out that small microbial constituents resulting from bacterial lysis or cell wall shedding passed through the filters and could have contributed to the immune responses observed. Although this was not tested here, it has been observed by others (Huang et al., 2011) and cannot be excluded as a possibility. Also, only a single time-point was selected for analysis (i.e., 24 h after last exposure), so cytokine, leukocyte and immunoglobulin levels were not all elevated at this time-point for all treatments. This was especially evident with the differential inflammation responses of HDM + MBP/HDM that showed greater eosinophil and neutrophil levels compared to that of MBP/HDM. Conversely, the MBP/HDM response was dominated by elevated expression of cytokines. It is possible that HDM + MBP/HDM caused a more rapid induction of allergic inflammation compared to MBP/HDM. With additional time-points the entire progression of allergic inflammation could have been better resolved. Another observation was that MBP/HDM was more inductive than HDM/MBP, which suggests that the effects from an abbreviated repeated HDM exposure of only five days may be transient. Nevertheless, examining multiple markers (cytokines/chemokines, leukocyte populations, and immunoglobulin levels) in the lung tissue and blood

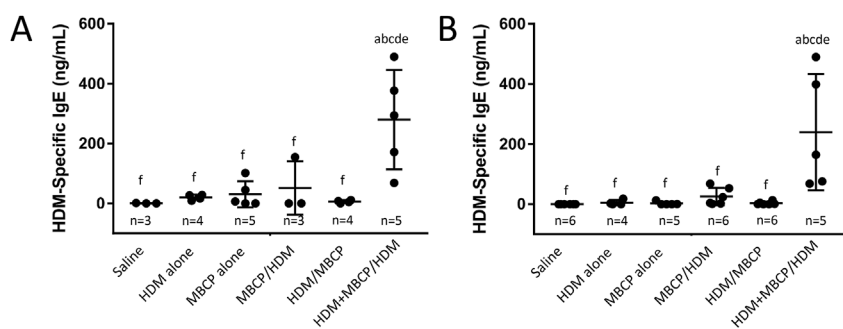


Fig. 8. Levels of HDM-specific IgE following MBP and HDM exposures. Blood was collected by cardiac puncture into EDTA tubes on day 12 following sensitization/challenge with the indicated treatments. After removal of cells, the plasma was used to measure HDM-specific IgE as described in the Methods section. Either whole MBP (A) or MBP acellular filtrate (B) was used in the exposures. Note that sufficient plasma was not available for all measurements, so numbers of replicates (n) are provided for each treatment. Data were compared using 1-way ANOVA followed by Tukey's multiple comparison test with a value of $p < 0.05$ indicating statistical difference. The 'a' denotes a difference from control, 'b' denotes a difference from HDM alone, 'c' denotes a difference from MBP alone, 'd' denotes a difference from MBP/HDM, 'e' denotes a difference from HDM/MBP, and 'f' denotes a difference from HDM + MBP/HDM.

enabled the interpretation that MBCP can function to potentiate the acute allergic lung inflammation to HDM.

5. Conclusions

We have demonstrated using a mouse model for HDM-induced allergic inflammation, that MBCP alone, which contained a mixture of *Bacilli* and proprietary formulation constituents, was not able to induce inflammation, since none of the MBCP challenges alone resulted in elevated pro-inflammatory/T_H2 cytokines or leukocytes involved in inflammation. The strongest reactivity was observed in the MBCP/HDM and HDM + MBCP/HDM groups, indicating that MBCPs could potentiate the acute allergic inflammation to HDM. The results presented here suggest that for the MBCP tested, the microbes present in the product do not pose an added hazard for early HDM-associated allergic inflammation compared to the other formulation components. Further studies could focus on screening other MBCPs, and on an extended exposure time-course to understand the effect of MBCPs on the chronic allergic inflammation response.

Acknowledgements

The authors would like to thank Drs. David Lefebvre and Dalibor Breznán for reviewing the manuscript. We appreciate the assistance of Douglas Parks, Dominique Patry, Scott Smith, and Julie Todd of the Health Canada Scientific Services Division for technical help, animal maintenance, dose administration, haematology analysis, and blood collection. Financial support for this research was provided by Health Canada intramural funds.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fct.2018.02.030>.

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