Biomedicine Hub

Research Article

Biomed Hub 2025;10:8–22
DOI: 10.1159/000542807

Received: May 24, 2024
Accepted: November 12, 2024
Published online: December 4, 2024

Meconium Influences Pulmonary Short-Chain Fatty Acid Concentration in Porcine Meconium Aspiration Model

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Keywords
Meconium aspiration syndrome · Microbiome · Short-chain fatty acids · Propionic acid

Abstract
Introduction: The factors influencing meconium aspiration syndrome (MAS) severity remain poorly understood. In a piglet model of MAS, we hypothesized the respiratory microbiome would reflect the bacterial signature of meconium with short-chain fatty acid (SCFA) accumulation as a by-product of bacterial fermentation. ***Methods:*** Cesarean section at approximately 115-day term was performed on two sows. Male (9) and female (3) piglets were delivered, instrumented, anesthetized, and randomized into a Control (n = 6) or MAS group (n = 6). MAS received a meconium slurry (3 mL/kg) aspiration injury. Experimental animals were monitored continuously, ventilated, and resuscitated for 24 h. BALF was collected for 16S microbiome sequencing and SCFA analysis by gas chromatography. Effects of SCFAs on A549 alveolar pulmonary epithelial in vitro cell viability and inflammation were assessed. ***Results:*** The MAS group had significantly higher fluid and vasopressor requirements than the Control group ($p < 0.05$) though both groups developed lung injury. The meconium microbiome demonstrated a difference in genus proportions as compared with the BALF of the Control and MAS groups. The MAS group had a relative increase in propionic acid-forming bacteria and higher BALF concentrations of propionic acid (0.6 ± 0.2 mmol/kg) than the Control group (0.2 ± 0.2 mmol/kg; $p >$ 0.05). Propionic acid was associated with decreased pulmonary epithelial cell viability and an upregulated pro-inflammatory response. ***Conclusions:*** Meconium may host a microbiome with byproducts that interact with the pulmonary epithelium and influence lung injury severity in MAS.

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Introduction

Meconium aspiration syndrome (MAS) is a disease of the newborn infant caused by the aspiration of meconium-stained amniotic fluid, which leads to localized inflammation and heterogeneous airway obstruction [1, 2]. This results in hypoxemia [1] and decreased lung compliance [3]. MAS causes respiratory distress that ranges from mild to severe. Up to 36% of infants require mechanical ventilation [4, 5], with ECMO cannulation being used in cases of severe refractory hypoxemia [6]. MAS afflicts up to 35,000 newborn infants in the USA annually [7] with mortality rates as high as 37% [8], and long-term morbidities including chronic pulmonary disorders [9, 10], and neurologic sequelae [11, 12].

It is unclear whether the meconium discharge into amniotic fluid should be viewed as a marker of fetal distress and subsequent lung injury or if meconium itself is the primary driver of MAS [1, 13]. Although multiple treatment strategies for MAS have been attempted, including surfactant therapy [14], tracheal suctioning [15–17], mechanical ventilation [18], thinning of the meconium with amnioinfusion [19], and pharmacotherapy [20–25], none have reduced either short-term mortality or long-term morbidity. This may reflect our current need for a better understanding of MAS pathogenesis, particularly regarding the molecular effects of meconium on the pulmonary epithelium.

Less than one-third of infants born with meconium-stained amniotic fluid go on to develop MAS [4] for reasons that remain unclear. However, the incidence of MAS is affected by the mode of delivery [26] and post-gestational age, which also influences the infant microbiome [6]. We hypothesize that microbiome-derived components in meconium may play a role in MAS pathogenesis and tested this idea in a piglet model of MAS through analysis of both the microbiome and its bacterial metabolic byproducts, the short-chain fatty acids (SCFAs) [27].

Methods

This study protocol was reviewed and approved by the SUNY Upstate Medical University Institutional Animal Care and Use Committee (Protocol #506) and in accordance with ARRIVE guidelines. All animal studies were performed at SUNY Upstate Medical University.

Cesarean Section

Cesarean section in two sows occurred at near full-term (115 days) to approximate the physiology of the typical infant delivered with MAS. Prior to cesarean section, each sow was premedicated by intramuscular injection with a mixture of atropine 0.01 mg/kg (MWI, Boise, ID, USA), xylazine 2 mg/kg (Bayer Healthcare Inc., Shawnee Mission, KS, USA), and Telazol™ 5 mg/kg (tiletamine hydrochloride + zolazepam; Dechra Vet Products, Overland Park, KS, USA). During the procedure, the sows were provided intravenous hydration in addition to respiratory support, while hemodynamics and respiratory status were monitored continuously.

Under sterile conditions, a tracheostomy was performed, following which a surgical plane of inhaled anesthesia was maintained with 2% isoflurane gas (Dechra Vet Products, Overland Park, KS, USA). Inhalational anesthetic was used to facilitate rapid recovery of the piglets from the effects of maternal anesthesia.

A standard cesarean section was performed via a ventral midline incision with exposure of a uterine horn. Piglets were removed individually and instrumented immediately (with one sow delivering 13 and the other delivering 14 piglets). Piglets were assigned a consecutive integer value according to the order of delivery and had been pre-randomized into a Control (n = 6) and MAS (n = 6) group. The veterinarian delivering the piglets was blinded to the grouping strategy and did not participate in the experimental protocol. To limit bias, no animals were excluded. Any additional piglets in the litter after the n = 6 per group was achieved served as a Naïve group (n = 15) and were euthanized with intravenous sodium pentobarbital 150 mg/kg (Akorn Inc., Lake Forest, IL, USA) injected through the umbilical vein, following which necropsy was performed. Following euthanasia, meconium was extracted from the intestines of the Naïve group in a sterile fashion, to be used for the MAS group. After all the piglets were delivered, maternal blood was obtained and the sow euthanized with intravenous sodium pentobarbital 150 mg/kg (Akorn Inc., Lake Forest, IL, USA).

Piglet Instrumentation

Upon the delivery of a piglet, the airway was suctioned, the piglet weighed, and placed on a heating mat under a heating lamp. One percent lidocaine was injected subcutaneously into the neck and a tracheostomy performed with a 3.0 Fr endotracheal tube. Piglets were immediately connected to a mechanical ventilator (Dräger Infinity V500 ventilator, Lübeck, Germany) with sterile tubing and ventilated with volume guarantee (VG) settings (as described under *Ventilation Settings*). An umbilical artery catheter (3.5 Fr Argyle™, Covidien, MA, USA) was placed for blood pressure monitoring and arterial blood sampling, and an umbilical vein catheter (3.5 Fr Argyle™, Covidien, MA, USA) was placed for fluid administration.

An esophageal catheter was passed into the stomach for decompression. Following instrumentation, the piglets were placed in a neonatal isolette (Dräger Babytherm 8010, Lübeck, Germany) with temperatures adjusted to maintain piglet body temperature at 37.5 ± 0.5°C.

Piglet Resuscitation and Intensive Care

Each piglet received a single prophylactic dose of cefazolin 50 mg/kg/dose (MWI Animal Health, Boise, ID, USA), administered via the umbilical vein catheter. Maternal plasma (5 mL/kg, IV) was administered through the catheter to provide passive immunity and compensate for the absence of colostrum. Each piglet was administered hourly fentanyl 2 μg/kg/h (MWI Animal Health, Boise, ID, USA) and midazolam 6 μg/kg/h (MWI Animal Health, Boise, ID, USA) titrated to maintain a continuous plane of anesthesia. Parenteral nutrition (Clinimix™ E2.75/10, Baxter, Deerfield, IL, USA) was provided continuously at a rate of 4 mL/kg/h, beginning immediately after placing the umbilical vein catheter.

Boluses of Lactated Ringer's 10 mL/kg (Baxter, Deerfield, IL, USA) or albumin 10 mL/kg (MWI Animal Health, Boise, ID, USA) were provided as necessary to maintain a goal mean arterial pressure of 35–45 mm Hg. Vasopressors (dopamine, epinephrine) were administered to piglets that became unresponsive to fluid resuscitation. Piglets demonstrating evidence of spontaneous breathing received rocuronium 10 μg/kg/min (X-GEN Pharmaceuticals, Big Flats, NY, USA) to ensure standardization of mechanical ventilation. Blood pressure, pulse, and oxygen saturation were monitored continuously (Dräger Infinity Delta XL, Lübeck, Germany) and recorded hourly. At baseline and every 4 h, arterial blood gases were measured (Cobas 221 blood gas analyzer, Roche Diagnostics, Indianapolis, IN, USA). The bladder was decompressed manually every 4 h. The piglets were repositioned every 4 h from one side to the other to avoid dependent edema.

Ventilation Settings

Upon receiving a tracheostomy, animals were initiated on VG with Vt of 6 mL/kg, inspiratory time of 0.3 s, respiratory rate of 40 breaths per min, PEEP 5 cm H_2O, and FiO_2 50%. Respiratory rate was titrated according to $PaCO_2$ (goal of 35–50 mm Hg), and PEEP and FiO_2 were titrated according to PaO_2, SpO_2 (goal SpO_2 88%–94%), and respiratory system compliance. If FiO_2 requirements increased in response to decreasing PaO_2 or SpO_2, PEEP was also incrementally increased.

Meconium Aspiration Injury

Meconium (30 mg) was retrieved from the intestines of Naïve animals in a sterile fashion, pooled, and diluted with 10 mL of normal saline. The meconium slurry (3 mL/kg) was administered by a suction catheter inserted through the endotracheal tube to animals in the MAS injury group, following a previously validated injury protocol [28, 29]. The Control group had normal saline (3 mL/kg) administered in a similar fashion. The animals were rotated to the right and left lateral decubitus positions, with half of the solution administered to each side.

Necropsy

After 24 h, the experimental protocol was terminated. The animals were euthanized with an overdose of 150 mg/kg sodium pentobarbital (Akorn Inc., Lake Forest, IL, USA) and necropsy performed. Lungs were removed under sterile conditions and inflated to 20 cm H_2O, following which the endotracheal tube was clamped to standardize the lung pressures. The bronchus to the right lower lobe of the lung was isolated and tied off, with the lobe then preserved in formalin by immersion for subsequent histopathologic analysis after hematoxylin and eosin staining. The right middle lobe bronchus was isolated and tied off, with removal of the right middle lobe. This right middle lobe was weighed (wet weight) and allowed to desiccate in an oven with daily weights. When there was a difference of less than 0.1 g between two consecutive daily weights, this was recorded as the final dry weight and compared as wet/dry weight. Bronchoalveolar lavage fluid (BALF) was collected by advancing the endotracheal tube into the left mainstem bronchus, instilling 10 mL of normal saline (as SCFAs are detectable in water samples), withdrawing the fluid and storing at −80°C [30]. BALF was collected from both experimental groups (Control and MAS) as well as from the Naïve piglets following euthanasia.

Microbiome

Microbiome sequencing was performed on the ventilator circuit, meconium, and BALF from the three piglet groups (Naïve, Control, and MAS). The ventilator circuit sample served as an environmental control and was obtained by collecting air from the inspiratory limb of the ventilator into a sterile cup filled with saline. Sequencing was performed by Microbiome Insights according to their protocol accredited by the College of American Pathologists. In this protocol, DNA is captured and extracted by magnetic beads (Qiagen MagAttract PowerSoil DNA KF Kit, Hilden, Germany) while excluding organic inhibitors. DNA quantification and quality checks are

performed by Qubit. High-fidelity Phusion polymerase is used to amplify 16S marker genes, following which PCR was performed with dual-barcoded primers targeting the prokaryotic regions. PCR reactions are normalized using high-throughput SequalPrep™ (ThermoFisher Scientific, Waltham, MA, USA) and samples pooled to create a single library that is subsequently quantified (KAPA qPCR Library Quant kit, Sigma-Aldrich Inc., St. Louis, MO, USA). Samples that contained a dominant bacterium (>80%) or had low α-diversity were excluded from analysis due to contamination concerns.

Short-Chain Fatty Acids

The three SCFAs: acetic acid, butyric acid, and propionic acid [31], as well as the branched-chain fatty acid, isobutyric acid, were analyzed by Microbiome Insights (Vancouver, BC). Fatty acids were extracted from each aqueous sample and analyzed in a gas chromatograph with a flame ionization detector using a Thermo TG-WAXMS A GC column (ThermoFisher Scientific, Waltham, MA, USA). The analytes were quantified against a series of stock standard solutions.

Epithelial Cell Culture

The immortalized human pulmonary cell line A549 alveolar pulmonary epithelial cells (AECs) were obtained from ATCC (CRM-CCL-185, Manassas, VA, USA). These cells were cultured in DMEM media (11965092, Gibco, Grand Island, NY, USA) containing 1% Penicillin-Streptomycin antibiotic solution (15070063, Gibco, Grand Island, NY, USA) and 10% fetal bovine serum (A4766801, Grand Island, NY, USA) under sterile conditions, and maintained at 37°C and 5% CO_2 gas. Varying concentrations of acetic acid (Fisher Scientific, Catalog # A38-500), isobutyric acid (Fisher Scientific, Catalog # AAL00AP), and propionic acid (Fisher Scientific, Catalog # A258-500) were added to the cultured pulmonary epithelial cells. The pH of each culture was measured with an Ultrabasic UB-5 pH meter (Denver Instruments, Bohemia, NY, USA). In vitro cell viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay (ab211091, Abcam, Waltham, MA, USA). Cytokine analysis of TNF-α (88-7346-88, ThermoFisher Scientific, Waltham, MA, USA), IL-1β (88-7261-88, ThermoFisher Scientific, Waltham, MA, USA), IL-6 (88-7066-88, ThermoFisher Scientific, Waltham, MA, USA), and HMGB1 (NBP2-62766, Novus Biologicals, Centennial, CO, USA) was performed by ELISA according to manufacturer's (ThermoFisher Scientific, Waltham, MA, USA) instructions (https://www.thermofisher.com).

Statistical Analysis

Sample sizes were designed based on our previous study of pre-term neonatal piglets in which $n = 5$/group yielded 90% power to detect a mean difference in PaO_2/FiO_2 of 30.8 mm Hg by 24 h using two-sample t test [32]. It was assumed that $n = 5$/group would also provide adequate power to detect accumulation of SCFAs with meconium aspiration. Since sows have litter sizes ranging from 12 to 28 piglets [33], two sows were deemed sufficient to provide enough piglets for the MAS, Control, and Naïve groups. Data are reported as mean ± SEM. Repeated measures ANOVA was used to compare differences within and between treatment groups for continuous parameters and post hoc Tukey's tests if significance was found in the group-time effect. One-way ANOVA was used to compare SCFAs in BALF as well as the effect of SCFAs on pulmonary epithelial cell cytokine upregulation. Time to vasopressors was compared with a log-rank test. p values <0.05 were considered significant. Analyses were performed using SPSS v. 26 software program (IBM, Armonk, NY, USA).

Results

Characteristics

Of the 12 piglets included in the study, 3 were female and 9 were male (Control: 1 female, 5 males; MAS: 2 females, 4 males). Fifteen piglets served as naïve controls (7 male, 8 female) and as sources of meconium to establish the meconium injury in the experimental groups. Piglets in the MAS group were smaller (1.3 ± 0.1 kg) than in the Control group (1.6 ± 0.2 kg), but this was not significant ($p > 0.05$).

Hemodynamics

All the animals survived the 24-h study duration. Heart rate ($p = 0.05$; Fig. 1a) and mean arterial pressure ($p = 0.05$; Fig. 1b; Table 1) were not significantly different between groups. However, the MAS group had significantly higher fluid requirements ($p < 0.0001$; Fig. 1c), with all animals requiring vasopressors by T8 compared with the Control group in which only half required vasopressors by T15 ($p < 0.05$; Fig. 1d).

Respiratory

By the end of the study, respiratory system compliance in the Control group was greater than in the MAS group, but not significantly so ($p > 0.05$; Table 1). The tidal volumes between the two groups were controlled, and thus similar ($p > 0.05$), and generated similar plateau

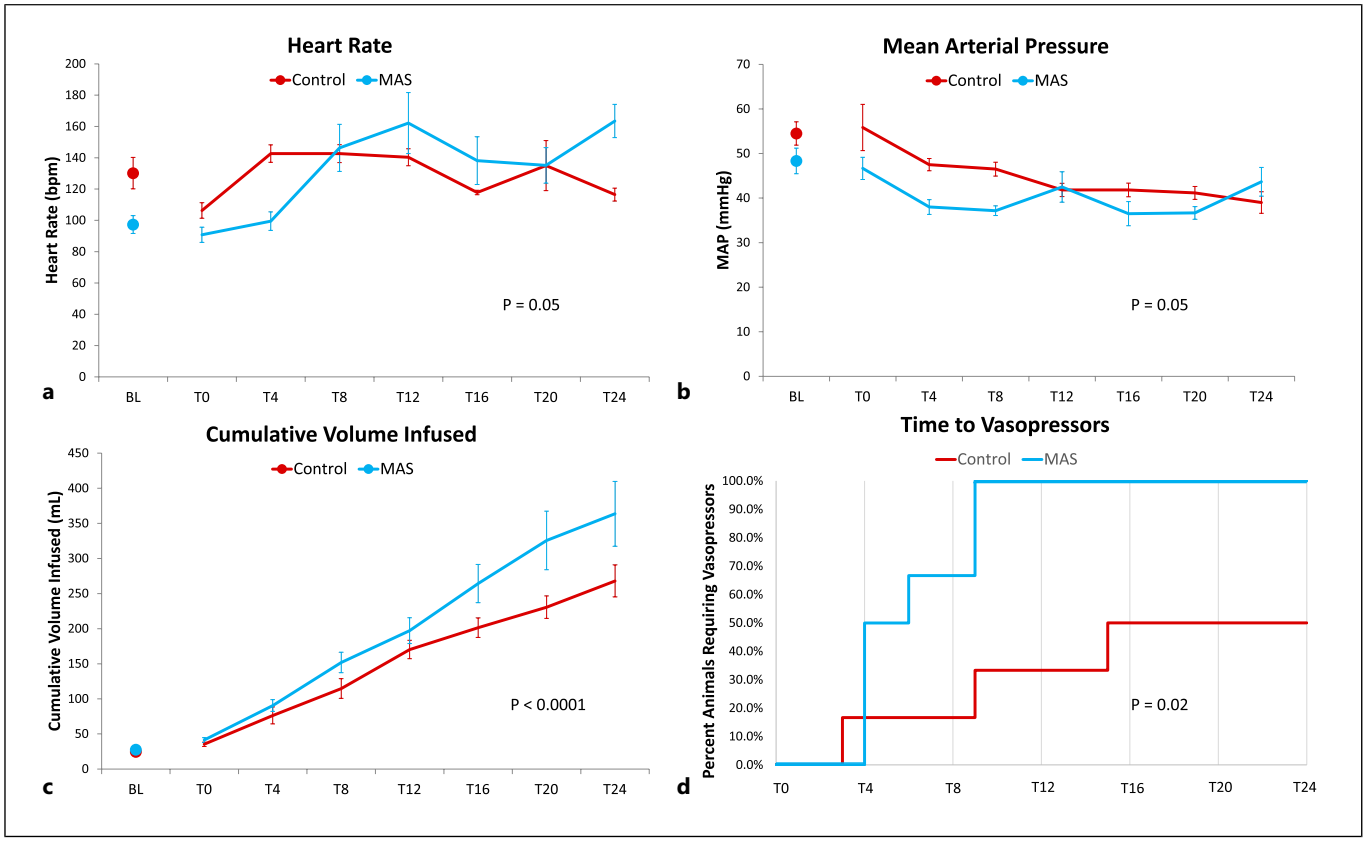


Fig. 1. Hemodynamics. Comparison of heart rate (**a**), mean arterial pressure (**b**), fluid (**c**), and vasopressor requirements (**d**) in the Control group (red) with no injury and the MAS group (blue; $n = 6$ per group). BL, baseline; T0, represents timepoint just after injury in the MAS group with recordings reported every 4 h until the experiment end (T24).

pressures ($p > 0.05$). Both groups developed mild acute respiratory distress syndrome by the end of the study, with a PaO_2/FiO_2 ratio of 277.3 ± 41.0 mm Hg in the Control group and 282.6 ± 55.6 mm Hg in the MAS group ($p > 0.05$), and with similar oxygen requirements ($p > 0.05$). Despite being uninjured, the Control group was unexpectedly more challenging to ventilate leading to a higher $PaCO_2$ (81.1 ± 14.2 mm Hg) as compared with MAS animals (63.8 ± 8.0 mm Hg; $p > 0.05$) though the groups had a similar pH (Control: 7.2 ± 0.1; VG: 7.3 ± 0.0; $p > 0.05$).

Histology

There was no significant difference in lung wet-dry weight ratio between the Control (6.5 ± 0.2) and MAS (6.0 ± 0.2) groups ($p > 0.05$), though the Naïve group had a significantly higher ratio (10.1 ± 0.1; $p < 0.0001$). There were qualitative differences in alveolar histology between the Control and MAS groups (Fig. 2). The MAS group had meconium plugs in bronchioles extending into alveoli. The associated lobules demonstrated concomitant atelectasis compared to the Control group, which had no meconium plugs and showed minimal atelectasis.

Microbiome

The genetic diversity (α-diversity) was measured in the BALF of Control and MAS groups, the ventilatory circuit, and the meconium. The α-diversity of BALF retrieved from all animals ranged from 0.4 to 4.2. The three samples with the lowest α-diversity were contaminated by >80% Staphylococcus and Streptococcus (one Control, two MAS) and were eliminated from the analysis (Fig. 3a).

The ventilator circuit served as an environmental control and was found to have a similar microbial profile as compared with the piglet BALF of all groups (Fig. 3b). BALF from Naïve animals had no detectable microbiome signature.

Table 1. Hemodynamic and respiratory parameters

	Baseline	T0	T6	T12	T18	T24	*p* value
Heart rate, beats/min							0.05
Control	130.2±10.1	106.3±4.9	136.5±4.2	140.3±5.4	134.5±10.2	116.5±4.1	
MAS	97.3±5.7	90.8±4.9	121.5±6.6	162.2±19.5	130.2±12.0	163.5±10.6	
Mean arterial pressure, mm Hg							0.05
Control	54.5±2.6	55.8±5.2	43.2±1.7	41.8±1.5	43.2±1.3	39.0±2.4	
MAS	48.3±2.9	46.7±2.5	40.2±0.8	42.5±3.4	39.5±3.1	43.7±3.2	
Plateau pressure, cm H_2O							0.33
Control	17.8±1.1	16.7±1.7	13.8±1.2	12.8±0.6	12.5±0.7	14.1±1.4	
MAS	17.3±1.0	19.0±1.8	15.0±1.9	12.6±1.8	13.2±1.7	14.5±1.3	
Tidal volume, mL/kg							0.68
Control	5.7±0.2	5.8±0.2	5.5±0.7	6.2±0.3	6.1±0.7	5.9±0.4	
MAS	6.0±0.1	5.8±0.2	5.9±0.1	6.3±0.2	6.2±0.2	6.1±0.2	
Compliance, L/cm H_2O							0.42
Control	0.8±0.2	0.8±0.2	1.4±0.4	1.8±0.3	1.9±0.4	1.8±0.4	
MAS	0.6±0.0	0.5±0.1	1.0±0.2	1.3±0.4	1.4±0.4	1.1±0.2	
Total fluids, mL							<0.0001
Control	24±2	35±3	96±12	170±13	216±15	268±23	
MAS	28±4	41±3	122±11	197±18	307±40	364±46	
FiO_2, %							0.002
Control	0.5±0.0	0.5±0.0	0.4±0.0	0.4±0.0	0.5±0.0	0.5±0.0	
MAS	0.5±0.0	0.5±0.0	0.4±0.0	0.4±0.0	0.3±0.0	0.5±0.1	
PaO_2/FiO_2 ratio, mm Hg							0.48
Control	288.7±20.2	307.5±17.1		264.3±59.0		277.3±41.0	
MAS	335.5±22.3	253.8±23.7		311.8±26.1		282.6±55.6	
pH							0.48
Control	7.2±0.1	7.2±0.1		7.3±0.1		7.2±0.1	
MAS	7.3±0.1	7.3±0.1		7.3±0.1		7.3±0.0	
$PaCO_2$, mm Hg							0.52
Control	79.0±11.4	81.4±12.0		75.1±18.0		81.1±14.2	
MAS	59.0±8.5	61.9±8.9		57.8±9.6		63.8±8.0	

$N = 6$ per group. $p < 0.05$ is considered significant; however, there were no significant differences at individual time points. MAS, meconium aspiration syndrome.

The meconium samples were the only ones with an appreciable difference in bacterial population. The meconium samples were found to have a lower α-diversity than the remaining samples, including both the ventilator circuit samples and those obtained by BALF from the piglet groups, suggesting that meconium has a microbial presence but that the range of microbes increased upon exposure to the extrauterine environment. Furthermore, bacterial isolates known to generate propionic acid fermentation byproducts (Veillonella, Fusobacterium, and Clostridiaceae) [34] were found in relatively higher concentrations in meconium (Fig. 3c). Specifically, meconium had a relatively higher abundance of Veillonella (11.7% versus 0.5% in the Control group and 0.3% in the MAS group), Fusobacterium (3.4% versus 0.3% in the Control group and 0.06% in the MAS group), and Clostridiaceae (3.6% versus 0% in the Control group and 0.07% in the MAS group). The MAS group had a relatively higher proportion of Muribaculaceae (6.3% compared with 1.4% in the Control group and 0.09% in meconium), which is also a known propionic acid producer [35]. Finally, the Control group had a relatively higher representation of Clostridiales (3.6% versus 1.7% in MAS and 0.3% in meconium) in the BALF. Other microbial species that generate propionic acid during fermentation, including Propionibacteria, Selenomonas, Megasphera, and Salmonella, were not observed in any of the samples [34].

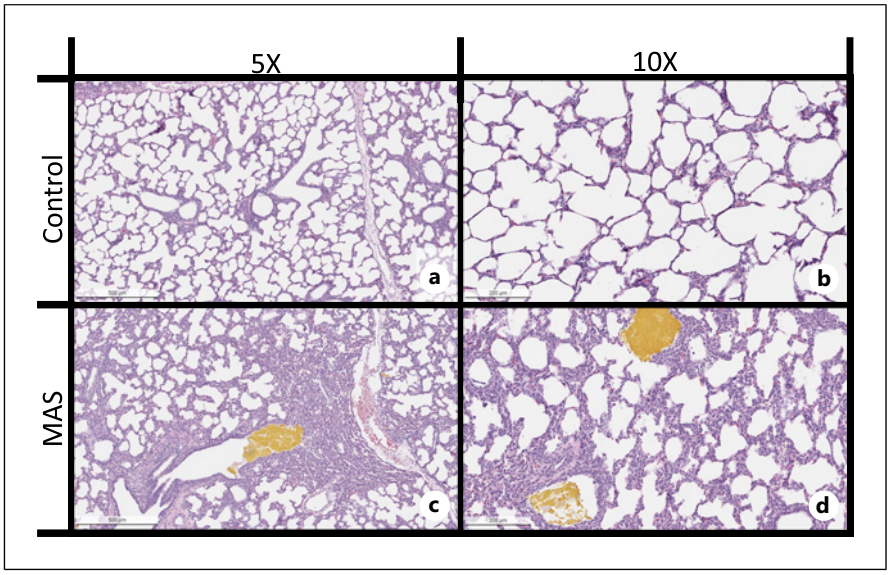


Fig. 2. Lung histology with meconium aspiration syndrome. Control group at lower-power (5×; **a**) and higher-power (10×; **b**) magnification demonstrating open bronchioles and minimal atelectasis in histopathology with hematoxylin-eosin staining. MAS group at lower-power (5×; **c**) magnification demonstrating meconium plugs (sepia) in bronchiole with alveolar collapse in the associated lobule and higher-power (10×; **d**) magnification demonstrating distal alveolar duct meconium plug with associated atelectasis.

SCFAs in BALF

Piglet meconium diluted with PBS to a concentration of 100 mg/mL was found to have a pH of 7.54. Acetic acid concentration was higher in the MAS group as compared with the BALF of the Naïve and Control groups ($p = 0.05$; Fig. 4a). Isobutyric acid was present in the MAS group, albeit at low levels, but was undetectable in the Naïve and Control animals ($p < 0.05$; Fig. 4b). Propionic acid was higher, but not significantly, in the MAS group as compared with the Control group and was not present in the Naïve unventilated piglets ($p > 0.05$; Fig. 4c). Butyric acid was present in low concentrations in all piglet BALF ($p > 0.05$; Fig. 4d).

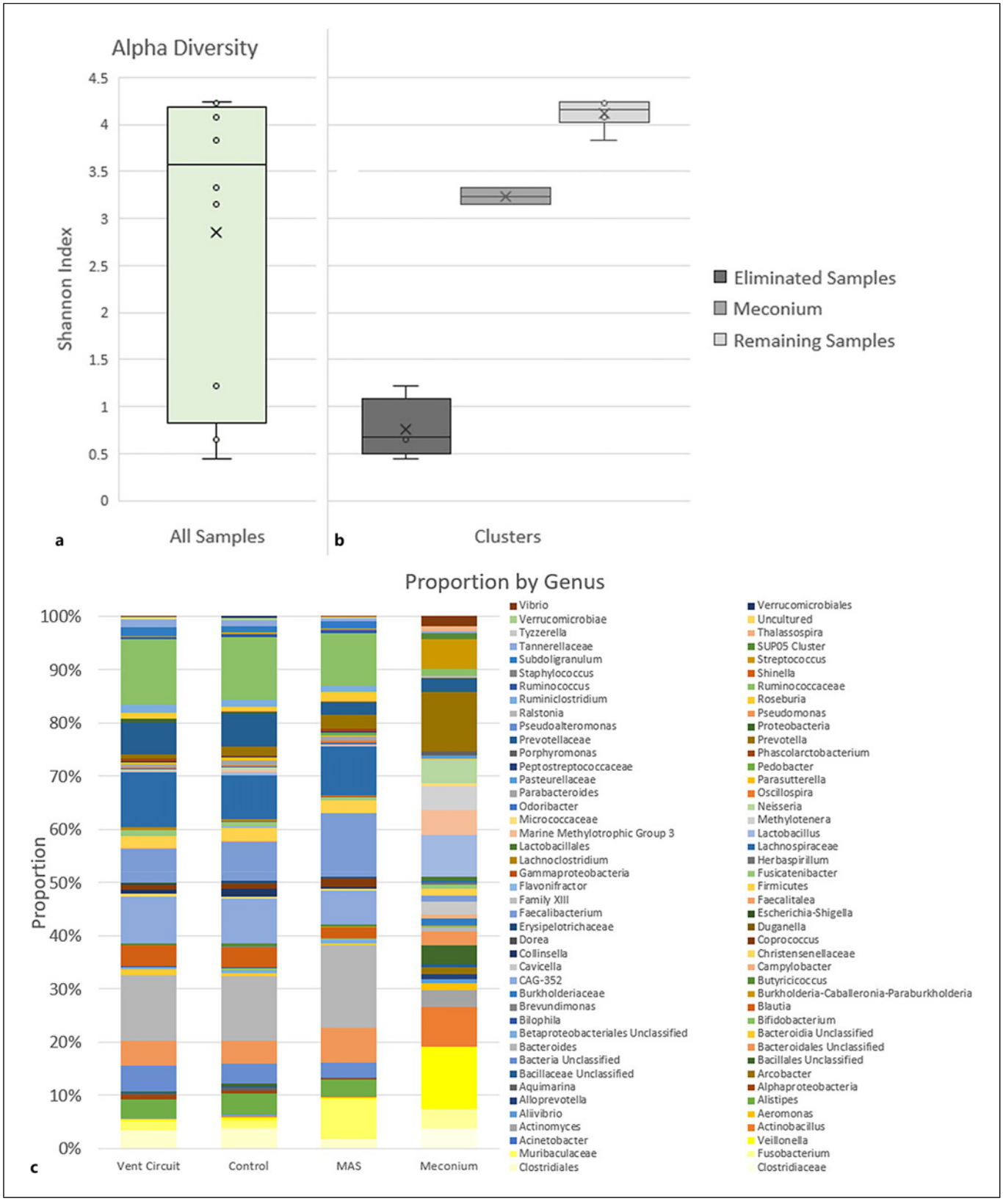
Effect of SCFAs on Pulmonary Epithelial Cell Viability and Inflammation

Isobutyric acid had a marginal effect on in vitro AEC viability with 98.5% viability at concentrations up to 10 mM (Fig. 5a). Acetic acid started impacting cell viability at concentrations above 25 mM, where there was a decrease in cell viability from 95% to 13% at 50 mM. Propionic acid had the most pronounced effect on AEC viability, with 85% cell viability at propionic acid concentrations as low as 10 mM and 13% cell viability at a propionic acid concentration of 25 mM. SCFA pH according to concentration was tested to determine whether cell viability was related primarily to underlying pH as opposed to other fatty acid properties. The pH of propionic acid at a concentration as high as 10 mM, which was affiliated with decreased in vitro cell viability, was 6.65. At the highest concentration of isobutyric acid tested (2.5 mM), the pH was measured at 7.35. Acetic acid produced the lowest pH at a concentration of 10 mM (pH 6.56) and 25 mM (pH 5.66).

Propionic acid had the greatest impact on TNF-α (Fig. 5b), IL-1β (Fig. 5c), and IL-6 (Fig. 5d). Isobutyric acid was associated with a more modest increase in IL-6, whereas acetic acid demonstrated a relatively large increase in IL-1β (Fig. 5). HMGB1 is a non-histone chromatin-associated protein that is actively released in the setting of inflammation (TLR-4- and RAGE-mediated) and passively released upon cell death [36]. Propionic acid upregulated HMGB1 to a greater extent than acetic or isobutyric acid (Fig. 5e).

Discussion

This study validates an established porcine model of MAS that replicates the clinical features of lung injury with PaO_2/FiO_2 <300 mm Hg and hemodynamic instability (Fig. 1), with histopathology demonstrating meconium plugs with associated atelectasis (Fig. 2). There are three important findings from this study. First, meconium harvested in a sterile fashion from naïve piglets was observed to have a microbial signature (Fig. 3). Second, though meconium aspiration did not markedly affect the piglet lung microbiome, propionic acid concentration was altered (Fig. 4). Third, SCFAs were found to both impact cell viability and induce a pro-inflammatory response (Fig. 5). Cumulatively, these findings suggest that a microbial presence and SCFA



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byproducts may exist in MAS and contribute to the evolution of lung injury.

Of all the samples sequenced, meconium was the only one that revealed a distinct microbial signature (Fig. 3). The remaining samples had similar microbiomes, suggesting they reflected the bacterial signature of the analysis kit and the laboratory environment rather than bacteria colonizing the samples. This is a particular risk in specimens with low bacterial abundance and was likely exacerbated by the dilution of the samples with saline in the process of obtaining BALF [37, 38]. The meconium microbiome finding is interesting, albeit historically controversial, and remains debated. Theodor Escherich first described meconium as "free of viable bacteria" in 1885 [39], a finding supported through the 20th century, where most meconium isolates were found to be sterile based on standard culture techniques [40, 41]. With 16S sequencing, this is no longer an incontrovertible fact. What is clear is that if meconium does indeed harbor a microbiome, it must have scarce microbes, which makes finding reliable evidence of its presence more challenging.

If meconium is sterile, it naturally follows that the womb must also be sterile. In support of this, the infant stool microbiome varies according to the delivery method, suggesting that it is the influence of the vaginal microflora, and not the womb, that plays a dominant role in the subsequent microbiome [26]. Another supporting argument is that germ-free rodents have been delivered from mothers with a normal microbiome. This would not be expected if bacteria were present in their meconium because this would lead to quick colonization [42]. Opposing the argument for a sterile meconium microbiome is that humans develop an immune cell repository while in the womb, and there is evidence that amniotic fluid hosts a microbiome [43]. One study suggesting that the womb may not be sterile revealed that 27% of placentas have intracellular bacteria present in the basal plate [44]. Most compelling, however, is that when pregnant mice were orally inoculated with genetically labeled *E. fecium*, the bacterium was found in both the amniotic fluid and meconium of their babies delivered by cesarean section [45, 46]. The presence of microbiota in meconium in utero could therefore be the result of microbial presence in amniotic fluid. As infants swallow amniotic fluid, the gut would be populated by the same microbiome, which would then be present in the meconium.

Free fatty acids have previously been implicated in meconium-induced pulmonary inflammation and surfactant dysfunction [47, 48]. The finding of SCFAs, a bacterial fermentation byproduct [27], in BALF (Fig. 4) is intriguing because they are not native to the lung but also offers a potential therapeutic target using fatty acid binding agents [48]. Propionic acid, in particular, was present in meconium and in the animals subjected to meconium aspiration but not in the Naïve group. Propionic acid was present at lower concentrations in the Control group, but this could have reflected colonization independent of the MAS injury. Also, organisms that ferment propionic acid, including Veillonella, Fusobacterium, and Clostridia [34], were more abundant in the meconium samples [37, 38]. Finally, propionic acid led to the greatest pro-inflammatory response and impairment in cell viability (Fig. 5). Thus, although a causal relationship between microbiome alterations and the changes noted in SCFAs, specifically propionic acid, has not been established, the relative increases in propionic acid forming bacteria suggest an association.

Bacterial fermentation byproducts have previously been established in conditions of pulmonary dysbiosis and as a modifier of disease pathology. In conditions such as cystic fibrosis where there is a relatively hypoxic environment associated with disease, facultative anaerobic bacteria have an opportunity to proliferate and produce SCFA byproducts [49]. Furthermore, anaerobic microorganisms with the ability to form biofilms might allow for survival of lung-resident bacteria and subsequent production of SCFAs [50]. Sputum neutrophil counts and alveolar epithelial cell derived pro-inflammatory cytokines have been shown to correlate with SCFA concentration, which is reduced in patients with cystic fibrosis after treatment with antibiotics [49]. Other conditions of pulmonary dysbiosis, such as in patients with HIV on antiretroviral therapy, have been associated with bacteria overgrowth and higher concentrations of SCFAs, specifically butyric and propionic acids [50].

Fig. 3. Microbiome. **a** Boxplot of α-diversity clusters of all samples. Bottom and top whiskers represent the minimum and maximum values. Box represents first and third quartile. Line within max is the median value. **b** The three eliminated samples due to contaminants (dark gray) had the lowest Shannon index as compared with meconium (medium gray) and the remaining samples including control samples and piglet BALF (light gray). **c** Microbial proportion by genus in the ventilator circuit (n = 5 samples) compared with meconium (n = 8 samples), Control animals mechanically ventilated for 24 h (n = 6), and animals that underwent a meconium aspiration injury (MAS; n = 6). Bacteria known to yield propionic acid fermentation byproducts are demarcated in yellow. The Naïve group did not demonstrate any microbial presence and so was not included.

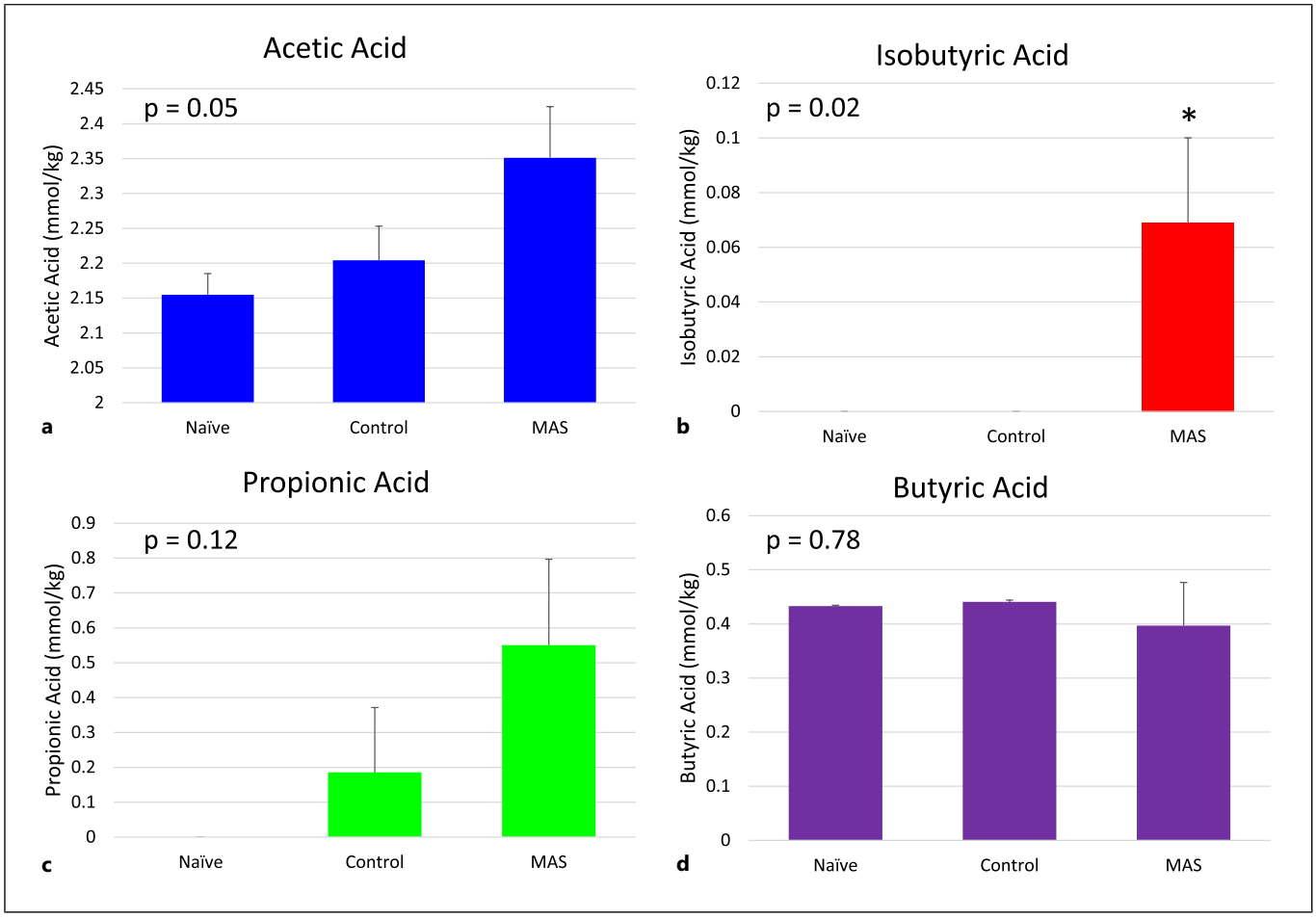

Fig. 4. SCFA quantified from bronchoalveolar lavage fluid (BALF). Acetic acid (**a**), isobutyric acid (**b**), propionic acid (**c**), and butyric acid (**d**) in BALF from Naïve animals (sacrificed immediately after birth; $n = 6$ samples), Control animals (ventilated for 24 h; $n = 6$), and animals with meconium aspiration injury (MAS; $n = 6$). Data reported as mean ± SEM. *$p < 0.05$ versus all other groups.

In aspiration lung injury, the pulmonary consequences are most often attributed to the low pH in gastric fluid that leads to pneumonitis with direct alveolar epithelial injury [51] and stripping of surfactant [52]. Specifically, pH ≤6.5 has been associated with a significant decrease in cell viability [51]. However, in this study, piglet meconium was found to have a relatively neutral pH, suggesting that acidity was not a driving factor in MAS development.

Surfactant dysfunction is a known driver of MAS, even in the early phases [53], though this was not specifically studied. Secretory phospholipases A_2 ($sPLA_2$) have also been implicated in MAS pathophysiology by directly damaging the epithelial cells [54], causing airway contraction likely through a cyclooxygenase-mediated pathway [55], and mediating surfactant dysfunction [53]. $sPLA_2$ is of particular interest in MAS because it is not only present in meconium as the pancreatic sub-type (IB) but is also found in BALF of neonates with MAS [56]. $sPLA_2$ has been implicated in surfactant phospholipid hydrolysis, and as a driver of inflammation by activating arachidonic acid release [53, 57]. Furthermore, sPLA2 (sub-type IIA), which has been correlated with oxygen impairment and edema in MAS [56], has also been noted to have bactericidal activity, and is involved in modifying both the gut and pulmonary microbiota [58]. Though phospholipases and surfactant were not specifically studied, this represents a future target area to better understand whether and how the phospholipases may be interacting with the pulmonary microbiome and byproducts.

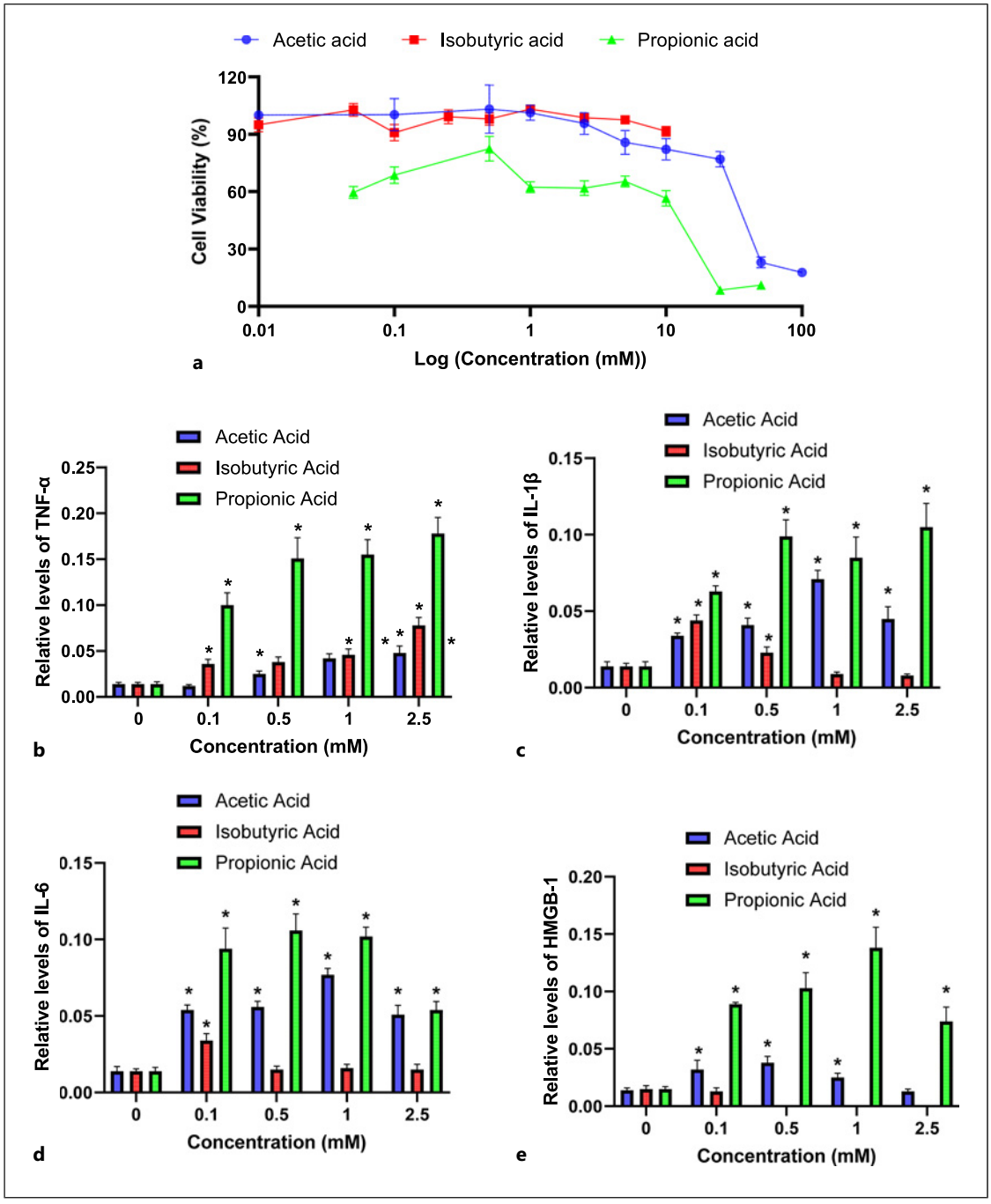

Fig. 5. Inflammatory effect and viability of alveolar pulmonary epithelial cells with SCFA exposure. Pulmonary alveolar epithelial cells were cultured in a 96-well plate with acetic acid (0.1–100 mM), isobutyric acid (0.01–10 mM), and propionic acid (0.05–50 mM) for 24 h in serum-free media to determine cell viability by MTT assay (**a**). The culture media were collected for the determination of TNF-α (**b**), IL-1β (**c**), IL-6 (**d**), and HMGB-1 (**e**) by ELISA. Data reported as mean ± SEM, n = 4–6/group. *$p < 0.05$ vs. control (0 mM).

SCFAs may play an essential role in lung immunometabolic tone [59] by way of cell activation [60], host defense regulation by histone deacetylase inhibition [50], and G protein-coupled receptor activation [61]. Although pulmonary dysbiosis influences SCFA concentration and increases infection susceptibility [62], this is likely

concentration dependent. Congruent with our findings, both acetic acid and propionic acid have been affiliated with IL-6 upregulation [49], where IL-6 has been associated with loss of epithelial barrier integrity [63, 64]. However, butyric and propionic acid have also been shown to have a protective effect on the lung, including maintenance and repair of epithelial barrier function [65]. Thus, SCFAs may regulate inflammation and influence bacterial growth in response to their concentration and affiliated disease state.

Limitations

This study is limited by the challenge of sequencing the meconium microbiome, recognizing that bacteria, if present, would occur in low abundance. Thus, our study was likely underpowered to detect meaningful changes in the piglet lung microbiome in response to meconium. The meconium was further diluted from its initial concentration with amniotic fluid and subsequent normal saline lavage to obtain BALF in just one lung. The influence of the environment of the sampling and analysis on subsequent meconium sequencing must also be considered. This was controlled for by eliminating samples of low integrity and avoiding overinterpretation of the microbiome signal.

Animals had meconium/normal saline slurry instilled to mimic meconium aspiration; however, a meconium/amniotic fluid slurry would more closely approximate the pathophysiology associated with human meconium aspiration. Normal saline was used instead of amniotic fluid because of initial challenges in collecting sufficient amniotic fluid. Additionally, the microbiome of amniotic fluid was not tested, which could have been helpful in interpreting the BALF signal as it would have provided an in utero microbiome baseline.

The study was designed with the intent to assess for microbial presence and metabolic byproducts, and not specifically the effects of meconium on the inflammatory response or surfactant function, both known to be impaired with MAS [1, 53]. Specifically, pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-8 were not tested in vivo, though these have been affiliated with local inflammatory responses in the alveolar epithelium as a response to meconium stained amniotic fluid [66]. In particular, IL-8 a chemokine that recruits neutrophils to the respiratory airways as a part of the inflammatory process [67], was not studied though meconium is known to influence its release [68], and has been associated with worsening lung function [68]. Meconium has several constituents that can potentially be harmful, including amniotic fluid, lanugo, desquamated epithelial cells, fatty acids, bile, hemoglobin, cholesterol [66], and proteolytic enzymes such as $sPLA_2$ [56]. With the focus of the study on the microbiome, we elected to study the isolated effects of SCFAs in vitro, in order to eliminate any confounding pro-inflammatory influence of these other meconium isolates, as well as mechanical ventilation.

Another limitation is that there was not a marked change in oxygenation between the MAS injury and Control groups despite using meconium aspiration protocols that have been validated by other investigators [28, 29]. Our model is distinguished from others in that newborn piglets were used to ensure that the microbiome results reflect the in utero environment, whereas other studies have used piglets from 1 day to 10 weeks of age [28, 29]. Thus, our animals were transitioning from intrauterine to extrauterine respiratory and hemodynamic physiology, which may have contributed to the lack of a difference between the two experimental groups. Finally, a longer study duration and/or the addition of an echocardiogram assessment may have revealed meaningful cardiac differences between the Control and MAS groups. Due to the coordination involved in instrumenting and monitoring multiple piglets at once, as well as to mitigate bias, animals were randomized ahead of time according to the order of delivery. Animals were therefore not matched for weight and gender.

Conclusions

This study demonstrated that while ventilation outcomes were similar, meconium aspiration substantially impaired hemodynamic stability compared with non-injured lungs. While additional exploration is needed regarding the meconium microbiome and its importance, bacterial fermentation byproducts in the form of SCFAs were evident to a greater degree in MAS lungs. This represents a novel finding and a potential therapeutic target for infants with MAS.

Statement of Ethics

This study protocol was reviewed and approved by the SUNY Upstate Medical University Institutional Animal Care and Use Committee (IACUC approval # 506) and in accordance with ARRIVE guidelines.

Conflict of Interest Statement

M.K.S. has received an educational research grant from Dräger Medical Systems, Inc. The authors maintain that industry had no role in the design and conduct of the study; the collection, management, analysis, or interpretation of the data; nor the preparation, review, or approval of the manuscript.

Funding Sources

This work was supported by NIH grant R01HL14270 (Jason H.T. Bates, Donald P. Gaver, Gary F. Nieman).

Author Contributions

M.K.S. contributed to the study conception and design, data acquisition, analysis and data interpretation, and manuscript drafting. H.R., G.L., E.S.C., M.L., J.S., and S.S. were involved in data acquisition. A.G. contributed to analysis and data interpretation, as well as critical revisions to the manuscript. Q.M. was involved in data acquisition, analysis and data interpretation, and critical revision of the manuscript. H.M.M. contributed to analysis and data interpretation, and critical revision of the manuscript. D.P.G., J.H.T.B., and G.N. were all involved in critical revision of the manuscript. All authors approved the final draft of the submitted manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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