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Effect of ionization, bedding, and feeding on air quality in a horse stable

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Background: Organic dust is associated with Equine asthma. Ionization should reduce airborne dust levels.

Objectives: To determine the effect of ionization of air, type of bedding, and feed on the levels of airborne dust, endotoxin, and fungal colonies in horse stables.

Animals: 24 healthy University-owned horses occupied the stables.

Methods: A randomized controlled cross-over study. Four units with 6 stables were equipped with an ionization installation (25 VA, 5000 Volt Direct Current). Horses were kept either on wood shavings and fed haylage (2 units), or on straw and fed dry hay (2 units). Measurements were performed with and without activated ionization, during daytime and nighttime, repeatedly over the course of a week and repeatedly during 4-6 weeks. Statistical analysis was performed using a mixed effect model with Akaike's Information Criterion for model reduction and 95% profile (log) likelihood confidence intervals (CI).

Results: Ionization did not alter concentrations of dust, endotoxin, or fungi, fewer. In the units with straw and hay, the concentration of dust, endotoxin, and fungi (difference in logarithmic mean 1.92 (95%CI 1.71-2.12); 2.86 (95%CI 2.59-3.14); 1.75 (95%CI 1.13-2.36)) were significantly higher compared to wood shavings and haylage.

Conclusions and Clinical Importance: The installation of a negative air-ionizer in the horse stable did not reduce concentrations of dust, endotoxin, and viable fungal spores. The substantial effect of low dust bedding and feed is confirmed.

KEYWORDS
airborne, dust, endotoxin, fungi

1 | INTRODUCTION

Airborne dust in horse stables contains high concentrations of bacterial endotoxin, molds, mite debris, plant debris, and inorganic dust.[1–3] Both stable workers and horses are exposed to high concentrations of these airborne irritants,[4,5] which can have negative health effects.[5] Type of bedding and feed in the barn significantly influences the concentration

Abbreviations: AIC, Akaike's information criterion; CI, confidence interval; CFU, colony-forming units; GSP, gesamtstaubprobenahme; HD unit, High dust unit; LD unit, low dust unit; LOD, limit of detection; RAO, recurrent airway obstruction; ULOD, upper limit of detection.

The work has been performed at Utrecht University (The Netherlands), Equine Hospital.

This study was presented as a poster at the 2015 VCRS congress, Edinburgh, United Kingdom.

of particulate matter, endotoxin, and fungi.[1,2,6–8] Based on aerodynamic particle diameter, airborne dust is divided into 3 human health-related size fractions: "inhalable," "thoracic," and "respirable" dust fractions.[7] Inhalable particles are particles which can enter the respiratory tract during normal, open-mouth breathing (nominally ≤100 μm diameter). Thoracic particles will pass through the nose and throat, reaching the lungs (nominally ≤10 μm diameter). Respirable dust particles (nominally ≤4 μm diameter) can penetrate the airways beyond the terminal bronchioles into the lower airways causing inflammation and irritation.[2,3,7] For horses, the exact size of particles that can enter the gas-exchange area of the lungs is currently not known. However, inhalation of smaller (respirable) dust particles is considered most detrimental in the equine lung.[2] Inhalation of dust particles is reported to play a major role in the pathogenesis of Equine Asthma, previously known as the non-septic chronic airway inflammatory diseases (inflammatory airway disease) and recurrent airway obstruction (RAO).[2,3,6,9,10] A higher concentration of airborne dust in the direct surroundings is associated with an increased risk of visible mucus accumulation in the trachea and inflammatory cell numbers in tracheal lavage, which is associated with reduced performance in racehorses, show-jumpers, and dressage horses.[2,10–12]

Ionizers have been suggested to reduce the concentration of dust particles and might have bactericidal effects. Ionizers release negatively charged ions from electric wires in the air.[13,14] This emission of negative ions is assumed to enhance the agglomeration of smaller airborne particles into larger particles, which settle through gravitation.[15] Ionization might also increase attraction between particles and earthed surfaces, resulting in enhanced electrostatic deposition. These mechanisms can result in removal of airborne particles and thereby a reduction of airborne dust concentrations.[13,16] Studies in hatching cabinets and broiler farms showed a significant reduction in airborne bacteria and dust concentrations when ionization devices were used.[15–18] The aim of the present study was to determine the effect of ionization of air and management system on the concentration of airborne inhalable dust, endotoxin, and fungi in horse stables.

2 | MATERIALS AND METHODS

2.1 | Animals and husbandry

The study was performed at the Department of Equine Sciences of the Faculty of Veterinary Medicine (Utrecht University, the Netherlands). Four identical stable units (unit I-IV; 12 m × 8 m × 4 m, 96 m^2, 384 m^3) were used, each consisting of 6 stalls which were all occupied by a total of 24 horses owned by the department (Figure 1). Stable units were physically separated. In unit I and II, horses were kept on straw and were fed dry hay (further referred to as HD, "High Dust" units). In unit III and IV, horses were kept on wood shavings and were fed haylage (further referred to as LD "Low Dust" units). The main door and windows in each stable unit were permanently opened to provide the best ventilation possible. Besides this natural ventilation, a low-pressure ventilation system was active, removing contaminated air from the building. All horses were turned out and trained on a daily basis. Most activities in the stables, feeding, cleaning, supply of fresh bedding, or sweeping, were performed between 0700 and 1500 hours. At night, the only activities performed were observations, feeding, and removal of manure.

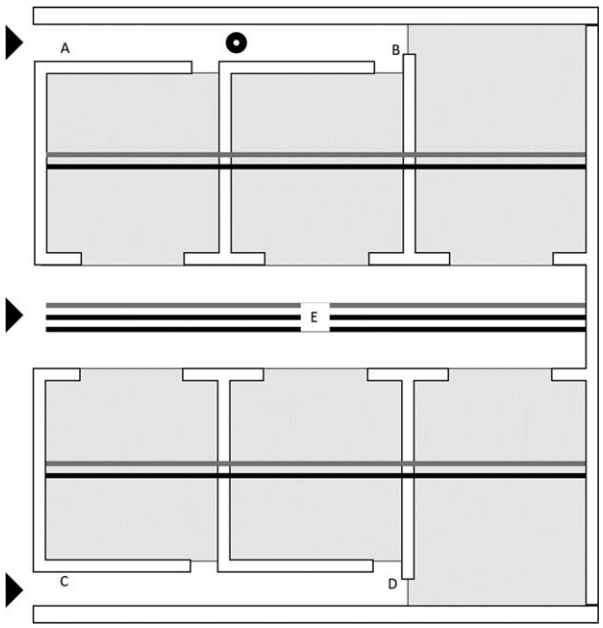

FIGURE 1 A map of 1 of the 4 identical stable units. The black circle indicates the standard location for sampling. The letters A-E indicate the locations where the variable samples were taken. The arrow heads indicate doors, with the middle door being an important inlet for fresh air. The gray lines indicate the corona wires, and the black lines indicate the iron wires required to create sufficient earthed surface

2.2 | Study design

The study was performed during a 6-week period. Ionization devices were installed in all 4 units, but alternately operated for 7 days, in 2 units of different management systems at the same time (see Table 1). Every week, for the first 4 weeks of the study, ambient airborne samples were collected for dust, endotoxin, and fungi in all units. In the first 2 weeks, on 5 consecutive days in both weeks, samples for dust and endotoxin were collected once daily, during the day. In week 3 and 4, dust and endotoxin samples were collected once, at night, on 3 consecutive days in both weeks. Fungal samples were collected on different times points. For the first 2 weeks, samples were taken 4 times a week, at 0900 and 1700 hours. In week 3 and 4, fungal samples were taken 3 times a week at 0100 hour.

After week 4, the ionization devices were modified in the HD units. An iron mesh was installed at the height of the corona wires to increase the earthed surface. In these units, samples for dust and endotoxin were collected once daily, during the day on 5 consecutive days in week 5 and week 6 (Table 1, Figure 1).

2.3 | Ionization device

All stable units were equipped with a custom-designed prototype negative ion generator (25 VA), fixed to the wall with ionizing wires (corona wires, 5000 Volts) attached to the ionizer extending over the length of

TABLE 1 Study design to examine the effects of ionization on air quality in horse stables. In week 1–6 samples for dust, endotoxin and fungi concentrations were collected. Unit I and II contained straw and dry hay, in unit III and IV shavings and haylage were used. HD Unit = straw and hay unit, LD Unit = wood shavings and haylage unit. 'On' and 'Off' indicates whether ionization was operating or not

	Sampling moment		Unit I HD regime	Unit II HD regime	Unit III LD regime	Unit IV LD regime
Week no.	Dust and endotoxin	Fungi	Ionization	Ionization	Ionization	Ionization
1	Daytime	0900 and 1700 hours	On	Off	On	Off
2	Daytime	0900 and 1700 hours	Off	On	Off	On
3	Nighttime	0100 hour	On	Off	On	Off
4	Nighttime	0100 hour	Off	On	Off	On
5	Daytime	-	On Extra mesh[a]	Off Extra mesh[a]	No measurements	No measurements
6	Daytime	-	Off Extra mesh[a]	On Extra mesh[a]	No measurements	No measurements

HD, High dust; LD, low dust.
[a]In weeks 5 and 6, extra mesh was added to the high dust units to increase the earthed surface.

the stable at a height of 3.20 m above the floor, over the corridor between individual horse stalls (horses could not reach these wires). Iron wires were placed at the same height, 30 cm from the corona wires, to create the required earthed surface. After 4 weeks, an iron mesh was installed in the HD units, in addition to the original iron wires, to increase the earthed surface (Figure 2).

2.4 | Collection and analysis of samples

2.4.1 | Inhalable dust and endotoxin

Inhalable dust samples were collected once daily, over an 8-hour period, from 0800 to 1600 hours (daytime samples, week 1 and 2) or from 0000 to 0800 hours (nighttime samples, week 3 and 4). In each unit, samples were always collected at 1 fixed location, 150 cm above floor level (Figure 1). Beside the standard location, additional samples were collected once during the experiment in each unit, at random times at 5 additional fixed locations, with or without ionization operating (Figure 1).

Inhalable dust samples were collected on 37 mm glass fiber filters (Whatman International Ltd, Maidstone, UK) using a Gillian Gil-Air 5 constant flow pump (Gillan, Sensidyne, Clearwater) and Gesamtstaub-probenahme an der Person (GSP) sampling heads (Sensidyne St. Petersburg, Florida). The pump flow rate was calibrated at 3.5 L/min using a rotameter (Brooks Instruments, Hatfield, Pennsylvania) before each measurement period and again checked after each period. The volume of air sampled was calculated based on the mean flow rate of the pump and the duration of sampling. One blank sample was taken each day to control for passive contamination of the filters. After collection, the dust samples were stored at −20°C until further processing to prevent growth and amplification of microbes. Mass of the collected dust was determined by weighing the filters before and after sampling in an acclimated room (temperature 22.6°C, relative humidity 35.2%, air pressure 1019 mbar), using a Mettler AX105 analytical scale (Mettler-Toledo GmBH, Greifensee, Switzerland). Before weighing, filters were conditioned for 24 hours in an acclimated room. The inhalable dust concentration was calculated by dividing the increase in weight of the filters by the volume of air that had run through the filter. Based on blank filters, the lower limit of detection (LOD) for dust weight was 0.01 mg. This corresponds to a dust concentration of 0.059 mg/m^3.

To determine the endotoxin concentration, endotoxin in the captured dust was extracted using the protocol described by Spaan et al[19] Briefly, the filters were transferred to sterile, endotoxin free 50 mL tubes (Greiner Bio One, Alphen aan de Rijn, the Netherlands) and 4 mL pyrogen-free water with 0.05% Tween-20 was added and rocked vigorously by an end-over-end roller for 1 hour at room temperature. Afterward, the tubes were centrifuged for 15 minutes at 1000 G (=2094 rpm), supernatant was harvested and stored in

FIGURE 2 Pictures of the ionization installation in 1 of the units (high dust unit using straw as bedding and dry hay as roughage), showing the ionization device (arrow), corona wires (arrowhead) and iron wires with additional mesh (*)

0.1 mL aliquots at −20°C until analysis. All extracts were analyzed with the kinetic chromogenic Limulus amoebocyte lysate (LAL; Lonza, Breda, the Netherlands) assay in a 1 : 500 dilution, without using Tween during the assay. The lower LOD for endotoxin was 30 EU per filter which corresponds with an endotoxin concentration of 17.82 EU/m^3.[19]

2.4.2 | Fungi

Airborne fungal cultivable samples were collected on dichloran-glycerol agar 18 (DG-18) plates using an Andersen one-stage 400 hole impactor (sKc Inc. Procare, Groningen, the Netherlands) connected to a pump with a flow rate of 28.3 L/min for 30 seconds. Between samples, the Andersen impactor was cleaned using ethanol wipes and the ethanol was allowed to evaporate before the next use of the impactor. At each sampling time, 5 samples were collected, 1 from each unit, plus 1 field blank. The field blank sample was collected without the pump operating. During each day of sampling, the samples were stored at 4°C. At the end of each day, the plates were placed in an incubator and incubated for 4 days at 24°C. The number of colonies on each plate was counted twice, the average of 2 counts, without positive hole correction applied, was divided by the volume of air sampled to determine the bio-aerosol concentration as colony-forming units per cubic meter of air (CFU/m^3). The number of colonies was corrected for blank values. Since a 400 hole impactor was used, the maximum number of fungal CFU that could be measured was 400. The upper limit of detection (ULOD) was 28.3×10^3 CFU/m^3.

2.5 | Statistical analysis

Statistical analyses were performed using R software (version 3.4.0). Inhalable dust and endotoxin measurements were log transformed in order to achieve a normal distribution. Repeated measurements in each unit were regarded as correlated, therefore a mixed effect model was used with stable unit as random effect and HD versus LD, use of ionization and week number (1 and 2; 3 and 4; 5 and 6) as fixed effects. Akaike's Information Criterion (AIC) was used for model reduction. From the final model, 95% profile confidence intervals were calculated. Residuals were checked for normality using normal probability plots.

In many samples, excessive growth of fungal colonies occurred and the ULOD was reached which precluded the quantification of the fungal concentration and thus parametric analysis. Therefore, it was first determined whether reaching the ULOD or not was related to the independent variables ionization, HD or LD unit and sampling moment of the day using logistic regression. Residuals versus fitted values plots were checked for fit of the model and the homogeneousness of the variance. Model reduction was performed using AIC. In a second analysis, using only the samples that did not reach the ULOD, the log of the fungal concentrations was analyzed with a linear model, conditional on the event that the upper limit was not reached, assuming the normal distribution holds approximately. This last assumption was checked with a normal probability plot. For all important effects, according to AIC, profile 95% confidence intervals (95% CI) were calculated.

3 | RESULTS

3.1 | Inhalable dust and endotoxin

A total of 127 samples and 28 field blanks were collected for dust and endotoxin measurements, of which 96 samples were collected at the standard location and 31 at alternative locations. Three dust and five endotoxin samples were lost during analysis and extraction. Dust and endotoxin concentrations in all field blanks were below the limit of detection. None of the dust samples had a dust content below the LOD. Two endotoxin samples were below the LOD, one because the pump stopped working during collection, this sample was excluded from analysis. For the other sample the lower LOD was used for analysis. The mean sampling time was 481 ± 12.7 minutes, with a range of 445–524 minutes.

The dust and endotoxin concentrations are shown in Figure 3. Ionization did not significantly affect the amount of dust and endotoxin in the different units. Therefore, ionization was not included in the final model, whereas type of management system was.

Dust and endotoxin concentrations were significantly reduced in the LD units compared to the HD units. Dust concentrations were reduced by 86% in the LD units, the difference in logarithmic mean between HD and LD units was 1.92 (95%CI 1.71-2.12). Endotoxin concentrations were 95% less in LD units compared to the HD units, the difference in logarithmic mean between the 2 management systems was 2.86 (95%CI 2.59-3.14). Dust concentrations were similar for daytime and nighttime sampling, whereas endotoxin concentrations were significantly lower during overnight sampling, with a reduction of 40% in endotoxin concentrations. The difference in logarithmic means between daytime and nighttime was −0.51 (95%CI −0.79 to −0.22) (Table 2).

The samples collected at the variable locations showed no consistent pattern with respect to airborne concentrations. There was no specific location within the stable units at which a higher concentration of dust or endotoxin was present (data not shown).

3.2 | Airborne culturable fungal colonies

A total of 88 samples were collected for quantification of airborne culturable fungal colonies. Culturable fungal colonies mainly consisted of *Penicillium spp*. and *Aspergillus spp*. The likelihood of reaching the ULOD was related to type of bedding and time of day (Table 3). In the HD units, the maximum number of 400 viable fungal CFU (28.3×10^3 CFU/m^3) was reached very often, with an odds ratio of 102.65 (95%CI 20.54–994.70) for HD versus LD units. In both HD and LD units, the likelihood of reaching the ULOD was greatest for the samples taken at 0900 hours, compared to 1700 hours (odds ratio 4.98; 95% CI 1.02–37.04) and 0100 hour (odds ratio 10.59; 95% CI 1.86–90.00). The second analysis, including only those samples that did not reach the ULOD, indicated a significant effect of management system on airborne fungal concentrations, with a significant reduction of fungal CFU/m^3 concentrations in the LD units of 81%. The difference in

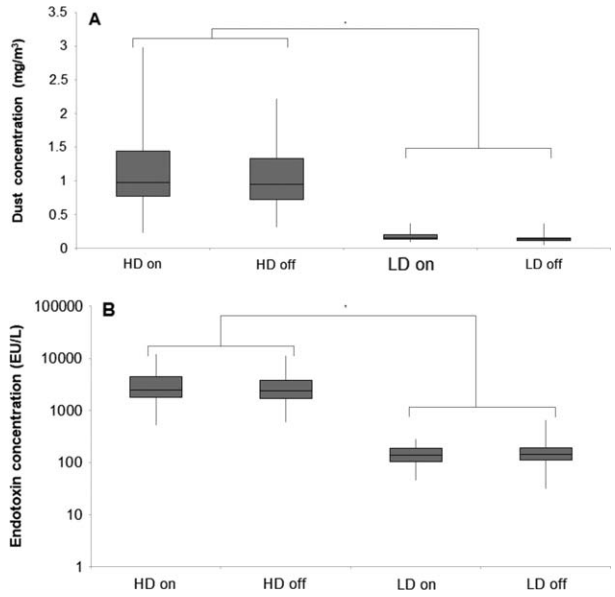

FIGURE 3 Boxplots of dust concentrations (A) and endotoxin concentrations (B) in horse stables with different types of bedding and feed and with and without ionization; "on" and "off" indicating whether ionization was operating or not; HD, high dust unit with straw bedding and dry hay feeding; LD, units with shavings bedding and haylage feeding; * = significant different to HD units

logarithmic mean was 1.75 (95% CI 1.13-2.36). In both analyses, ionization did not have a significant effect on the fungal concentrations (Table 3).

4 | DISCUSSION

In the present study, the effect of negative ionization on area inhalable dust, endotoxin, and airborne culturable fungi was studied. No benefits of air ionization were observed with regard to concentrations of dust, endotoxin, or fungi, neither in both in HD nor in LD units.

Our study investigated the effect of ionization in horse stables. The effects of ionization units have been studied in homes and poultry stable environments, with conflicting results.[13,16–18,20]. A significant reduction in airborne dust (particle size 0.3–100 μm) concentrations, using real-time area measurements, as well as reduced bacterial growth in hatching cabinets was demonstrated.[16,17] Several studies showed a significant reduction of inhalable,[21] thoracic, and respirable[15,18] dust concentrations in broiler breeding flocks, when ionization is used in a closed room. A significant reduction in breathing area aerosols and respirable dust particles in confined indoor spaces was demonstrated.[13] However, a large review explored the clinical effect of ionization installations on chronic asthma variables in humans and failed to identify any significant improvement.[20] The fact that no reduction of airborne dust and endotoxin concentrations was detected in the current study could be related to the size of the compartment studied. Previous studies that demonstrated a positive effect of ionization were mostly conducted in small, confined indoor spaces, whereas the present experiment took place in relatively large horse stall units with open doors, windows, and ample ventilation. Ionization causes small particles to aggregate into larger particles. Possibly respirable particles have transformed into thoracic or inhalable particles as a result of ionization. The concentration of respirable particles might have been reduced while inhalable particle concentrations remained unchanged. Further studies to evaluate the effect of ionization on respirable dust particles in the horse's breathing zone are necessary to evaluate the ultimate benefit of ionization in horse stables.

Several studies have been performed to investigate the relationship between sources of feed and types of bedding and air quality in horse stables. In our study, inhalable dust concentrations were 86% lower in the units with wood shavings and haylage (LD) compared to those with straw and hay (HD). Previous studies found comparable reductions in breathing area total and respirable dust concentrations of 60%-94%.[2,7] Wood shavings compared to straw as bedding in horse stalls has been shown to reduce airborne dust, endotoxin, and fungi matter significantly.[2,3,22] One study demonstrated area total dust concentrations of 0.70 mg/m^3 for horses kept on wood shavings and fed a complete pelleted feed, compared to 0.14 ± 0.07 mg/m^3 in our study, and 2.55 mg/m^3 for horses kept on straw and fed dry hay, compared to our 0.97 ± 0.7 mg/m^3.[22] These differences might be explained by different ventilation in the stable units used or differences in the dust content of the bedding and feed used. An experimental study reported higher respirable dust and fungal concentrations when wood shavings were used as

TABLE 2 Mean (geometric mean) of dust and endotoxin concentration per type of bedding in relation to time of sampling; 'on' and 'off' indicating whether ionization was operating or not. HD = units with straw bedding and dry hay feed; LD = units with shavings bedding and haylage feed

	Ionization on				Ionization off			
	HD		LD		HD		LD	
Unit	Dust (mg/m^3)	Endotoxin (EU/m^3)	Dust (mg/m^3)	Endotoxin (EU/L)	Dust (mg/m^3)	Endotoxin (EU/m^3)	Dust (mg/m^3)	Endotoxin (EU/m^3)
Total	1.2 (1.0)	3476 (2698)	0.17 (0.16)	149 (137)	1.1 (0.9)	2853 (2351)	0.14 (0.13)	176 (143)
Daytime	1.1 (1.0)	3940 (3256)	0.18 (0.16)	171 (164)	1.2 (1.2)	3686 (3206)	0.12 (0.11)	152 (134)
Nighttime	0.73 (0.61)	1990 (1578)	0.15 (0.15)	90 (87)	0.69 (0.61)	1664 (1305)	0.18 (0.16)	215 (159)
Extra mesh	1.7 (1.5)	4013 (3188)	n/a	n/a	1.1 (1.0)	2536 (2301)	n/a	n/a

HD, High dust; LD, low dust.

TABLE 3 Mean (geometric mean) of concentration × 10^3 of fungal colonies (CFU/m^3) per type of bedding in relation to time of sampling; 'on' and 'off' indicating whether ionization was operating or not. HD = units with straw bedding and dry hay feed; LD = units with shavings bedding and haylage feed. Mean is only based on samples that did not reach the upper limit of 28.3 × 10^3 CFU/m^3

	Ionization on				Ionization off			
	HD		LD		HD		LD	
Unit	$N_{<UL}/N^a$	Mean CFU/m^3 (GM)	$N_{<UL}/N^a$	Mean CFU/m^3 (GM)	$N_{<UL}/N^a$	Mean CFU/m^3 (GM)	$N_{<UL}/N^a$	Mean CFU/m^3 (GM)
Total	7/24	24.0 (23.7)	21/23	3.4 (2.9)	5/20	20.1 (17.9)	21/21	9.7 (4.8)
Morning	0/9	–	8/9	5.1 (4.5)	1/9	6.3 (6.3)	9/9	11.0 (6.3)
Afternoon	3/9	26.0 (25.9)	8/8	2.4 (2.2)	2/7	27.5 (24.3)	8/8	6.6 (3.6)
Midnight	4/6	24.3 (24.0)	5/6	2.5 (2.2)	2/4	19.7 (19.7)	6/6	10.8 (4.6)

CFU, colony-forming units; HD, High dust; LD, low dust.
[a]Number of samples below upper limit of detection ($N_{<UL}$)/total number of samples (N); mean and GM are based on samples below upper limit of detection only.

bedding compared to straw.[6] In that study, feed and bedding samples were placed in a sample chamber of 0.6 m^3, through which there was a standardized flow rate of air passed. This methodology is very different to studies in horse stables and this might explain the different outcome. Interestingly a different study reported lower dust concentrations when horses were fed haylage and kept on straw bedding compared with dry hay and wood shavings. A change in feeding regime might even be more important than the type of bedding to improve the air quality in a horse stable.[7] A more than 5-fold decrease in total endotoxin concentrations was demonstrated when a management system using wood shavings and silage was applied in a horse stable, the decrease of 95% found in the present study even exceeds this positive result in endotoxin reduction.[8]

The finding of higher concentrations of fungi in stables with straw compared to (wood) shavings is consistent with previous results.[3] The fungal and endotoxin burden was highest in the morning, and at night. The lower concentrations during the night are probably due to reduced horse and personnel activity. Horses susceptible to equine asthma showed clinical signs of RAO after exposure to moldy hay containing, among others, *Aspergillus fumigatus*.[23] High concentrations of *Penicillium spp*. and *Aspergillus spp*. were visible on microscopic examination of the agar plates in samples from both types of stable bedding. Subspecies differentiation was not performed.

A major difference in study design of the present compared to other studies[6,7] is that samples were collected at fixed locations within the stable units, while other studies collected the samples close to the horses' or humans' nostrils. Measuring in the direct surroundings of the nose of the horse gives a more precise indication of the particulates that the horse actually inhales[24] and such samples yield higher results for dust since horses are constantly exploring their feeding bins and stalls looking for food.[22,24] Area measurements using stationary sampling equipment allows continuous monitoring of dust particles in the stable.[24] The methods used in the present study were aimed at investigating the effect of ionization, bedding and feed on the total stable air quality, and therefore, area measurements were preferred.

Overall it can be stated that the installation of an air purifier in the form of a negative ionizer in the horse stable, under the conditions used in our study has no effect on the reduction of inhalable dust, inhalable endotoxin, and fungi. The substantial effect of reduced dust bedding and feed is confirmed.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

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