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Effects of nondigestible oligosaccharides on inflammation, lung health, and performance of calves

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ABSTRACT

Our objective was to determine the effects of nondigestible oligosaccharides (NDO) on lung health and performance. Three hundred male Holstein-Friesian calves aged 18.0 ± 3.6 d received 1 of 6 treatments for 8.5 wk (period 1). Treatments included a negative control (CON), galacto-oligosaccharides (GOS) administered as a spray via the nose once daily (SPR), GOS administered via the milk replacer (MR) at 1% (GOS-L) and 2% (GOS-H), fructo-oligosaccharides administered via the MR at 0.25% (FOS) and a combination of GOS and fructo-oligosaccharides administered via the MR at 1% and 0.25%, respectively (GOS-FOS). Milk replacer was fed twice daily. Feeding levels were equal between calves and increased progressively in time. Body weight was measured every 4 wk and clinical health was scored weekly. Blood and broncho-alveolar lavage fluid (BALF) samples were collected bi-weekly from a subset of calves (n = 120). After period 1, all calves received the same control MR for 18 wk until slaughter (period 2), during which general performance and clinical health were measured. Generally, infection pressure was high, with clinical scores and BALF proinflammatory TNFα concentrations increasing with time in period 1, which resulted in a high number of required group antimicrobial treatments (6 group antimicrobial treatments in 13 wk, supplied to all calves). Average daily gain adjusted to equal solid feed intake was increased for GOS-L (+61 g/d) compared with CON calves from experimental wk 1 to 5. Plasma white blood cell concentration tended to be lowered by GOS-L, plasma IL-8 concentration was reduced by all orally supplemented NDO, plasma IL-6 was reduced by all NDO treatments except GOS-FOS and plasma IL-1β was reduced by all NDO treatments compared with CON, although this differed per time point for SPR. The neutrophil percentage in BALF was reduced by GOS-L in wk 6, which was associated with a relative increase in macrophages. The BALF concentration of TNFα and IL-8 was reduced or tended to be reduced by GOS-L and GOS-H, while IL-6 was or tended to be reduced by SPR, GOS-L, GOS-H, and GOS-FOS, and IL-1β was reduced by SPR, GOS-L, GOS-H, and FOS. Generally, feeding the combination of GOS and FOS was not more effective than feeding GOS or FOS alone, because feeding GOS-FOS resulted in higher concentrations of plasma and BALF cytokine and chemokine concentrations compared with feeding GOS-L alone, and resulted in higher plasma cytokine concentrations compared with feeding FOS alone. None of the BALF and plasma cytokine or chemokine concentrations differed between the GOS-L and GOS-H treatment. Performance and clinical scores in period 2 did not differ among treatments. Altogether, all tested NDO reduced systemic and lung inflammation in calves under high natural infection pressure and for GOS-fed calves, this increased performance during the first 4 wk. Combining GOS and FOS did not have a synergistic effect. The intranasal administration of GOS also lowered systemic and lung inflammation, but tended to negatively affect performance. Overall, this study demonstrates the potential of NDO to alleviate systemic and respiratory inflammation in calves.

Key words: calf, oligosaccharides, lung health, inflammation

INTRODUCTION

Bovine respiratory disease (**BRD**) is a major health issue in dairy and veal calves. Clinical symptoms of BRD include coughing, nasal discharge, increased respiratory rate and rectal temperature, loss of appetite, apathy, and reduced growth. Mortality due to BRD varies between 1.3% and 3.1% (Brscic et al., 2012; Pardon et al., 2012b, 2013; Lava et al., 2016) and BRD accounted for 56% of all morbidity cases in veal calves (Pardon et al., 2012b). Furthermore, BRD in early life in Holstein calves, as assessed by lung consolidation

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during the first 8 wk of life, was associated with a reduced milk production during the first lactation (Dunn et al., 2018).

Bovine respiratory disease is a multifactorial disease, with different pathogens involved, such as *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, *Mycoplasma bovis*, bovine viral diarrhea virus, bovine coronavirus, bovine respiratory syncytial virus, parainfluenzavirus type 3, bovine herpesvirus type 1, and bovine adenovirus (Autio et al., 2007; Pardon et al., 2011, 2020). Due to the involvement of pathogenic bacteria in BRD, antimicrobial treatments are applied to combat or prevent BRD, and respiratory disease is the main cause for antimicrobial treatment supply in veal calves (Pardon et al., 2012a). Many pathogens involved in BRD are resistant to one or more antimicrobial agents (Catry et al., 2005; Pardon et al., 2011; Rérat et al., 2012), which is a major concern for both animal (Bengtsson and Greko, 2014) and human health (Ma et al., 2021). Therefore, strategies that prevent or reduce BRD are required.

One strategy is the use of oligosaccharides, such as bovine milk oligosaccharides or synthetic oligosaccharides, including oligofructose (fructo-oligosaccharides), galacto-oligosaccharides (**GOS**), and mannan-oligosaccharides (**MOS**). In general, oligosaccharides are not digested by the host digestive enzymes and are fermented by the microbiota, resulting in changes in the microbial composition, such as promoting the growth of bifidobacteria and lactobacilli (Smiricky-Tjardes et al., 2003; Alizadeh et al., 2016; Castro et al., 2016; Boudry et al., 2017). Furthermore, supplementation with nondigestible oligosaccharides (**NDO**) increases the concentration of fermentation products such as short-chain fatty acids and can increase intestinal barrier function (Alizadeh et al., 2016; Boudry et al., 2017). In addition to the well-known prebiotic effects of NDO on gut health, effects of NDO on lung health have also been demonstrated. A reduction in lung inflammation and mortality was found in C57BL/6 mice fed pectin-derived acidic oligosaccharides after a *Pseudomonas aeruginosa*-induced lung infection (Bernard et al., 2015). Supplementation of a mixture of GOS, long-chain fructo-oligosaccharides, and low-viscosity pectin prevented the development of lung emphysema following nasal lipopolysaccharide administration in BALB/c mice (Janbazacyabar et al., 2019). In pigs, supplementation with MOS reduced the increase in rectal temperature following infection with porcine reproductive and respiratory syndrome virus compared with pigs fed the control diet, although MOS supplementation did not reduce lung lesions caused by the infection (Che et al., 2011). Furthermore, GOS and alginate-oligosaccharides inhibited directly the growth of *Escherichia coli* in vitro (Asadpoor et al., 2021). Therefore, in addition to oral NDO supplementation, direct intranasal NDO supplementation provides an interesting strategy to reduce BRD as well. Although the exact mechanisms by which NDO can affect lung health are not entirely known, the existence of a bidirectional gut-lung axis seems evident (Anand and Mande, 2018) providing a target for influencing lung health.

Veal calves provide an interesting model to study the effects of NDO on lung health. Veal calves originating from many different dairy farms are transported and mixed at a young age, resulting in a high infection pressure after arrival, with peak incidences of BRD between 2 and 6 wk after arrival (Pardon et al., 2012b). In the current study, this natural exposure of veal calves to BRD pathogens was used to evaluate the effects of supplementation of NDO. The objective of this study was to determine the effects of NDO supplementation, either via the MR or via intranasal application, on lung health and performance of calves. We hypothesized that NDO would reduce lung inflammation and thereby improve performance of calves.

MATERIALS AND METHODS

A project license was granted by the Central Committee for Animal Experimentation (the Hague, the Netherlands) after approval by the Animal Care and Use Committee of Wageningen University (AVD1040020185828, Wageningen, the Netherlands). This experiment was approved by the Animal Welfare Body of Wageningen University (2017.W-0017.001) and was conducted at the research facilities of the VanDrie Group (Scherpenzeel, the Netherlands). The study described in this paper was part of a larger project. Effects of NDO, using calf primary bronchial epithelial cells, combined with selected parameters obtained in vivo as a reference, have been presented elsewhere (Cai et al., 2021, 2022a,b).

Experimental Design

The experiment consisted of 2 periods. Period 1 started when calves arrived at the experimental facilities and lasted 8.5 wk (experimental wk 1 to 9), in which NDO treatments were applied daily. Measurements were performed to evaluate effects of NDO treatments on (lung) health and performance. In period 2, no NDO treatments were applied and all calves received the same dietary treatment. Period 2 lasted from experimental wk 10 to slaughter at experimental wk 28 and included general (performance) measurements.

Animals, Housing, and Feeding

Calves were housed in a mechanically ventilated barn. Light was provided by daylight, and artificial light was switched on between 0600 and 1800 h. Calves were housed in pens (9 m^2) containing wooden-slatted floors. In the first 6 wk after arrival, individual housing was applied (1.2 m^2/calf) by placing stainless steel fences within the pens, allowing for individual monitoring of fecal consistency. After 6 wk, the individual fencing was removed and calves were housed in groups of 5. In the first 3 wk, a minimum temperature of 15°C was maintained and heat canons were used when needed. Thereafter, ventilation rates were adjusted to maintain a maximum change in temperature of 5°C compared with the outside temperature and a maximum relative humidity of 80%.

A total of 300 male Holstein-Friesian calves of German origin were included in the study. This sample size was estimated based on Pardon et al. (2015), who calculated that in a similar setting of natural exposure to BRD pathogens, 56 calves per groups would be needed to demonstrate a reduction in BRD incidence by 25% (2 sided, 95% confidence, power = 0.8) in calves receiving either adequate or inadequate colostrum intake. We calculated the standard deviation of the work of Pardon et al. (2015) and used this in a power analysis. We estimated that 46 calves per group were needed based on a 25% reduction in BRD incidence and a standard deviation of 33% (one sided, 95% confidence, power = 0.8). We rounded the minimal group size to 50, because calves are housed in groups of 5.

Calves arrived at the facilities at 18.0 ± 3.6 d of age and 43.3 ± 3.2 kg of BW (mean ± SD) and were randomly assigned to pens. Pens were evenly distributed throughout the barn and the 6 treatments were blocked over pens to have an equal distribution of treatments throughout the barn. Calves received electrolytes solution upon arrival in the evening, and treatments started the next day. Treatments (n = 50 calves per treatment) included a negative control (**CON**) and 5 NDO supplementations; GOS administered as a spray via the nose (**SPR**), GOS administered via the milk replacer (**MR**) at 1% (**GOS-L**) and 2% inclusion (**GOS-H**), fructo-oligosaccharides administered via the MR at 0.25% inclusion (**FOS**) and a combination of GOS and fructo-oligosaccharides administered via the MR at 1% and 0.25%, respectively (**GOS-FOS**). The NDO of the oral NDO treatments were included in all MR feedings, which was twice daily. The SPR was administered once daily. The fructo-oligosaccharides used in the experiment was Frutalose OFP chicory oligofructose (Sensus, Roosendaal, the Netherlands), containing 97.6% DM and 92% fructo-oligosaccharide on DM basis. The GOS used was Vivinal GOS Syrup (FrieslandCampina DOMO, Borculo, the Netherlands) containing 74% DM and 59% GOS on DM basis (see Logtenberg et al., 2020, for a typical composition of the GOS in Vivinal GOS). These inclusion levels were based on our previous ex vivo studies, in which lower levels of fructo-oligosaccharides compared with GOS were required to reduce the *Mannheimia haemolytica*-induced release of cytokines and chemokines from calf primary bronchial epithelial cells (Cai et al., 2021, 2022a). For the SPR treatment, Vivinal GOS Syrup was diluted with saline to reduce the viscosity and 10 mL warm GOS solution (providing 0.99 g of GOS DM) was sprayed into the nasopharynx once per day with an intranasal applicator (Rispoval, Zoetis B.V., The Netherlands) attached to a syringe. The researchers, except for the main researcher, were blinded to the treatment allocation during the study, except to the SPR treatment, as all researchers were involved in the application of the SPR.

The ingredient and nutrient composition of the MR in period 1 and 2 are in Table 1. Nondigestible oligosaccharides administered via the MR were included at the expense of lactose, corrected for the purity and DM of the NDO products used. Solid feed was composed of chopped wheat straw and concentrates. Solid feed was supplied from 1 wk after arrival onward. Straw and concentrates were supplied at a ratio of 16:84 for 2 wk and was thereafter supplied at a ratio of 13:87. Concentrates comprised corn (412 g/kg), corn flakes (200 g/kg), lupines (195 g/kg), barley (102 g/kg), molasses (50 g/kg), vitamin-mineral mix (25 g/kg), urea (6 g/kg), palm oil (5 g/kg), and sodium bicarbonate (5 g/kg) and were provided as flakes. Analyzed crude protein content of the concentrates was 140 g/kg DM. Feeding levels of both MR and solid feed were equal between calves and were based on a practical feeding scheme. Milk replacer increased progressively from 426 to 1,300 g/d in period 1 and from 1,300 to 2,711 g/d in period 2. The MR was mixed with warm water (66°C) and supplied a temperature of approximately 42°C. In period 1, the MR concentration was 125 g/L and in period 2 it increased progressively from 125 to 182 g/L. Solid feed supply increased progressively from 174 to 984 g/d in period 1 and from 1,019 to 2,865 g/d in period 2.

Calves were fed 2 equal MR meals at 0600 and 1600 h and were allowed access for at least 15 min, after which MR refusals were quantified. In case of MR refusals, calves were supplied with water to prevent dehydration. After wk 6 onwards, calves were restrained in the headlock for a maximum of 60 min. to enable individual MR feeding. Solid feed was provided after the morning MR meal. Solid feed refusals were removed twice a week and quantified weekly per pen. From wk 6 onwards, water was available through water nipples.

Table 1. Ingredient and nutrient composition of the experimental milk replacers

Ingredient (g/kg)	Period 1	Period 2
Lactose[1]	35	
Whey powder	527	560
Delactosed whey powder	52	93
Whey protein concentrate	50	
Soy protein concentrate	60	55
Soluble wheat protein	50	41
Pea fiber	3	
Extruded wheat flour		41
Fat		
Lard	43	48
Tallow	68	77
Coconut oil	54	34
Lecithin	7.2	5
Emulsifier	7.2	5
Premix	10[2]	10[3]
Calcium formate	9.7	10
Citric acid	2.0	2
Sodium bicarbonate	4.0	4
Monoammonium phosphate	3.5	2
Lysine	9.8	8
Methionine	2.4	3
Threonine	1.3	2
Aroma	0.2	
Nutrient (g/kg of DM unless noted)		
DM (g/kg)	975	976
Crude ash	82	74
CP (N × 6.25)	231	191
Crude fat	193	184
Lactose[4]	453	455
Iron (mg/kg of DM)	48	10

[1]In period 1, calves fed the control milk replacer received lactose with the milk replacer. For the nondigestible oligosaccharide milk replacer treatments, (part of) the lactose was replaced with nondigestible oligosaccharides according to their allocated treatment.

[2]The premix in period 1 provided (per kg of experimental diet) CP, 0.7 g; crude fat, 0.2g; starch, 5.1 g; crude ash, 1.5 g; calcium, 16.6 mg; phosphorus, 7.6 mg; sodium, 0.7 mg; potassium, 7.3 mg; chloride, 13.1 mg; magnesium, 0.5 g; iron, 37.2 mg; copper, 7.4 mg; zinc, 109 mg; manganese, 43 mg; selenium, 0.3 mg; iodide, 6.9 mg; sulfur, 84 mg; vitamin A, 25,011 IU; vitamin D3, 4,002 IU; vitamin E, 142 IU; vitamin C, 0.3 g; vitamin K3, 2.1 mg; vitamin B1, 8.2 mg; vitamin B2, 10.2 mg; vitamin B3, 34.9 mg; vitamin B5, 18.0 mg; vitamin B6, 9.4 mg; vitamin B12, 0.1 mg; biotin, 0.1 mg; choline, 0.4 g; and folic acid, 0.7 mg.

[3]The premix in period 2 provided (per kg of experimental diet) CP, 0.8 g; crude fat, 0.1g; starch, 5.5 g; crude ash, 1.3 g; calcium, 17.0 mg; phosphorus, 8.1 mg; sodium, 0.8 mg; potassium, 7.8 mg; chloride, 13.2 mg; magnesium, 0.5 g; iron, 0.3 mg; copper, 4.0 mg; zinc, 100 mg; manganese, 43 mg; selenium, 0.3 mg; iodide, 1.0 mg; sulfur, 78 mg; vitamin A, 25,000 IU; vitamin D3, 4,000 IU; vitamin E, 100 IU; vitamin C, 0.1 g; vitamin K3, 2.1 mg; vitamin B1, 8.2 mg; vitamin B2, 10.2 mg; vitamin B3, 34.8 mg; vitamin B5, 18.0 mg; vitamin B6, 6.3 mg; vitamin B12, 0.1 mg; biotin, 0.1 mg; choline, 0.4 g; and folic acid, 0.7 mg.

[4]Calculated content.

Measurements

General health of the calves was monitored daily. Calves were weighed at arrival (wk 1) and in wk 5, 8, 13, 17, 21, and 25 with a digital BW scale. All calves were weighed on the same day. Hemoglobin (**Hb**) concentration was determined in jugular blood at arrival and in experimental wk 10, 14, and 18. A minimum hemoglobin concentration of 5.5 mmol/L was targeted. Iron (iron dextran, 20%) was injected intramuscularly when required, preceded by supplying acetylsalicylic acid (Preventiron, Dopharma, Raamsdonksveer, the Netherlands) with the MR the day before iron injections to prevent iron shock. Medical treatment was applied when required based on clinical signs of illness. Individual antimicrobial treatments included trimethoprim with sulphamethoxazol or paromomycin against diarrhea, oxytetracycline against leg problems and depocillin, florfenicol, ampicillin, amoxicillin, or marbofloxacine against respiratory problems. Group medical treatment to all calves was applied when 10% of the calves had been treated within 5 d, or 5% of the calves had become ill within 24 h or when the situation required group treatment in the expert judgment of a veterinarian. Group antimicrobial treatments included doxycycline or tilmicosin against respiratory problems, and ampicillin or tylosin against serositis. All individual and group medical treatments were recorded. Calves that died were sent to the Animal Health Service (Deventer, the Netherlands) for autopsy.

Period 1. Measurements were performed for all calves or for a subset of calves. This subset of calves included 2 calves per pen (n = 20 per treatment), selected on BW at arrival closest to the average BW of all calves at arrival. Blood samples were collected by venipuncture in the jugular vein at arrival before the first MR feeding from all calves, and in experimental wk 3, 5, and 7 from the subset of calves. Blood was collected in 9-mL and 4-mL K_2-EDTA tubes and were kept on ice for collection of plasma or kept at room temperature for analysis of hematological parameters, respectively. Plasma was collected after centrifugation at 2,000 × *g* and 4°C for 20 min and was stored at −20°C pending cytokine and chemokine analyses.

Feces of individual calves were scored for consistency daily for the first 9 d after arrival. After d 9, fecal consistency was scored weekly until 6 wk after arrival. Fecal consistency was scored on a 5-point scale, ranging from firm feces (score 1) to liquid diarrhea (score 5). Clinical scoring was performed weekly, according to a BRD-scoring system (Pardon, 2012), in which a score from 0 to 3 was provided for rectal temperature, coughing, nasal discharge and behavior (Table 2). Clinical score was calculated as the sum of these 4 scores. In experimental wk 7, fecal samples were collected directly from the rectum of the subset of calves and pH was measured in fresh fecal samples (Mettler Toledo, SevenGo SG23). Broncho-alveolar lavage fluid (**BALF**) samples were collected from the subset of calves in experimental wk 2, 4, 6, and 8. To collect a BALF sample, the calf was restrained in the headlock. The nostrils

Table 2. Clinical scoring used for assessing lung health in calves[1]

Item	Score			
	0	1	2	3
Rectal temperature, °C	<38.5	38.5–39.0	39.0–39.5	>39.5
Cough	None	Single cough	Occasional cough	Repeated cough
Nasal discharge	Normal, slightly serous	Excessively serous, unilateral cloudy serous	Excessive mucus, bilateral cloudy or mucus	Copious bilateral, mucopurulent
Behavior	Normal (standing or standing up upon approach)	Slightly depressed (laying down longer)	Mildly depressed (laying down, away from pen mates)	Severely depressed (refuses to stand up upon stimulus)

[1]Adapted from Pardon (2012).

were cleaned with cotton wool and 70% ethanol. Thereafter, a sterile flexible silicon tube was inserted via the nasal cavity into the trachea until the tube was fixed in a bronchus. Placement of the silicon tube in the lungs was verified before BALF sampling by a coughing reflex or sticking out of the tongue. In case the tube was inserted into the esophagus (i.e., no reflexes), the tube was retrieved, passing the epiglottis and a second attempt was performed. If the tube was swallowed and inserted into rumen, the tube was retrieved completely, cleaned and flushed with saline before the tube was inserted again. After appropriate fixation in the lungs, saline (30 mL, ~37°C) was inserted completely and immediately retrieved. The BALF samples were stored on ice in 30-mL tubes until further processing in the laboratory the same day.

In experimental wk 9, 1 calf per pen was dissected from all NDO treatments supplemented with the MR and the control treatment (n = 50 in total), with the objective to evaluate degradation kinetics of oligosaccharides along the intestinal tract (not part of this manuscript). At dissection, lungs were scored for lesions, with scores ranging from 0 (no pneumonia) to 3 (severe pneumonia), according to Leruste et al. (2012).

Period 2. Clinical scoring was performed weekly as in period 1, except that rectal temperature was not measured. In addition, general performance (BW, MR, and solid feed refusals, medical treatments, Hb) was measured as mentioned previously. In experimental wk 28, calves were slaughtered, carcasses were weighed and lungs were scored for lesions, with scores ranging from 0 (no pneumonia) to 5 (severe pneumonia).

Analytical Procedures

Blood samples were analyzed for hematological parameters within 24 h by fluorescence flow cytometry using a Sysmex 1800iV (Sysmex Europe GmbH, Norderstedt, Germany). Plasma samples from wk 5 and 7 were analyzed using ELISA kits for TNFα (R&D Systems, Minneapolis, MN), IL-8 (Mabtech, Nacka Strand, Sweden), IL-1β (Invitrogen, Thermo Fisher Scientific), and IL-6 (Invitrogen, Thermo Fisher Scientific) according to the instructions of the manufacturer.

The BALF samples were filtered using a 70-μm cell strainer (Corning). Subsequently, the BALF suspension was centrifuged at 400 × *g* and 4°C for 5 min. Supernatant was collected and stored at −80°C pending cytokine and chemokine analyses. The cell pellets were resuspended with 1 mL of cold fetal bovine serum. Cells were counted using a Cellometer Bright Field cell counter (Nexcelom Bioscience, Lawrence, MA). After cell counting, 0.5×10^6 cells were used for making cytospins, which were stained with Diff-Quick (Medion Diagnostics, Medion Diagnostics International Inc., Miami, FL) according to manufacturer's description. For differential BALF cell counts, a minimum of 400 cells was counted.

The BALF supernatants were analyzed for TNFα at all time points and for IL-1β, IL-6, and IL-8 concentrations in wk 6 using the same ELISA kits as used for the plasma samples.

Statistical Analyses

Calves that died were removed from the dataset. Statistical analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC). Except for feed intake and adjusted ADG data (see below), all observations were performed on individual calves. Calves were, however, housed in groups of 5 per pen, and a random effect of pen was included in all models. In each model, treatment differences were evaluated using pre-set contrasts (CON vs. SPR, CON vs. GOS-L, CON vs. GOS-H, CON vs. FOS, CON vs. GOS-FOS, FOS vs. GOS-FOS, GOS-L vs. GOS-FOS, and GOS-L vs. GOS-H), using the LSMESTIMATE statement including Bonferroni adjustment. Studentized residuals of each model were checked visually for homogeneity of variance and data transformations were applied to obtain homogeneity

of variance when needed based on visual inspection of quantile–quantile (QQ) plots.

For performance data, calves that died in period 1 (n = 9) or in the first 3 wk of period 2 (n = 6) and 1 calf with excessive MR refusals (>40 L, i.e., 9.5% of supply), were removed from the dataset for period 1. For period 2, 2 calves with excessive milk refusals (>100 L, i.e 5.8% of supply) were removed from the performance dataset, in addition to the calves that died in period 2 (n = 9). Refusals of solid feed were recorded per pen. To account for this, ADG was adjusted to the average realized level of solid feed intake for each period. To obtain this adjusted ADG, first the residuals of the GLM model ADG = solid feed intake were obtained. These residuals represent the difference between the observed and predicted ADG based on the model. Subsequently, these residuals were added to the mean ADG of the specific period. This adjusted ADG is the ADG at equal solid feed intake. Performance data per period, blood Hb, and white blood cell (**WBC**) concentration at arrival, and fecal pH (wk 7) were analyzed for the treatment contrasts as described above using the MIXED procedure. The ADG and carcass weight data were squared to obtain homogeneity of variance. For BW data and ADG in period 2, BW at arrival was included as a covariate in the model.

Continuous parameters measured over time (blood, BALF, rectal temperature) were analyzed using the MIXED procedure with treatment, time and treatment × time interaction as fixed effects, and included time as a repeated statement with calf as unit. Treatment contrasts were evaluated as described above, but if the interaction effect of the main model was significant, these treatment contrasts were evaluated per time point separately. The covariance structure per model was selected based on fit statistics (lowest Akaike information criterion and Bayesian information criterion). For blood parameters, the values at d 1, before the first feeding, were included as a covariates. Blood concentrations of IL-6 were inverse-transformed and blood concentrations of IL-8, and WBC were log-transformed. Blood percentages of neutrophils were arc-sin transformed, percentages of lymphocytes and eosinophils were squared, and percentages of monocytes and basophils were angular-transformed. The BALF cell concentrations were square-root transformed, and BALF macrophage and neutrophil percentages were arc-sin transformed. Percentages of BALF lymphocytes were very low with many calves having 0% lymphocytes, and these data were analyzed for treatment effects per week separately, using the Kruskal-Wallis test including the Dwass-Steel-Critchlow-Fligner method for evaluating treatment comparisons. Concentrations of TNFα and IL-6 in BALF were square-root transformed, and BALF IL-8 and IL-1β concentrations were log-transformed. Rectal temperature data were inverse-transformed.

Discrete parameters measured over time (clinical and fecal scores) were analyzed for treatment contrasts per week using the GLIMMIX procedure with a Poisson distribution. Fecal scoring was performed daily for the first 9 d, and the median fecal score for wk 1 and 2 was obtained and used in the model. Lung lesion scores at dissection and slaughter were analyzed for treatment contrasts using the GLIMMIX procedure with Poisson distribution as well. Individual antimicrobial treatment supply was analyzed for treatment contrasts for period 1 and 2 separately, using the GLIMMIX procedure with a negative binomial distribution, including all calves. Mortality was analyzed for treatment contrasts using the GLIMMIX procedure with a binary distribution. Treatment contrasts were always evaluated using Bonferroni adjustment.

Principal component analysis (**PCA**) was performed on blood (wk 7) and BALF (wk 6) inflammatory parameters to evaluate interrelations. The FACTOR procedure with the principal axis method was used and prior communality estimates were set at 1. The extracted principal components (**PC**) were subjected to varimax rotation, and PC with an eigenvalue >1.5 were retained. Variables with a loading ≥0.40 or ≤−0.40 in the rotated factor pattern, were considered to load on that PC.

Differences were considered significant when $P < 0.05$ and considered a trend when $P < 0.10$. Results are expressed as nontransformed means and their pooled SEM. The *P*-values of treatment contrasts represent Bonferroni-adjusted *P*-values.

RESULTS

At arrival, calves weighed 43.3 ± 3.2 kg of BW (mean ± SD), and this did not differ among treatments (Table 3). Blood Hb and WBC concentration at arrival did not differ among treatments ($P > 0.10$ for all treatment contrasts), and averaged 5.5 ± 0.08 mmol/L and 8.1 ± 0.17 × 10^9/L, respectively.

Performance and Clinical Health

Adjusted ADG in the first 4 wk after arrival was higher for the GOS-L (+ 61 g/d; $P = 0.001$) compared with the CON calves (662 ± 11 g/d; Table 3). Body weight at arrival explained variation in BW at later time points ($P < 0.001$). The GOS-L calves (65.3 ± 0.6 kg; $P = 0.002$) were heavier and GOS-H calves (64.0 ± 0.6 kg; $P = 0.06$) tended to be heavier compared with CON calves (62.6 ± 0.7) at experimental wk 5. The GOS-L calves (86.7 ± 0.8 kg) were also heavier

Table 3. Performance of veal calves not supplemented with nondigestible oligosaccharides (CON) or supplemented with nondigestible oligosaccharides (SPR, galacto-oligosaccharides [GOS] applied with a spray into the nose; GOS-L, low [1%] supplementation of GOS via the milk replacer [MR]; GOS-H, high [2%] supplementation of GOS via the MR; FOS, supplementation of fructo-oligosaccharides [FOS, 0.25%] via the MR and GOS-FOS, GOS [1%] and FOS [0.25%] supplemented via the MR)

	Treatment							*P*-value of treatment contrast[1]							
Item	CON	SPR	GOS-L	GOS-H	FOS	GOS-FOS	Pooled SEM	CON vs. SPR	CON vs. GOS-L	CON vs. GOS-H	CON vs. FOS	CON vs. GOS-FOS	FOS vs. GOS-FOS	GOS-L vs. GOS-FOS	GOS-L vs. GOS-H
BW (kg)															
Arrival	43.6	43.7	44.1	43.4	42.8	42.7	0.46	1.00	1.00	1.00	1.00	1.00	1.00	0.65	1.00
Wk 5[2]	62.6	62.9	65.3	64.0	62.6	62.9	0.60	1.00	0.002	0.063	1.00	0.24	1.00	0.70	1.00
Wk 8[2]	83.7	82.4	86.7	85.3	83.5	84.4	0.78	0.55	0.031	0.24	1.00	0.33	1.00	1.00	1.00
Wk 25[2]	252.9	253.5	258.8	253.2	252.6	252.6	3.91	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Carcass weight[2]	154.3	156.3	158.2	156.4	156.0	155.7	2.44	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ADG (g/d)															
Wk 1–5	656	662	732	708	680	698	13.6	1.00	0.001	0.063	1.00	0.26	1.00	0.48	1.00
Wk 5–8	1,007	930	1,018	1,016	995	1,022	20.8	0.080	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Wk 1–8	803	775	852	838	812	834	12.4	0.66	0.020	0.24	1.00	0.37	1.00	1.00	1.00
Wk 8–25[3]	1,433	1,448	1,458	1,419	1,435	1,422	29.3	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Adjusted ADG[4] (g/d)															
Wk 1–5	662	666	724	704	691	691	12.9	1.00	0.011	0.20	0.98	1.00	1.00	0.60	1.00
Wk 5–8	1,012	951	998	1,009	998	1,013	19.8	0.27	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Wk 1–8	809	786	838	832	820	826	10.9	1.00	0.51	1.00	1.00	1.00	1.00	1.00	1.00
Wk 8–25	1,437	1,457	1,452	1,421	1,433	1,422	18.3	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

[1]*P*-values of LSM differences were estimated following Bonferroni adjustment.
[2]Body weight at arrival was included as a co-variable in the model ($P < 0.001$).
[3]Body weight at arrival was included as a co-variable in the model ($P = 0.004$).
[4]Average daily gain adjusted for solid feed intake per pen of calves.

than CON calves (82.4 ± 0.9 kg) in wk 8 ($P = 0.03$). Adjusted ADG in period 2 (1,437 ± 18.3 g/d on average) and BW at the end of period 2 (254 ± 2.4 kg on average) did not differ among treatments.

Fecal consistency scores did not differ among treatments ($P > 0.10$), but did change over time ($P < 0.001$), which was generally reflected by higher fecal scores in experimental wk 1 to 3, compared with fecal scores in wk 4 to 6. In wk 1, fecal consistency score averaged 3.8 ± 0.10, whereas in wk 6, fecal consistency averaged 2.6 ± 0.10. When categorizing fecal consistency score 4 and 5 as diarrhea, 59% of the calves had diarrhea in wk 1 and 8% of the calves had diarrhea in wk 6. Fecal pH (wk 7) averaged 6.5 ± 0.12 and did not differ among treatments ($P > 0.10$).

Rectal temperatures and clinical scores over time in period 1 are presented in Figure 1. For rectal temperature, there was treatment × time interaction ($P < 0.001$), related to a numerically lower rectal temperature for SPR (38.5 ± 0.07°C) and GOS-H calves (38.4 ± 0.07°C) in wk 4, but a numerically higher rectal temperature for SPR calves in wk 8 (39.6 ± 0.12°C) compared with the CON calves (38.7 ± 0.06°C in wk 4 and 39.3 ± 0.10°C in wk 8). Both rectal temperature and clinical score generally increased with time ($P <$ 0.001), with the highest scores in wk 6 (39.1 ± 0.08°C and 3.1 ± 0.18) and 8 (39.2 ± 0.09°C and 3.1 ± 0.18). When categorizing a clinical score >4 as BRD, 0.4%, 4.6%, 12.4%, and 13.5% of the calves had BRD in wk 1, 3, 6 and 8, respectively. Clinical scores in period 2 did not differ among treatments ($P < 0.10$) and averaged 1.3 ± 0.01, but did change over time ($P < 0.001$). When categorizing a clinical score >4 as BRD, in most weeks in period 2 no calves had BRD, except in wk 15, 17, 18, 20, and 21 when 1 calf (0.4%) had BRD. Lung lesion scores at dissection ($P < 0.10$) and slaughter ($P < 0.10$) did not differ among treatments.

In period 1, 66 individual antimicrobial treatments were administered, of which 78.8% related to respiratory problems, 13.6% related to diarrhea, 1.5% related to (poly)serositis, and 6.1% related to other problems (e.g., leg problems). Individual antimicrobial treatments did not differ among treatments in period 1 ($P > 0.10$). In this period, 4 antimicrobial group treatments were applied. Three of these group treatments were related to respiratory infections and the last one to (poly)serositis. In the same period, 7 calves died and 2 were euthanized (2 from the SPR, 5 from the GOS-L, 1 from the GOS-H, and 1 from the FOS treatment) and 2 calves removed from the trial (due to excessive milk refusals and poor health; 1 from the SPR and 1 from the FOS treatment). In period 2, 88 individual antimicrobial treatments were administered, of which 48.9% related to respiratory problems, 1.1% related to

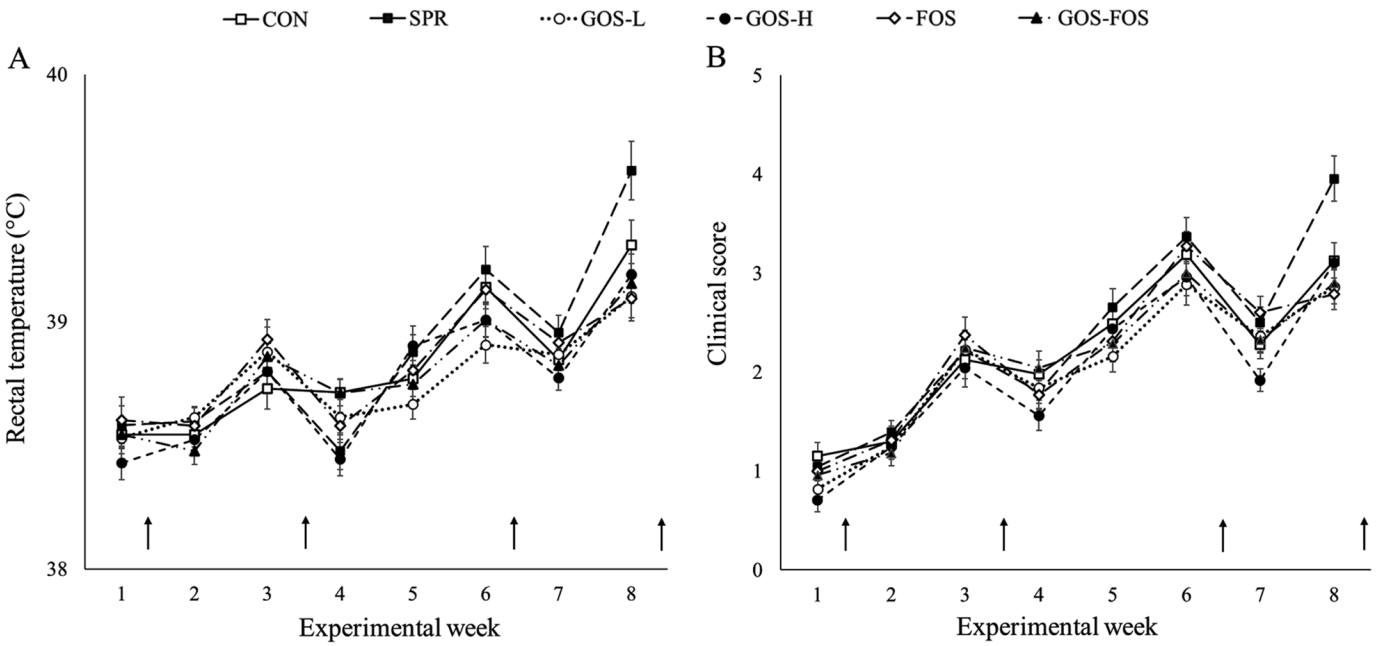

Figure 1. The development of rectal temperatures (A) and clinical scores (B) over time of veal calves not supplemented with nondigestible oligosaccharides (CON) or supplemented with nondigestible oligosaccharides (SPR, galacto-oligosaccharides [GOS] applied with a spray into the nose; GOS-L, low [1%] supplementation of GOS via the milk replacer [MR]; GOS-H, high [2%] supplementation of GOS via the MR; FOS, supplementation of fructo-oligosaccharides [FOS, 0.25%] via the MR and GOS-FOS, supplementation of GOS [1%] and FOS [0.25%] via the MR). Arrows indicate group antimicrobial treatments. Error bars represent SE.

diarrhea, 14.8% related to (poly)serositis, and 35.2% related to other problems. Individual antimicrobial treatments did not differ among treatments in period 2 ($P > 0.10$). Two antimicrobial group treatments were applied related to (poly)serositis in the first 3 wk of period 2. In period 2, 9 calves died (4 from the CON, 1 from the SPR, 1 from the GOS-L, 2 from the GOS-H, and 1 from the GOS-FOS treatment), of which 6 calves died in the first 3 wk of period 2. Mortality did not differ among treatments ($P > 0.10$). For 12 out of 18 calves that died, the cause of death was likely (poly)serositis, related to infection with *Mannheimia haemolytica*.

Inflammatory Parameters in Blood and BALF

Blood inflammatory parameters are presented in Table 4. White blood cell concentration changed over time ($P < 0.001$), with the highest concentration in wk 7 ($9.3 \pm 0.56 \times 10^9$ cells/L), and WBC values at arrival explained variation in WBC concentration later in time ($P < 0.001$). Overall, WBC concentration of GOS-L calves tended to be lower compared with CON calves ($P = 0.06$). Differential cell counts were not affected by treatments but changed over time ($P < 0.001$), and values upon arrival explained variation in neutrophil ($P = 0.03$), monocyte ($P < 0.001$), and basophil percentages ($P = 0.09$) later in time.

Plasma TNFα was not affected by treatment, but increased with 43% from wk 5 to 7 ($P < 0.001$). Plasma IL-8 was 66 times higher in wk 7 than in wk 5 ($P < 0.001$) and orally supplemented NDO ($P < 0.001$ for all) lowered plasma IL-8 compared with the CON treatment, but SPR treatment did not ($P = 1.00$). Feeding GOS-L separately resulted in a lower plasma IL-8 concentration compared with feeding GOS-L and FOS together ($P < 0.001$). Feeding FOS separately tended to lower plasma IL-8 concentration compared with feeding GOS-L and FOS together in wk 5 ($P = 0.08$), but not in wk 7 ($P = 0.39$), which resulted in a treatment × time effect ($P = 0.01$). Plasma IL-6 decreased 12% from wk 5 to 7 ($P < 0.001$) and was lower for all NDO treatments compared with the CON treatment. Feeding GOS-L ($P = 0.002$) or FOS ($P = 0.07$) separately resulted in a lower plasma IL-6 concentration compared with feeding them together. Plasma IL-1β decreased 80% from wk 5 to 7 ($P < 0.001$), and generally all NDO treatments lowered plasma IL-1β concentration compared with CON. The treatment × time effect ($P = 0.01$) was caused by a lower IL-1β concentration for SPR compared with CON in wk 5 ($P = 0.01$), but not in wk 7 ($P = 0.19$), and because feeding GOS-

Table 4. Blood parameters of veal calves not supplemented with nondigestible oligosaccharides (CON) or supplemented with nondigestible oligosaccharides (SPR, galacto-oligosaccharides [GOS] applied with a spray into the nose; GOS-L, low [1%] supplementation of GOS via the milk replacer [MR]; GOS-H, high [2%] supplementation of GOS via the MR; FOS, supplementation of fructo-oligosaccharides [FOS, 0.25%] via the MR and GOS-FOS, supplementation of GOS [1%] and FOS [0.25%] via the MR)

	Treatment							Model P-value[1]				P-value of treatment contrast[2]							
Item	CON	SPR	GOS-L	GOS-H	FOS	GOS-FOS	Pooled SEM	Treat	Time	Treat × time	$Time_0$	CON vs. SPR	CON vs. GOS-L	CON vs. GOS-H	CON vs. FOS	CON vs. GOS-FOS	FOS vs. GOS-FOS	GOS-L vs. GOS-FOS	GOS-L vs. GOS-H
White blood cells ($\times 10^9$/L)								0.083	<0.001	0.59	<0.001	0.14	0.061	1.00	1.00	1.00	1.00	0.58	1.00
Arrival	8.1	9.0	7.7	8.5	7.8	7.6	0.41												
Wk 3	9.0	8.2	7.6	8.3	8.6	8.9	0.49												
Wk 5	8.6	7.6	7.0	7.9	7.5	8.7	0.68												
Wk 7	10.2	8.9	8.6	9.4	10.0	8.5	0.56												
Neutrophils (%)								0.37	<0.001	0.48	0.028	1.00	1.00	1.00	1.00	0.99	1.00	0.80	1.00
Arrival	63.5	58.2	60.4	61.8	56.3	55.4	3.7												
Wk 3	38.8	33.3	28.3	36.2	30.2	34.8	3.7												
Wk 5	29.2	29.0	25.4	26.8	25.3	31.1	2.3												
Wk 7	39.0	42.4	39.8	41.9	43.5	41.0	1.4												
Lymphocytes (%)								0.67	<0.001	0.76	0.37	1.00	1.00	1.00	1.00	1.00	1.00	0.93	1.00
Arrival	32.5	38.4	35.9	34.4	40.1	40.5	3.6												
Wk 3	52.3	58.7	61.8	53.0	59.3	55.2	3.5												
Wk 5	55.0	53.7	56.5	55.3	55.2	52.1	2.2												
Wk 7	47.9	43.4	39.8	44.1	43.5	41.0	1.8												
Monocytes (%)								0.34	<0.001	0.15	<0.001	1.00	1.00	1.00	0.73	1.00	1.00	1.00	1.00
Arrival	1.3	1.3	1.5	1.3	1.7	1.9	0.3												
Wk 3	6.5	6.1	7.5	8.7	8.5	8.3	1.1												
Wk 5	13.1	14.9	15.9	15.9	17.6	14.9	1.1												
Wk 7	11.3	12.3	47.5	12.3	43.3	45.7	1.3												
Eosinophils (%)								0.95	<0.001	0.31	0.96	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Arrival	1.3	1.1	0.9	1.3	0.6	1.1	0.2												
Wk 3	0.2	0.3	0.5	0.4	0.4	0.3	0.1												
Wk 5	1.0	0.6	0.6	0.5	0.6	0.6	0.2												
Wk 7	0.8	0.9	11.2	1.0	11.5	11.6	0.6												
Basophils (%)								0.22	<0.001	0.50	0.090	1.00	1.00	1.00	0.91	0.20	1.00	0.64	1.00
Arrival	1.2	0.9	1.2	1.0	1.2	1.2	0.1												
Wk 3	1.9	1.5	1.9	1.7	1.6	1.4	0.2												
Wk 5	1.7	1.7	1.6	1.5	1.5	1.3	0.1												
Wk 7	1.0	1.0	0.7	0.8	0.9	0.9	0.1												
TNFα (pg/mL)								0.73	<0.001	0.58		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Wk 5	654	633	864	656	682	607	86.7												
Wk 7	1,517	1,463	1,661	1,622	1,737	1,556	122.3												
IL-8 (pg/mL)								<0.001	<0.001	0.010[3]		1.00	<0.001	<0.001	<0.001	<0.001	1.00	<0.001	1.00
Wk 5	36.8	41.5	8.7	8.9	16.3	21.1	3.8												
Wk 7	2,394	2,363	730	632	1,618	1,112	208.4												
IL-6 (pg/mL)								<0.001	<0.001	0.65		0.022	<0.001	<0.001	<0.001	0.40	0.067	0.002	1.00
Wk 5	938	770	700	775	756	877	24.4												
Wk 7	810	727	623	664	635	768	39.9												

Continued

Table 4 (Continued). Blood parameters of veal calves not supplemented with nondigestible oligosaccharides (CON) or supplemented with nondigestible oligosaccharides (SPR, galacto-oligosaccharides [GOS] applied with a spray into the nose; GOS-L, low [1%] supplementation of GOS via the milk replacer [MR]; GOS-H, high [2%] supplementation of GOS via the MR; FOS, supplementation of fructo-oligosaccharides [FOS, 0.25%] via the MR and GOS-FOS, supplementation of GOS [1%] and FOS [0.25%] via the MR)

	Treatment						Model P-value[1]			P-value of treatment contrast[2]						
Item	CON	SPR	GOS-L	GOS-H	FOS	GOS-FOS	Pooled SEM	Treat	Time	Treat × time	Time$_0$	CON vs. SPR	CON vs. GOS-L	CON vs. GOS-H	CON vs. FOS	CON vs. GOS-FOS	FOS vs. GOS-FOS	GOS-L vs. GOS-FOS	GOS-L vs. GOS-H
IL-1β (pg/mL)								<0.001	<0.001	<0.001[4]		<0.001	<0.001	<0.001	<0.001	0.032	0.003	0.008	1.00
Wk 5	246	148	146	163	141	196	11.9												
Wk 7	58	40	20	18	18	44	4.1												

[1]Treat = treatment; Time$_0$ = value at d 1, before the first feeding.
[2]P-values of LSM differences were estimated following Bonferroni adjustment.
[3]IL-8 tended to be lower for FOS than for GOS-FOS calves in wk 5 ($P = 0.080$), but did not differ in wk 7 ($P = 0.39$), resulting in a treatment × time effect ($P = 0.010$).
[4]IL-1β was lower for SPR compared with CON in wk 5 ($P < 0.001$), but not in wk 7 ($P = 0.19$); IL-1β did not differ between FOS and GOS-FOS in wk 5 ($P = 0.11$), but was lower for FOS compared with GOS-FOS in wk 7 ($P = 0.018$); and IL-1β did not differ between GOS-L and GOS-FOS in wk 5 ($P = 0.25$), but was lower for GOS-L compared with GOS-FOS in wk 7 ($P = 0.030$); resulting in an interaction effect ($P < 0.001$).

L ($P = 0.03$) or FOS ($P = 0.02$) separately resulted in a lower plasma IL-1β concentration compared with feeding them together, but only in wk 7. None of the plasma cytokine or chemokine concentrations differed between the GOS-L and GOS-H treatment ($P > 0.10$).

Inflammatory parameters in BALF are presented in Table 5. Concentrations of total cells in BALF were not affected by treatment and were higher in wk 6 and 8 compared with wk 2 and 4 ($P < 0.05$). Differential cell counts in BALF changed over time ($P < 0.001$), with macrophages being the most prevalent cell type in wk 2 (78.0 ± 4.8%) and 4 (71.4 ± 5.5%), decreasing to 45.1 ± 5.6% and 46.6 ± 6.2% in wk 6 and 8, respectively. This coincided with a relative increase in neutrophils from 21.9 ± 4.8% and 28.5 ± 5.5% in wk 2 and 4, respectively, to 54.2 ± 5.7% and 52.9 ± 6.2% in wk 6 and 8, respectively. Lymphocytes represented a small fraction of the cells in BALF, with 0.1 ± 0.07% in wk 2 and 0.6 ± 0.21% in wk 6. Macrophage percentage (interaction effect $P = 0.03$; Figure 2) was higher for GOS-L than for CON ($P = 0.005$), but only in wk 6, and tended to be higher for GOS-FOS compared with FOS ($P = 0.08$), but only in wk 2. This coincided with a lower percentage of neutrophils (interaction effect $P = 0.01$; Figure 2) for GOS-L ($P = 0.002$) compared with CON calves in wk 6. Concentrations of TNFα in BALF increased in time ($P < 0.001$) and were highest in wk 6 and 8. Concentrations of TNFα were lower for GOS-H ($P = 0.02$) and tended to be lower for GOS-L ($P = 0.06$) compared with CON. In wk 6, IL-8 concentration was lower for GOS-L and GOS-H ($P < 0.001$ for both) compared with CON and it was lower for GOS-L than for GOS-FOS calves ($P = 0.005$). Concentrations of IL-6 in BALF were lowered by SPR ($P = 0.02$), GOS-L ($P < 0.001$) and GOS-H ($P = 0.01$) and tended to be lowered by GOS-FOS ($P = 0.07$) compared with CON. Concentrations of IL-1β were lowered by SPR ($P = 0.01$), GOS-L ($P < 0.001$), GOS-H ($P = 0.005$) and FOS ($P = 0.02$) compared with CON and it was lower for GOS-L than for GOS-FOS calves ($P = 0.01$). None of the BALF cytokine or chemokine concentrations differed between the GOS-L and GOS-H treatment ($P > 0.10$).

Interrelations Between Blood and BALF Inflammatory Parameters

Loadings per PC extracted from PCA on BALF (wk 6) and blood parameters (wk 7) to evaluate interrelations are presented in Table 6. Four PC with an eigenvalue >1.5 were retained, accounting for 54% of the total variance. Total cell concentration, neutrophil percentage, and TNFα concentration in BALF loaded positively and percentage of macrophages and lym-

Table 5. Broncho-alveolar lavage fluid parameters of veal calves not supplemented with nondigestible oligosaccharides (CON) or supplemented with nondigestible oligosaccharides (SPR, galacto-oligosaccharides [GOS] applied with a spray into the nose; GOS-L, low [1%] supplementation of GOS via the milk replacer [MR]; GOS-H, high [2%] supplementation of GOS via the MR; FOS, supplementation of fructo-oligosaccharides [FOS, 0.25%] via the MR and GOS-FOS, supplementation of GOS [1%] and FOS [0.25%] via the MR)

	Treatment							Model *P*-value			*P*-value of treatment contrast[1]							
Item	CON	SPR	GOS-L	GOS-H	FOS	GOS-FOS	Pooled SEM	Treat[2]	Time	Treat × time	CON vs. SPR	CON vs. GOS-L	CON vs. GOS-H	CON vs. FOS	CON vs. GOS-FOS	FOS vs. GOS-FOS	GOS-L vs. GOS-FOS	GOS-L vs. GOS-H
Total cells (× 10^6/mL)								0.094	<0.001	0.41	1.00	0.12	1.00	0.91	1.00	1.00	0.18	0.17
Wk 2	3.5	2.5	2.8	2.1	1.7	1.9	0.76											
Wk 4	1.3	1.9	3.3	2.1	3.4	2.3	0.60											
Wk 6	4.3	3.5	2.7	4.6	4.8	3.6	0.88											
Wk 8	3.6	3.5	4.6	4.0	3.0	3.6	0.66											
Macrophages (%)								0.13	<0.001	0.035[3]	1.00	1.00	1.00	1.00	0.49	0.061	1.00	1.00
Wk 2	83	77	71	75	71	90	4.82											
Wk 4	74	79	65	76	64	70	5.51											
Wk 6	30	46	63	45	40	48	5.64											
Wk 8	41	41	50	51	42	55	6.17											
Neutrophils (%)								0.087	<0.001	0.012[3]	1.00	0.94	0.99	1.00	0.16	0.091	1.00	1.00
Wk 2	17.4	22.6	28.7	24.6	28.4	9.7	4.81											
Wk 4	25.6	21.4	34.6	23.8	36.1	29.6	5.53											
Wk 6	69.3	53.8	36.7	54.4	59.9	51.3	5.72											
Wk 8	58.3	58.9	49.7	48.6	57.2	44.9	6.24											
Lymphocytes[4] (%)								0.34	<0.001	—	1.00	0.92	1.00	0.37	0.95	0.91	1.00	0.94
Wk 2	0.0	0.0	0.1	0.1	0.1	0.1	0.07											
Wk 4	0.1	0.1	0.1	0.1	0.3	0.2	0.07											
Wk 6	0.6	0.7	0.6	0.6	0.6	0.9	0.21											
Wk 8	0.4	0.5	0.4	0.4	0.5	0.4	0.15											
TNFα (pg/mL)								0.031	<0.001	0.55	0.18	0.064	0.020	1.00	0.54	1.00	1.00	1.00
Wk 2	270	320	169	203	216	259	46											
Wk 4	659	569	674	478	709	594	113											
Wk 6	903	532	575	559	683	678	83											
Wk 8	1,070	681	685	602	868	688	146											
IL-8 (pg/mL)																		
Wk 6	93	63	20	21	44	45	12	<0.001			1.00	<0.001	<0.001	0.16	0.24	1.00	0.005	1.00
IL-6 (pg/mL)																		
Wk 6	1,539	852	624	814	1,058	966	119	<0.001			0.022	<0.001	0.010	0.26	0.066	1.00	0.34	1.00
IL-1β (pg/mL)																		
Wk 6	507	211	158	186	206	295	48	<0.001			0.011	<0.001	0.005	0.019	0.22	1.00	0.013	0.42

[1]*P*-values of LSM differences were estimated following Bonferroni adjustment.

[2]Treat = treatment.

[3]Treatment contrasts are analyzed per week separately, because of the treatment × time effect, which is visualized in Figure 2.

[4]Percentage of lymphocyte data were analyzed for a treatment and time effect separately using a Kruskal-Wallis test. Contrasts were evaluated using the Dwass, Steel, Critchlow-Fligner method.

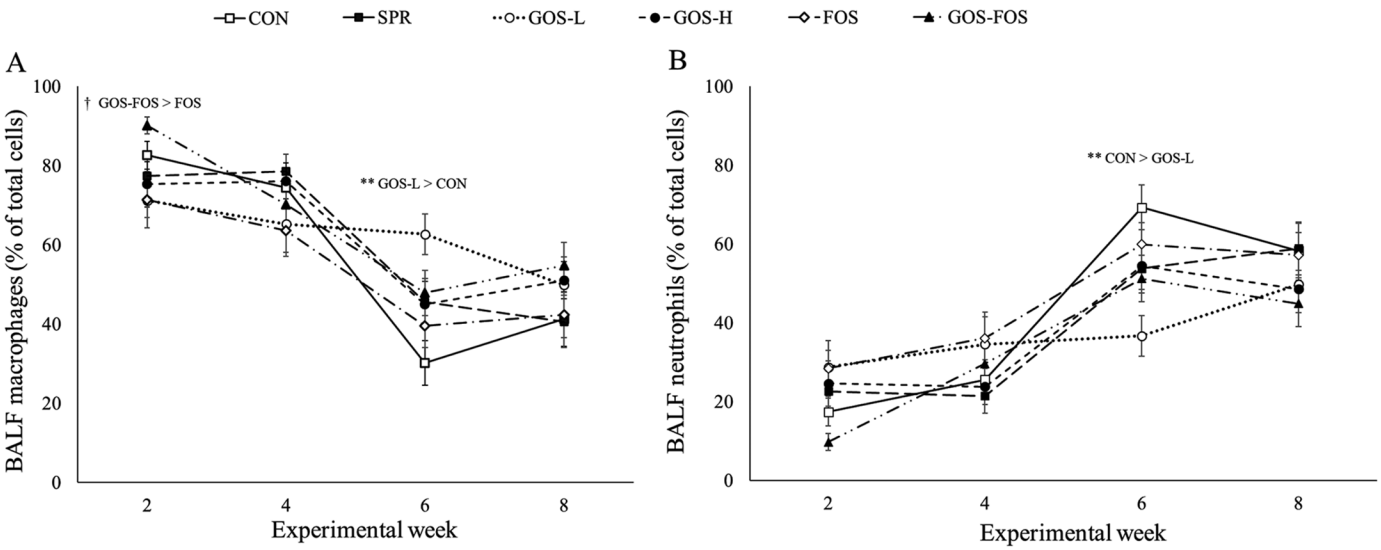

Figure 2. Percentage of macrophages (A) and neutrophils (B) in broncho-alveolar lavage fluid (BALF) of veal calves not supplemented with nondigestible oligosaccharides (CON) or supplemented with nondigestible oligosaccharides (SPR, galacto-oligosaccharides [GOS] applied with a spray into the nose; GOS-L, low [1%] supplementation of GOS via the milk replacer [MR]; GOS-H, high [2%] supplementation of GOS via the MR; FOS, supplementation of fructo-oligosaccharides [FOS, 0.25%] via the MR and GOS-FOS, supplementation of GOS [1%] and FOS [0.25%] via the MR). ** indicates treatments differ from each other, with $P < 0.01$; † indicates a trend for a difference at $P < 0.10$, where treatment contrasts are evaluated per week separately because of a treatment × week interaction ($P = 0.03$ for panel A and $P = 0.01$ for panel B). Error bars represent SE.

phocytes in BALF loaded negatively on PC1. Plasma WBC concentration and neutrophil percentage loaded positively and percentages of lymphocytes and basophils loaded negatively on PC2. Interleukin-1β and IL-8 in both BALF and plasma loaded positively on PC3. The BALF IL-6 and blood IL-1β and IL-6 loaded positively on PC4.

Table 6. Loadings per principal component (PC) extracted by principal component analysis with varimax rotation performed on inflammatory parameters in broncho-alveolar lavage fluid (BALF) and blood samples of veal calves[1]

Item	PC1	PC2	PC3	PC4
BALF total cells (× 10^6/mL)	**86**	9	1	4
BALF neutrophils (%)	**89**	8	14	13
BALF macrophages (%)	**−88**	−9	−14	−13
BALF lymphocytes (%)	**−51**	1	11	4
BALF TNFa (pg/mL)	**69**	2	21	17
BALF IL-1β (pg/mL)	16	−3	**89**	−1
BALF IL-8 (pg/mL)	10	3	**92**	1
BALF IL-6 (pg/mL)	17	−9	6	**80**
Plasma white blood cells (× 10^9/L)	3	**67**	11	6
Plasma neutrophils (%)	4	**93**	−4	3
Plasma monocytes (%)	7	−8	10	0
Plasma lymphocytes (%)	−7	**−90**	0	−3
Plasma eosinophils (%)	15	9	−10	−8
Plasma basophils (%)	−24	**−55**	24	10
Plasma TNFa (pg/mL)	−6	2	−1	−6
Plasma IL-1β (pg/mL)	−2	−1	**63**	**41**
Plasma IL-8 (pg/mL)	−1	6	**42**	33
Plasma IL-6 (pg/mL)	15	22	8	**74**
Eigenvalue	3.2	2.5	2.4	1.6
Explained variance (%)	18	14	13	9

[1]The eigenvalue and percentage of variance explained per PC are provided. Loadings >40 and <−40 are in bold.

DISCUSSION

In the current study, the effects of NDO on lung health and performance of calves were studied. Veal calves provided a suitable model for this, because of the high natural exposure to BRD pathogens, generally resulting in peak incidences of BRD between 2 and 6 wk after arrival (Pardon et al., 2012b). Similarly, in the current study, clinical scores, measured using a BRD-scoring system, increased in time, indicating the presence of BRD in veal calves. Proinflammatory TNFα concentrations in BALF also increased in time, which points to lung inflammation. Hence, 6 group antimicrobial treatments were applied in 13 wk to prevent potential excessive morbidity and mortality. This was high compared with the number of group antimicrobial treatments typically required at this facility (i.e., 2–3 group antimicrobial treatments). Overall, this indicates that (respiratory) infection pressure was sufficiently high to evaluate NDO effects on lung health.

The inclusion levels of NDO were based on our previous ex vivo observations using calf primary bronchial epithelial cells. Lower levels of fructo-oligosaccharides compared with GOS were required to reduce the *Mannheimia haemolytica*-induced release of cytokines

and chemokines (Cai et al., 2021, 2022a). In addition, the inclusion levels were chosen to prevent NDO-induced diarrhea, because diarrhea has been observed when supplementing GOS at 3.35% in MR fed to Holstein calves (Castro et al., 2016). Fecal consistency was monitored closely during the first 9 d of the experiment to adjust NDO inclusion levels if needed. However, fecal consistency was not higher for any of the NDO treatments compared with the CON treatment, indicating that the current NDO inclusion levels did not induce diarrhea. Generally, fecal consistency decreased in time, which is in line with veal calf practice, where diarrhea mainly occurs in the first 3 wk after arrival, as demonstrated by Pardon et al. (2012b).

Blood and BALF samples were used to evaluate the effects of NDO on inflammatory responses. Principal component analysis was used to evaluate interrelations between inflammatory parameters in BALF and blood. The PCA retained 4 PC with few cross-loadings. Interestingly, WBC concentrations and relative shifts in cell types loaded on separate PC for BALF (PC1) and blood (PC2). These loading patterns within sample type represented well-known relations during inflammation, with increased total WBC concentrations being related to an increase in neutrophils. For instance, in mice with LPS-induced acute lung injury, the total cell increase in BALF was mainly caused by neutrophil recruitment (Tang et al., 2010). Cytokine and chemokine concentrations in BALF and plasma did load together on PC, with positive loadings for IL-1β and IL-8 in BALF and plasma (PC3), for IL-6 in BALF and IL-6 and IL-1β in plasma (PC4). This suggests that these cytokine and chemokine concentrations simultaneously respond in the same direction in both BALF and blood during inflammation. These loading patterns from the PCA depict the immunological cascade following invasion of respiratory pathogens. Respiratory infections can lead to an imbalance in the composition of immune cells and the accumulation of inflammatory mediators in the lungs. Excessive inflammatory mediators can release into the blood and, therefore, induce systemic inflammation. In addition, toxins released into the blood by respiratory pathogens can also induce inflammatory responses through the systemic circulation (Caswell, 2014; Cai, 2021). In addition to blood and BALF parameters, clinical scoring was used in this study to evaluate the effects of NDO. Clinical scoring can be insensitive to diagnosing (subclinical) lung infections (van Leenen et al., 2020), and these scores were done only once a week, indicating that disease can be missed. Principal components were obtained using BALF and blood parameters of wk 6 and 7, respectively, and interestingly, PC2 correlated with clinical scores in wk 7 ($r = 0.34$, $P > 0.001$) and PC3 correlated with clinical scores in wk 6 ($r = 0.23$, $P = 0.01$).

Feeding GOS improved the performance of calves, mainly in the first 4 wk after arrival. Solid feed intake differed between pens of calves and, therefore, ADG was adjusted for solid feed intake. This adjusted ADG is the ADG at equal solid feed intake and is therefore a measure of feed efficiency. The GOS supplemented at 1% via the MR increased adjusted ADG in the first weeks after arrival and resulted in a higher BW in wk 5 and 8. Similarly, in piglets, on-top supplementation of GOS also improved growth performance, associated with improved gut function (Tian et al., 2018) and reduced systemic inflammation (Xing et al., 2020). In addition to performance, GOS reduced systemic and respiratory inflammation. Orally supplemented GOS reduced IL-6, IL-8, and IL-1β concentrations in both plasma and BALF, and GOS-L also reduced the percentage of neutrophils in BALF in wk 6, a week with high clinical scores. Generally, the GOS dose (1% or 2%) had little effect on inflammatory parameters. Several mechanisms might underlie the effects of GOS. Galacto-oligosaccharides can change the composition of gut microbiota (Castro et al., 2016) and exert anti-inflammatory effects via gut-lung axis (Anand and Mande, 2018; Cai et al., 2020). Although short-chain fatty acids have been identified as key mediators of the gut-lung axis (Dang and Marsland, 2019), it can be questioned whether they also played an important role in the current study. Calves received solid feed, resulting in fermentation in the rumen and fermentation of ruminal undegraded material in the hindgut. For comparison, in experimental wk 9, GOS-H calves received 25 g/d GOS compared with 984 g/d solid feed, which is composed mainly of carbohydrates, suggesting that NDO supplementation only marginally contributed to total short-chain fatty acid production. Microbiota-independent mechanisms could also underly the effects of GOS. Part of the GOS may be absorbed into the systemic circulation (Difilippo et al., 2015) and directly inhibit the activation of the proinflammatory signaling pathway caused by pathogens in the lungs, including the TLR4 pathway (Cai et al., 2022a).

Several NDO exhibit antibacterial effects, either by inhibiting the growth of pathogens or by killing them directly (Asadpoor et al., 2021). We found that GOS can lower the viability of *Mannheimia haemolytica* in vitro (Cai et al., 2022b). Therefore, GOS were also locally administered via intranasal application in the current study, to evaluate the direct effects of GOS on lung health. The intranasal GOS dosage (0.99 g of DM/d) was based on the dilution required to obtain a spray flow of the GOS syrup, which is viscous

when undiluted, and on a maximum volume of 10 mL to minimize fixation time of calves to administer the spray. The amount of GOS provided via nasal spray was lower compared with GOS provided via the MR (e.g., GOS-SPR provided 4.1 times and 12.2 times less GOS compared with the GOS-L treatment at the start and in wk 8 of the experiment, respectively). Likely, a lower dosage is required to enable effects in the lung directly compared with indirectly via the gut. Indeed, intranasal GOS application did reduce some of the systemic and respiratory inflammatory parameters in a comparable manner to orally supplemented GOS. Whether this GOS-spray effect is due to GOS reaching the lungs directly, or GOS being ingested after mucociliary clearance from the trachea or bronchus cannot be separated. A preliminary test (approved by the Animal Welfare Body of Wageningen University, 2017.W-0017.002) using 4 male Holstein-Friesian calves and spray containing Evans blue, revealed that the intranasal administration likely supplied GOS into the trachea and esophagus (unpublished data). Despite the reduction in lung and systemic inflammation, the GOS-SPR treatment tended to decrease ADG from wk 5 to 8. Likely, this reduction in clinical performance with GOS-SPR is due to the stress of daily handling the calves during the spray administration.

Feeding 0.25% FOS in the MR reduced inflammatory cytokine and chemokine concentrations in plasma and reduced IL1β concentrations in BALF, but did not improve performance or clinical health. In contrast, supplementation of fructo-oligosaccharides (10 g/d) to Simmental × Red Holstein veal calves tended to improve BW gain between 10 and 13 wk of age (Kaufhold et al., 2000). Supplementation of fructo-oligosaccharides was higher compared with the current study (where fructo-oligosaccharide supply increased from 1.1 to 3.2 g/d from experimental wk 1 to 9, respectively), and fructo-oligosaccharide was supplied on top and calves were not fed any solid feed, potentially explaining the difference with our study. Mechanisms underlying the effects of fructo-oligosaccharides, besides effects on the microbiota (Philippeau et al., 2010), could include the prevention of epithelial barrier dysfunction by pathogens. We showed that fructo-oligosaccharides prevented *Mannheimia haemolytica*-induced epithelial barrier dysfunction and inflammatory responses in vitro, which might be related to the inhibition of TLR5-mediated proinflammatory signaling (Cai et al., 2021), demonstrating the different mechanisms of action between GOS (TLR4-mediated; Cai et al., 2022a) and fructo-oligosaccharides (TLR5-mediated; Cai et al., 2021).

The effect of a combination of GOS (1%) and FOS (0.25%) on lung health and performance was also evaluated in the current study. Feeding GOS-FOS reduced inflammatory cytokine and chemokine concentrations in plasma and tended to reduce IL6 concentrations in BALF. However, feeding the combination of GOS and FOS was not more effective than feeding GOS or FOS alone (e.g., calves fed GOS-FOS had higher plasma and BALF cytokine and chemokine concentrations compared with calves fed GOS-L and had higher or tended to have higher plasma cytokine and chemokine concentrations compared with calves fed FOS). This suggests that even though the modes of action of GOS and FOS likely differ, this GOS-FOS combination does not exhibit synergistic effects in calves. Combinations of GOS and fructo-oligosaccharides are already included in infant formulas within Europe (Akkerman et al., 2019). Infants receiving formula with GOS and long-chain fructo-oligosaccharides (9:1) had increased numbers of fecal bifidobacteria and lactobacilli (Moro et al., 2002) and had reduced incidences of acute diarrhea and respiratory tract infections (Bruzzese et al., 2006). In mice, the combination of GOS and long-chain fructo-oligosaccharides decreased ovalbumin-induced lung inflammation (Vos et al., 2007). In such studies, often long-chain fructo-oligosaccharides are used, typically with a 9:1 ratio between GOS and fructo-oligosaccharides, to resemble the molecular size distribution of human milk oligosaccharides (Knol et al., 2005). In the current study, shorter chain FOS (degree of polymerization 2–10) in a 4:1 GOS:FOS ratio were used, based on our previous ex vivo studies using calf primary bronchial epithelial cells (Cai et al., 2021, 2022a). In humans, immune responses differed depending on the chain length of fructo-oligosaccharides/inulin (Vogt et al., 2017). Whether different structures of fructo-oligosaccharides or different ratios between GOS:FOS are more suitable for calves requires further research.

Potential carry-over effects of feeding NDO on clinical health and performance were also assessed. Infection pressure was lower in period 2, with clinical scores averaging 1.3 ± 0.01 in period 2 compared with 3.1 ± 0.18 in wk 6 of period 1 and clinical scores and performance in the 18 wk following NDO supplementation were not affected.

CONCLUSIONS

All tested NDO reduced systemic and lung inflammation in calves under high natural infection pressure. For GOS supplemented via the MR at 1%, this resulted in an increased performance during the first 4 wk after arrival. There was no dose-dependent effect of GOS. Combining GOS (1%) and FOS (0.25%) was generally less effective in reducing inflammation compared with feeding GOS (1%) or FOS (0.25%) alone, suggesting the absence of a synergistic effect. Galacto-oligosaccha-

rides were also administered intranasally, and this also reduced inflammation, but tended to negatively affect performance, likely due to the stress of daily handling of the calves. Overall, this study demonstrates the potential of NDO to alleviate systemic and respiratory inflammation in calves.

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