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RESEARCH ARTICLE

The Effects of Germanium Biotite Supplement as a Prophylactic Agent against Respiratory Infection in Calves

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ABSTRACT

Germanium biotite, a natural mineral, is comprised of mainly silicate. This mineral showed activities of increase in feed efficiency and non-specific immunostimulation in previous studies. The aims of the present study were to evaluate the prophylactic effects of germanium biotite against respiratory diseases in calves as a feed supplement and investigate the possibilities of the substitution of antibiotics with germanium biotite as feed additive. To achieve these purposes, bovine herpesvirus-1 (BHV-1) and *Mannheimia haemolytica* serotype A1 were experimentally inoculated into the calves. After challenge, germanium biotite showed a lower cumulative clinical score (CCS) than the control group. In accordance with these clinical results, enhanced clearance of BHV-1, a low infection rate of *Mannheimia haemolytica* serotype A1, tempered superficial lesions, and moderated histopathological signs were observed in the germanium biotite group, compared with the control group. The results of the present study indicated that germanium biotite had prophylactic effects against bovine respiratory disease and could be a candidate for a new alternative feed supplement in calves, through its effects as a non-specific immune stimulator.

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INTRODUCTION

The consumption of livestock products has been increasing along with increased per capita income. This growth in consumption has driven an increase in the importance of the safety of different food commodities. Antibiotics have been used as feed supplements to improve the rate of gain and feed efficiency and to prevent infectious diseases (Muhl and Liebert, 2007). However, due to concern about antibiotic residues and emerging antibiotic resistance (van den Bogaard and Stobberingh, 1999), use of antibiotics as feed additives has been banned in the European Union (Kamphues, 1999) and Korea. For this reason, studies seeking for substances which can activate the non-specific immune ability in place of antibiotics are actively in progress.

Biotite, a well known feed supplement, is a common phyllosilicate mineral containing potassium, magnesium, iron, aluminum, and silicate (Sarker *et al.*, 2010; Vondruskova *et al.*, 2010). It has been reported that immune cells stimulated by silicate were associated with the non-specific immunostimulating ability of biotite. Fibrogenic silicate (SiO_2) activated proinflammatory macrophages (Holian *et al.*, 1997), and aluminosilicate (Al_2SiO_5) improved immune-cell differentiation (Jung *et al.*, 2010). These previous studies suggested that germanium biotite has potential as a new alternative feed supplement for non-specific immunostimulators, prophylactic agents, and remedial agents.

Bovine respiratory disease (BRD) is a major economic problem in the cattle industry around the world due to morbidity, mortality, low feed efficiency, prevention costs, and treatment (Fulton, 2009; Irsik *et al.*, 2006). The disease is caused by not only multi-factorial pathogens but also environmental condition, and Bovine herpesvirus type 1 (BHV-1) and *Mannheimia haemolytica* serotype A1 have been described as major etiologic agents (Fulton, 2009). *Mannheimia haemolytica* serotype A1 causing BRD in young calves has been detected in the respiratory tract of healthy cattle. Although the microorganism naturally exists as commensal of the respiratory tract, it is also considered the major bacterial agent of BRD in calves (Rice *et al*., 2007). As host immunity becomes weaker as a result of stress or infection with other pathogens, the microorganism may become infective and play an important role in BRD, also known as shipping fever in calves. BHV-1 is also a major etiological agent of BRD along with *Mannheimia haemolytica* serotype A1 (Autio *et al*., 2007). BHV-1 is an α-herpesvirinae subfamily member that causes significant economic problems in the cattle industry (Irsik *et al*., 2006; Fulton, 2009). Following infection with BHV-1, the virus could be latent in sensory ganglia and reactivated both by stressful environments and administration of glucocorticoids. In addition, BHV-1 depresses cell-mediated immunity in the host by repressing expression of major histocompatibility complex (MHC) class I that acts in association with CD8+ T-cell recognition of infected cells and transporter associated with antigen presentation (Jones and Chowdhury, 2007). The suppression of the immune function renders the host susceptible to secondary infection like pneumonic mannheimiosis, thereby causing BRD more easily.

The aims of the present study were to investigate characteristics of germanium biotite, known to the non-specific immune stimulating mineral, about prophylactic effect in BRD infected calves. A BHV-1 and *Mannheimia haemolytica* serotype A1 challenge experiment in calves was performed to examine the prophylactic effect of germanium biotite against BRD. Finally, an aim was to explore the possibilities of the substitution of germanium biotite for antibiotics as a feed supplement.

MATERIALS AND METHODS

Source of feed supplements: Germanium biotite (under solto Bio) provided by Seobong Biobestech (Seoul, Korea) was comprised of silicon dioxide (SiO_2, 61.90%), aluminum dioxide (Al_2O_3, 23.19%), iron oxide (Fe_2O_3, 3.97%), calcium oxide (CaO, less than 2%), magnesium oxide (MgO, less than 2%), and titanium oxide (TiO_2 less than 2%).

Calves and challenge experiment design: Korean native calves of three months of age were used for this study. The calves were randomized into two groups: the control group (n=3), fed with a normal commercial feed without any antibiotics; and the germanium biotite group (n=3), fed with the commercial feed supplemented with 0.5% germanium biotite. The calves of two groups were housed in individual rooms and allowed easy access to water and feed. Before the challenge, calves were confirmed as negative against respiratory pathogens to be challenged. After feeding with those fodders for two weeks, the calves were challenged with BHV-1 (5.0×10^9 PFU) and *Mannheimia haemolytica* serotype A1 (1.0×10^{10}CFU) three times at 24 hours intervals. To equalize natural infectious condition, all calves were challenged through nasal spray.

Preparation of pathogens: The BHV-1 used was isolated from a calf naturally suffering from shipping fever (Animal, Plant and Fisheries Quarantine and Inspection Agency, QIA; Anyang, Korea). Before challenge, the virus was propagated and titrated in MDBK cells (Abril *et al*., 2004). *Mannheimia haemolytica* serotype A1 used in the study was also provided by QIA. The identity of the bacterium was confirmed by using multiplex PCR as previously described (Alexander *et al*., 2008). The pathogen was grown on a blood agar plate (BAP) in a CO_2 incubator at 37℃ and subcultured in tryptic soy broth (TSB; Difco, MD, USA) to obtain a sufficient quantity of challenge agent. Before challenge, the cultured bacteria were pelleted by centrifugation, washed three times in sterile phosphate buffered saline (PBS), and resuspended in PBS. The final concentration of approximately 1.0×10^{10} CFU was confirmed according to the standard curve of CFUs versus optical density (Hanzlicek *et al*., 2010). After each challenge, plate counts were used to confirm bacterial concentration.

Clinical monitoring after pathogens challenge: The body conditions and clinical signs of calves were recorded each 24 hr throughout the experiment period. Rectal temperature, degree of nasal discharge, cough frequency degree, and respiratory rate were scored using the criteria established by Hodgson *et al.* (1995) (Table 1). The measured scores of each calf were accumulatively added and considered as cumulative clinical score (CCS).

Table 1: The criteria for the scoring of clinical signs

Signs	Description	Score
Temperature	38.0-39.5℃	0
	39.5-40.0℃	1
	40.0-40.5℃	2
	40.51-41.0℃	3
	41.0℃ >	4
Degree of nasal discharge	Absent	0
	Mild	1
	Moderate	2
	Severe	3
Cough frequency degree	Absent	0
	Mild	1
	Moderate	2
Respiratory rate	< 50	0
	50-60	1
	60-70	2
	70-80	3
	80 >	4

These criteria were modified from Hodgson *et al.* (1995).

Measurement of challenged pathogens clearance: After challenge, nasal swab samples were collected after every two days and each sample was resuspended in 1 ml PBS and diluted in 10 fold serial dilutions up to 10^{-4}. Aliquots (50μl) of each dilution were used for experiment samples. Real-time PCR was used in measurement of virus clearance. DNA extraction from each sample was performed using the Accuprep genomic DNA extraction kit (Bioneer, Seoul, Korea) according to the manufacturer's instructions and used template for real-time PCR. Primer and probes for quantitative real-time TaqMan PCR, real-time PCR standard of BHV-1 genomes, and condition were developed and validated as TaqMan PCR, real-time PCR standard of BHV-1 genomes, and condition were developed and validated as

Table 2: Primers and probes for PCR

Pathogens	Oligonucleotide		Sequences (5′-3′)	Product size
BHV-1	BHV-1 (F)[a]		TGTGGACCTAAACCTCACGGT	97 bp
	BHV-1 probe[b]		AGGACCGCGAGTTCTTGCCGC	
	BHV-1 (R)[c]		GTAGTCGAGCAGACCCGTGTC	
Mannheimia haemolytica serotype A1	Lkt	(F)	GCAGGAGGTGATTATTAAAGTGG	206 bp
	HP	(R)	CAGCAGTTATTGTCATACCTGAAC	
		(F)	CGAGCAAGCACAATTACATTATGG	90 bp
	16s	(R)	CACCGTCAAATTCCTGTGGATAAC	
		(F)	GCTAACTCCGTGCCAGCAG	~304 bp
		(R)	CGTGGACTACCAGGGTATCTAATC	

[a] Forward primer; [b] 6-Carboxyfluorescein-labeled probe; [c] Reverse primer.

described previously (Abril *et al.*, 2004). Multiplex PCR was used in confirmation of bacterial infection using specific primers (Table 2). DNA extraction from aliquots of sample was performed according to a previous study (Suh and Song, 2005). DNA extracts were used for the PCR template as previously described (Alexander *et al.*, 2008). The total isolated bacteria counts were measured by CFU counting. Aliquots (50μl) of each sample dilution were applied to BAP and incubated at 37℃ for 16 hr. The viable counts were determined and expressed as CFU/swab. The area under curve (AUC) of bacteria counting graph was calculated for confirmation of total shedding bacteria throughout the experiment.

Necropsy: On day 12th post challenge, all calves were humanely euthanized and their lungs were collected. The percentages of superficial lesion area were calculated by comparison with total lung area. The lungs and trachea were collected from calves, and then representative samples were placed in 10% formal-saline for histopathological examination using standard techniques. Microscopic lesion scores were calculated using a described scoring system (Opriessnig *et al.*, 2004). For viral and bacterial examination, tissue samples (approximately 1 g) were homogenized in 9 ml PBS and diluted in 10-fold steps up to 10^{-4} and then aliquots (50μl) of each dilution were used for experiment samples for pathogen examination.

Statistical analysis: The data were expressed as mean ± standard deviation (SD), and statistical differences between the groups were analyzed with student's t-test; whereas, for microscopic lesion scores, a nonparametric test (Mann-Whitney U test) was performed using SPSS version 17.0 software (SPSS, Chicago, IL, USA). Differences were considered significant when probability values of P<0.05 were obtained.

RESULTS

Clinical signs after challenge in experiment calves: The CCSs of all calves were 0 before challenge. The CCSs, however, appeared to increase after challenge and lower CCSs than those of the control group were observed in the germanium biotite group throughout the experimental period (Fig. 1).

Viral and bacterial clearance: On D+2 following the challenge, BHV-1 was detected in all calves. After D+6, the BHV-1 genomic quantity of the germanium biotite group decreased, showing a lower level than the control group (Fig. 2a). The viral genome load in trachea of the germanium biotite group was lower than the control group at post-mortem, while higher level than in the control group was observed in lungs of the germanium biotite group (Fig. 2c). *Mannheimia haemolytica* serotype A1 was detected on D+2 by multiplex PCR in all calves except for two of the germanium biotite group (Fig. 3). Infection of two calves in the control group and one calf in the germanium biotite group was confirmed until D+10. *Mannheimia haemolytica* serotype A1 was identified in lungs from a calf of the control group at post-mortem, but not identified in other calves. The numbers of total bacteria isolated from nasal discharge in the germanium biotite and control groups showed no significant differences, but higher AUC than in the germanium biotite group was observed in the control group. The AUC of control and germanium biotite groups was 41.3±0.5 and 38.3±0.7, respectively (Fig. 2b).

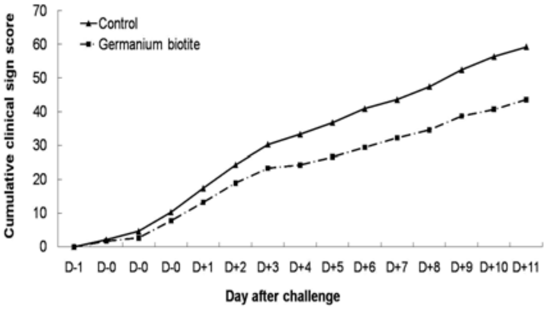

Fig. 1: Cumulative total clinical score was calculated from each clinical sign. The control group showed higher level than the germanium biotite group.

Gross pathology and histopathologic analysis: Lung lesions of calves varied from brown to purple dark pink often associated with congestion and most of the lesions were present in apical lobes. The germanium biotite group showed lower distribution of surface lesions and their severity as compared to the control group. Compared with the control group, low hepatization of lung was confirmed in the germanium biotite group by palpation. Calves in the germanium biotite group showed a significant decrease in the percentages of superficial lesion area compared with the control group (P<0.01). The percentages of lesion area in the control and germanium biotite groups were 34.8%±4.6 and 10.2%±3.0, respectively (Fig. 2d). In microscopic lung lesions, type 2 pneumocyte hypertrophy and hyperplasia, alveolar wall thickening, and neutrophils filtration were observed in all lungs of challenged calves, showing moderate signs in the germanium biotite group (Fig. 4). The microscopic lesion score of the germanium

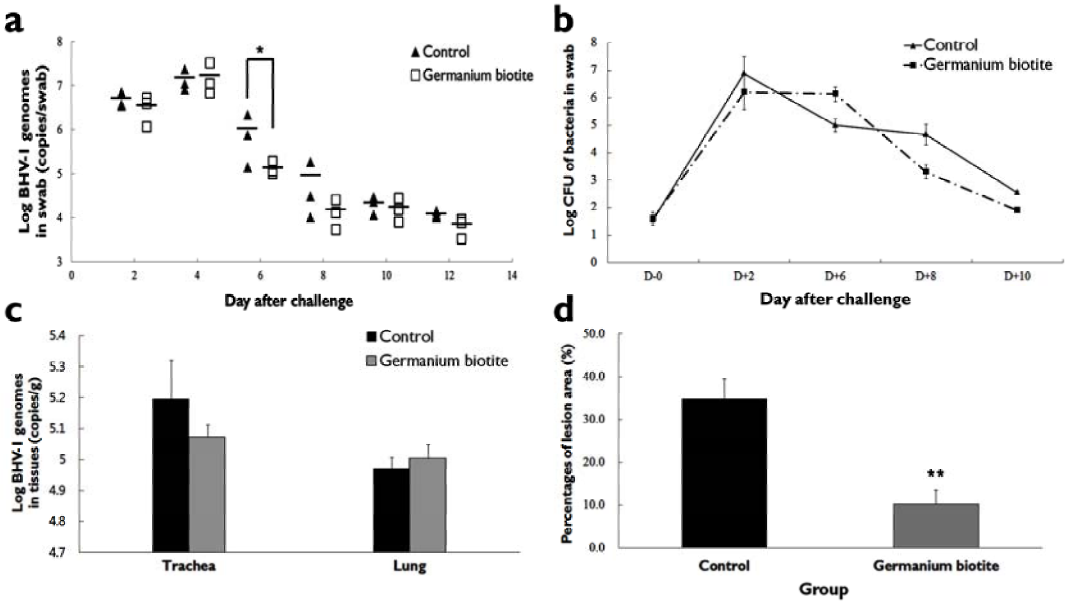

Fig. 2: Clearance of challenge pathogens and percentage of lesion area. (a) Clearance of BHV-1 in experimentally infected calves. (b) Clearance of total bacteria in experimentally infected calves. The higher AUC than in the germanium biotite group was observed in the control group. (c) Quantity of BHV-1 genomes in trachea and lung at post-mortem. While germanium biotite group showed higher quantity of BHV-1 genomes than control group in lung, a lower quantity than in the control group was observed in trachea of germanium biotite group. *Mannheimia haemolytica* serotype A1 was not detected at post-mortem, except for in a lung from a calf in the control group. (d) Percentages of superficial lesion area in experimentally infected calves. The germanium biotite group showed significantly lower percentages of lesion area than the control group. **Highly significant at P<0.01.

Table 3: Microscopic lesion scores in challenged calves

Groups	Control	Germanium biotite
Scores (± SD)	5.00 (± 0.26)	2.50 (± 0.23)
	4.30 (± 0.34)	1.75 (± 0.30)
	4.36 (± 0.28)	1.92 (± 0.38)
Means (± SD)	4.55 (± 0.22)	2.06 (± 0.23) **

The microscopic lesion scores were calculated using a previous scoring system (Opriessnig *et al.*, 2004). The control group shows higher lesion scores than the germanium biotite group significantly (P<0.01).

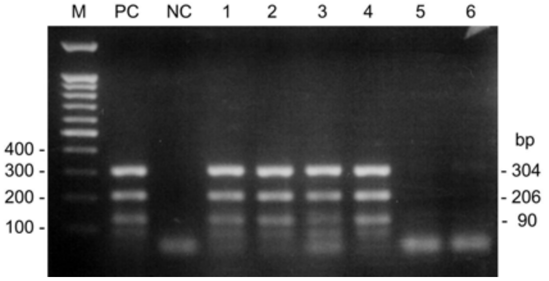

Fig. 3: The confirmation of *Mannheimia haemolytica* serotype A1 infection of claves on D+2 using multiplex PCR. Lines PC and NC, Postive control and negative control, respectively, lines 1-3; calves in control group and lines 4-6; calves in germanium biotite administrated group. PCR amplification regions of 304-bp, 206-bp, and 90-bp account for 16S rDNA gene, leukotoxin gene, and unknown hypothetical protein of *Mannheimia haemolytica* serotype A1. *Mannheimia haemolytica* serotype A1 is detected in all calves of the control group (1-3) and one calf of the germanium biotite group (4-6).

biotite group (2.06±0.23) was significantly lower, compared with the control group (4.55±0.22) (Table 3).

DISCUSSION

BRD, along with diarrhea a major cause of calf deaths, is caused by multi-factors mainly immune depression. BHV-1 and *Mannheimia haemolytica* serotype A1 are usually isolated from calves suffering from BRD (Irsik *et al.*, 2006; Fulton, 2009). In the cattle industry, antibiotics have been used as a feed supplement to prevent both BRD and secondary infections. Virus infection, however, cannot be prevented by using antibiotics, and the use of antibiotics has been gradually limited because of antibiotic residues and emergence of antibiotic resistance. In addition, there are restrictions on vaccination against viruses especially, live vaccines, in view of vaccine infection, latent infection, pathogen carrier and serological differentiation from wild infection (Bosch *et al.*, 1996). For these reasons, activation of non-specific immunity has been considered for controlling BRD in place of antibiotics.

Silicate, the major constituent of germanium biotite, has been studied for its non-specific immune enhancing effects. It was reported that macrophages could be stimulated and release large amounts of TNF-α by silicate *in vitro* (Holian *et al.*, 1997). It was also reported that relative mRNA expression levels of IFN-γ, IL-4 and TNF-α produced mainly by T cell and macrophages could increase significantly in splenocytes of aluminosilicate (Al_2SiO_5) orally primed mice. In addition, aluminosilicate (Al_2SiO_5) primed mice also showed high antibody production levels when they were exposed to formalin-killed *Pasteurella multocida* type A antigen. Moreover, oral ingestion of aluminosilicate (Al_2SiO_5) showed enhancing effects on reinforcing clearance of porcine circovirus type 2 in experimentally infected pigs (Jung *et al.*, 2010). The results of these studies of immune activities of silicate make us postulate that germanium

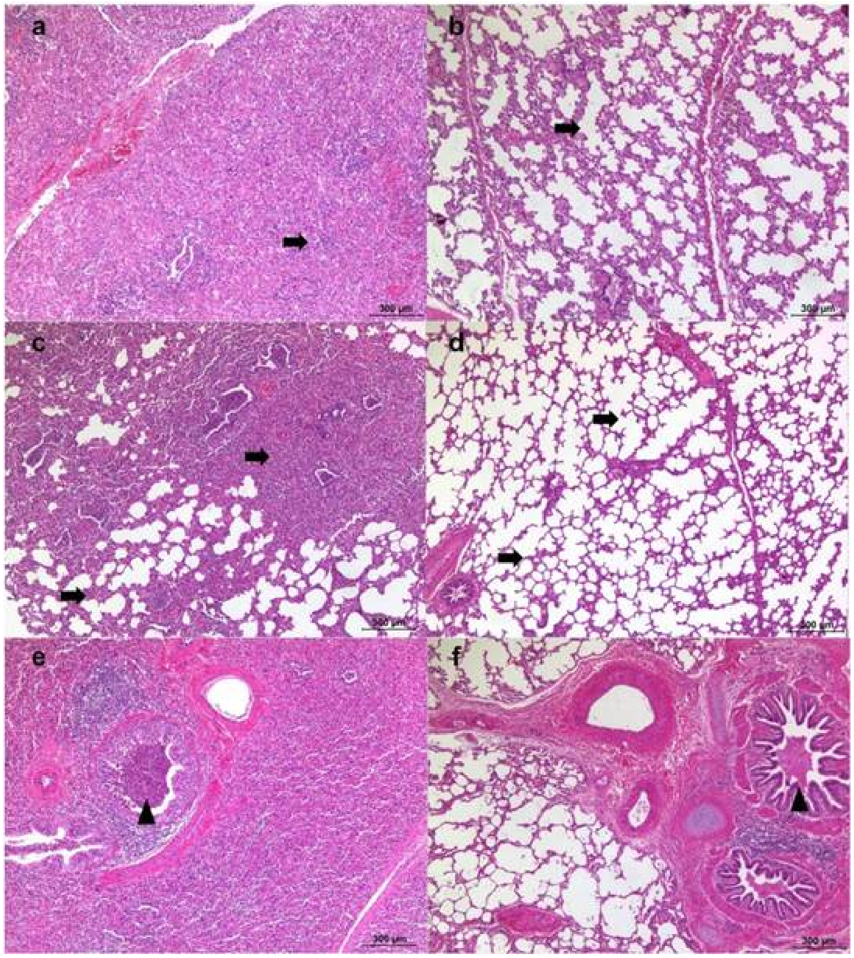

Fig. 4: Histopathological features of lung tissue samples in challenged calves. Severe neutrophils filtration and thickened alveolar septa are observed in lungs of the control group (arrows in **a** and **c**) compared with the germanium biotite group (arrows in **b** and **d**). Bronchiole of the control group also contains more numerous neutrophils (arrowhead in **e**) compared with the germanium biotite group (arrowhead in **f**).

biotite could have prophylactic effects on BRD occurred mainly with relevance to immunosuppression. However, a natural mineral like germanium biotite has not been studied for its prophylactic effect against BRD. In this study, the first analysis of the prophylactic effect of germanium biotite against BRD in experimentally infected calves was conducted.

To investigate exact clinical signs, the normal temperature limit (39-40°C) confirmed in a previous study (Hodgson *et al*., 1995) was modified because situations in the present experiment, like rearing environments and breed of calves, differed from those in the previous study. Based on this information, the normal temperature limit was modified in the present experiment according to the mean of temperatures measured in preinoculated calves.

Following the challenge, the germanium biotite group showed alleviated clinical signs compared with the control group. In accordance with these clinical results, the germanium biotite group showed enhanced clearance of BHV-1 and a low infection rate of *Mannheimia haemolytica* serotype A1 compared with the control group. The germanium biotite group also showed lower AUC of clearance of total bacteria in nasal swab samples. At post-mortem, however, the germanium biotite group showed a higher quantity of BHV-1 in lungs than the control group and a lower quantity in trachea than the control group. It appeared that there was a greater amount of virus shedding in the trachea in the control group, even though a high quantity of BHV-1 was detected in lungs of the germanium biotite group compared with the control group. Moreover, it was reported that severe clinical signs were usually accompanied by a high titer of virus shedding (Song *et al*., 2011). These clinical and clearance results correspond with superficial lesion area data and microscopic lesion analysis in the present study. Significantly higher percentages of normal area than the control group were observed in the germanium biotite group ($P<0.01$). In microscopic lung lesions, the germanium biotite group showed moderate signs of neutrophils filtration and bronchiolar exudates compared with the control group. Neutrophils filtration in alveoli and bronchial exudates are typical signs of bronchopneumonias, which are generally caused by bacteria. Therefore, it could be inferred that germanium biotite primed calves were protected from secondary infections caused mainly by bacteria like *Mannheimia haemolytica* serotype A1.

These results suggested that ingestion of germanium biotite had prophylactic effects against BHV-1 and *Mannheimia haemolytica* serotype A1 in calves. It can be presumed that the prophylactic effects of germanium biotite against challenged BRD pathogens are associated with its stimulating activities on non-specific immune response. In conclusion, results of this study indicated that germanium biotite has the potential to activate innate immunity thereby could be a good alternative to antibiotics as a feed supplement for calves. Future research is needed focusing on the mechanism of absorption of germanium biotite in calves' gastrointestinal tracts and the mechanism of non-specific immune stimulating effects.

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REFERENCES

Abril C, M Engels, A Liman, M Hilbe, S Albini, M Franchini, M Suter and M Ackermann, 2004. Both viral and host factors contribute to neurovirulence of bovine herpesviruses 1 and 5 in interferon receptor-deficient mice. J Virol, 78: 3644-3653.

Alexander TW, SR Cook, LJ Yanke, CW Booker, PS Morley, RR Read, SP Gow and TA McAllister, 2008. A multiplex polymerase chain reaction assay for the identification of *Mannheimia haemolytica*, *Mannheimia glucosida* and *Mannheimia ruminalis*. Vet Microbiol, 130: 165-175.

Autio T, T Pohjanvirta, R Holopainen, U Rikula, J Pentikainen, A Huovilainen, H Rusanen, T Soveri, L Sihvonen and S Pelkonen, 2007. Etiology of respiratory disease in non-vaccinated, non-medicated calves in rearing herds. Vet Microbiol, 119: 256-265.

Bosch J, M Kaashoek, A Kroese and J Van Oirschot, 1996. An attenuated bovine herpesvirus 1 marker vaccine induces a better protection than two inactivated marker vaccines. Vet Microbiol, 52: 223-234.

Fulton RW, 2009. Bovine respiratory disease research (1983-2009). Anim Health Res Rev, 10: 131-139.

Hanzlicek GA, BJ White, D Mosier, DG Renter and DE Anderson, 2010. Serial evaluation of physiologic, pathological, and behavioral changes related to disease progression of experimentally induced *Mannheimia haemolytica* pneumonia in postweaned calves. Am J Vet Res, 71: 359-369.

Hodgson JC, GR Barclay, LA Hay, GM Moon and IR Poxton, 1995. Prophylactic use of human endotoxin core hyperimmune gammaglobulin to prevent endotoxaemia in colostrum deprived, gnotobiotic lambs challenged orally with *Escherichia coli*. FEMS Immunol Med Microbiol, 11: 171-180.

Holian A, MO Uthman, T Goltsova, SD Brown and RF Hamilton Jr, 1997. Asbestos and silica-induced changes in human alveolar macrophage phenotype. Environ Health Perspect, 105: 1139-1142.

Irsik M, M Langemeier, T Schroeder, M Spire and J Roder, 2006. Estimating the effects of animal health on the performance of feedlot cattle. Bovine Pract, 40: 65-74.

Jones C and S Chowdhury, 2007. A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex and development of improved vaccines. Anim Health Res Rev, 8: 187-205.

Jung BG, NT Toan, SJ Cho, J Ko, YK Jung and BJ Lee, 2010. Dietary aluminosilicate supplement enhances immune activity in mice and reinforces clearance of porcine circovirus type 2 in experimentally infected pigs. Vet Microbiol, 143: 117-125.

Kamphues J, 1999. Antibiotic growth promoters for the view of animal nutrition. Berl Munch Tierarztl Wochenschr, 112: 370-379.

Muhl A and F Liebert, 2007. Growth and parameters of microflora in intestinal and faecal samples of piglets due to application of a phytogenic feed additive. J Anim Physiol Anim Nutr, 91: 411-418.

Opriessnig T, E Thacker, S Yu, M Fenaux, XJ Meng and P Halbur, 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. Vet Pathol, 41: 624-640.

Rice J, L Carrasco-Medina, D Hodgins and P Shewen, 2007. *Mannheimia haemolytica* and bovine respiratory disease. Anim Health Res Rev, 8: 117-128.

Sarker M, G Kim and C Yang, 2010. Effect of green tea and biotite on performance, meat quality and organ development in ross broiler. Egypt Poult Sci, 30: 77-88.

Song DS, HJ Moon, K Jung, MJ Yeom, HK Kim, SY Han, DJ An, JS Oh, JM Kim and BK Park, 2011. Association between nasal shedding and fever that influenza A (H3N2) induces in dogs. Virol J, 8: 1-4.

Suh DK and JC Song, 2005. Simultaneous detection of *Lawsonia intracellularis*, *Brachyspira hyodysenteriae* and *Salmonella* spp. in swine intestinal specimens by multiplex polymerase chain reaction. J Vet Sci, 6: 231-237.

van den Bogaard AE and EE Stobberingh, 1999. Antibiotic usage in animals: impact on bacterial resistance and public health. Drugs, 58: 589-607.

Vondruskova H, R Slamova, M Trckova, Z Zraly and I Pavlik, 2010. Alternatives to antibiotic growth promoters in prevention of diarrhoea in weaned piglets: a review. Vet Med (Praha), 55: 199-224.