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Effect of *Artemisia apiacea* Hance on growth performance, cecal opportunistic bacteria, and microbicidal peptides in rabbits

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ABSTRACT - This study aimed to investigate the effect of *Artemisia apiacea* Hance supplementation on growth performance, cecal opportunistic bacteria, and antimicrobial defense using 120 rabbits. There were four experimental diets containing a control and *A. apiacea* Hance added at doses of 25, 50, and 75 g kg^{-1} of feed. The trial lasted for 70 days. The results showed that diets supplemented with *A. apiacea* Hance improved feed intake, body weight gain, and feed efficiency. Linear and quadratic responses were found between feed intake and herbal meal doses. For cecal opportunistic pathogenic bacteria, compared with the control treatment, the herb decreased cecal *C. perfringens*, Gram-negative bacteria, and *Salmonella* spp. by 9.5 to 56.8%. Linear responses of herb doses were found on the four bacteria and a quadratic response on *Salmonella* spp. In addition, the herb increased the mRNA levels by 12.6 to 57.8% of cecal defensive peptides, including neutrophil peptide defensing-3a, regenerating family member-3 gamma defensin beta-1, and galectin-4. These genes linearly responded to the herb doses. The obtained data suggest that *A. apiacea* Hance is effective to improve animal growth by beneficially regulating gut opportunistic bacteria and microbicidal peptide activity.

Keywords: *C. perfringens*, gene expression, *Salmonella*

Introduction

Genus *Artemisia* consists of about 350 species, some of which, including *Artemisia apiacea* Hance, are widely used as herbal medicines against oxidation, inflammation, and immune and hepatic disease through their secondary metabolites, mainly including flavonoids and terpenoids (Lee et al., 2002, 2003; Pellicer et al., 2018). *In vitro* or *in rat* studies found that fractions from *A. apiacea* Hance suppressed serum transaminase activity, malondialdehyde, and proinflammatory chemokine production (Kim et al., 2003; Ryu et al., 2013; Yang et al., 2018). In farm animals, dietary *Artemisia vulgaris*, *Artemisia argyi*, *Artemisia annua*, or their extracts showed a significant improvement in growth, antioxidation, antiinflammation, meat quality, and immune function (Wan et al., 2016, 2017; Zhang et al., 2017; Baghban-Kanani et al., 2019; Wang et al., 2019).

Moreover, *A. apiacea* Hance was found effective against *A. niger*, *C. albicans*, *B. subtilis*, and *S. aureus in vitro* (Trinh et al., 2018). *Artemisia vulgaris* regulated gut microbes including *Lactobacilli*, *Bifidobacteria*, *E. coli*, *C. perfringens*, and *Salmonella* in rabbits (Wang et al., 2019). Additionally, gut epithelial antimicrobial peptides and proteins, such as alpha- or beta-defensins, calprotectin,

cathelicidins, C-type lectins, galectins, lipocalin, and peptidoglycan recognition proteins, have an essential role in allowing epithelial surfaces to cope with pathogenic microbial challenges (Gallo and Hooper, 2012). The expression, secretion, and activity of most epithelial antimicrobial peptides are tightly controlled by a complex network of developmental, microbial, and nutritional signals (Gallo and Hooper, 2012).

A. apiacea Hance extract, as a natural antimicrobial herb, is increasingly popular for humans. Considering its high yield and wide geographical distribution in wasteland and river beaches, hypothetically, *A. apiacea* Hance stem and leaf meal can be used as a natural and cost-effective growth-promoting additive for farm animals, especially herbivores. Furthermore, whether *A. apiacea* Hance phytochemicals regulate the activity of gut endogenous antibacterial peptides has not yet been elucidated.

The present study aimed to investigate the effect of supplemental *A. apiacea* Hance stem and leaf meal on growth performance, cecal opportunistic bacteria, and mRNA profiles of antimicrobial peptides of rabbits.

Material and Methods

The experimental protocol of the present study was approved by the local Institutional Committee for Animal Use and Ethics (No. 2018016). The study was carried out in Luoyang, China.

A. apiacea Hance was botanically identified by Prof. F. M. Dong, Henan University of Science and Technology, Luoyang, China. This plant–at vegetative period from Funiu mountains in Songxian of China (112°10' N, 34°+15' E)–was cut above the ground, air dried, ground into meal (40-mesh sieve), and added at 25, 50, and 75 g kg^{-1} to diets (Table 1).

The nutrition levels of experimental diets and animal management were as recommended by Technical Specification for Feeding and Management of Rex Rabbits (NY/T2765-2015, Ministry of Agriculture of China, 2015; Table 2). Diets were fed as cold formed pellets (3.5×8.0 mm, diameter × length) with water contents under 140 g kg^{-1}. All diets were stored in a cool, dry, dark, and well-ventilated place. No antibiotics were used either in feed or water throughout the experiment.

One hundred twenty weaned male Rex rabbits at approximately 35 days old with initial body weight 751±3.62 g (mean±SD) were randomly assigned to the four dietary treatments. There were six replicates in a treatment and five rabbits housed in a continuous row cage consisting of five independent spaces (35×45 cm, length × width) as a replicate. All replicates were uniformly distributed in the rabbit house. The rabbits had free access to diet and water. The feeding trial lasted for 70 days. Rabbits and feed in each replicate were weighed at 35, 70, and 105 days old. Average daily feed intake (ADFI), average daily body weight gain (ADG), and feed conversion ratio (FCR) were immediately adjusted when mortality occurred. All rabbits were monitored for general health twice a day.

At the end of the trial, five rabbits per replicate were weighed, and then cecal content was collected and stored at −40 °C for gut microbe enumeration. The cecum was cleaned with phosphate-buffered saline (0 to 4 °C), and 2 cm length of cecum was cut from proximal end and immediately stored in an RNAlater solution (Dalian TaKaRa Co., Ltd., Liaoning, China) for gene expression analysis.

Chemical analysis of proximate nutrients and minerals in *A. apiacea* Hance was carried out according to method by Zhang et al. (2018). Total flavonoids in *A. apiacea* Hance were detected by Folin-Ciocalteu method (Fan et al., 2012) using gallic acid (Chinese National Institute for the Control of Pharmaceutical

Table 1 - Chemical compositions of *Artemisia apiacea* Hance meal (g kg^{-1} of dry matter)

Composition	Content	Composition	Content
Dry matter	875.5	Ca	1.52
Crude protein	123.8	P	2.13
Crude fiber	104.7	Flavonoids	12.64
Crude fat	53.3	Total triterpenoid	9.44
Crude ash	74.1		

and Biological Products, Beijing, China) as a standard. For triterpenoid content detection, *A. apiacea* Hance (0.1 g) was measured and soaked in an ethanol-water solution at a 1:20 solid-liquid ratio. Triterpenoid was extracted by the ultrasonic method (100 W) using ethanol (80%) at 60 °C for 20 min. The supernatant was collected by centrifugation at 3,000 × *g* for 5 min, and then, the supernatant (0.16 mL) was pipetted into a tube and dried at 70 °C in water bath. Newly mixed vanillin-glacial acetic acid solution (0.2 mL, 5%) and perchlorate (0.8 mL) were added and mixed. The solution was heated and reacted in 70 °C water bath for 20 min and then rapidly cooled. The solution volume was adjusted to 10 mL with ethyl acetate. Absorptions for flavonoids and triterpenoids were measured at 765 and 551 nm, respectively, using a Varian Carys 3C spectrophotometer (Varian Analytical Instruments, Harbor City, California, USA). Total flavonoid and triterpenoid contents were expressed as equivalent in g kg^{-1} of dry matter.

Each cecal content (1 g) was diluted with sterile buffered peptone water (0.1%, 9 mL, 0-4 °C) and mixed as described by Liu et al. (2018). The suspension of each sample was serially diluted between 10^{-1} to 10^{-7} dilutions, and each diluted sample (100 µL) was subsequently spread onto duplicate selective agar plates for bacterial count. The number of cfu was expressed as a logarithmic (log_{10}) transformation per gram of cecal digesta. Cecal bacterial populations were detected using commercial media including chromogenic medium (HB7001) for *E. coli*, sulfite polymixin sulphadiazine agar base (HB0256) for *C. perfringens*, deoxycholate hydrogen sulfide lactose agar (HB4087) for *Salmonella* spp., and Gram-negative bacteria ($Gram^-$) selection medium (HB8643). The media were purchased from Qingdao Hope Bio-Technology Co., Ltd. (Shandong, China).

Total mRNA isolation and cDNA synthesis for cecal samples were carried out as described by Liu et al. (2010), and the transcript levels were expressed as the relative expression to actin-beta gene. Quantitative PCR reaction was set at 10 µL with 5 µL of SYBR Green Master Mix, 1 µL of primer, and 4 µL of 10 × diluted cDNA or DNA. Plates were run on the ABI Prism 7900HT Fast Real-Time PCR System. All qPCR were run in triplicates on the same thermal cycles (50 °C for 2 min, 95 °C for 10 min, 40 cycles of

Table 2 - Ingredients and nutrient levels of diets

Item	*A. apiacea* Hance meal (g kg^{-1} as fed)			
	0	25	50	75
Ingredient (g kg^{-1} as fed)				
Artemisia apiacea Hance meal	0	25.0	50.0	75.0
Alfalfa meal	400.0	375.0	350.0	325.0
Corn	228.5	228.5	228.5	228.5
Soybean meal	140.0	140.0	140.0	140.0
Corn germ meal	100.0	100.0	100.0	100.0
Wheat bran	100.0	100.0	100.0	100.0
Dicalcium phosphate	20.0	20.0	20.0	20.0
Limestone	0	0	0	0
Choline chloride	1.5	1.5	1.5	1.5
Premix[1]	10.0	10.0	10.0	10.0
Nutrition (g kg^{-1} of dry matter)[2]				
Crude protein	172.0	171.5	171.0	170.6
Digestible energy (MJ kg^{-1})	10.95	10.94	10.93	10.93
Crude fat	25.1	25.9	26.7	27.5
Crude fiber	144.4	139.6	134.8	129.9
Lysine	8.0	8.0	8.0	8.0
Met + Cys	4.8	4.8	4.8	4.8
Ca	11.3	11.0	10.7	10.4
P	6.0	6.0	6.0	6.0

[1] The premix provided the following per kg of diets: vitamin A, 12,000 IU; vitamin D, 2,000 IU; vitamin E, 30 IU; Cu, 12 mg; Fe, 64 mg; Mn, 56 mg; Zn, 60 mg; I, 1.2 mg; Se, 0.4 mg; Co, 0.4 mg; NaCl, 6.4 g.
[2] Calculated by Chinese Feed Database, version 25, 2014.

Table 3 - Information of primers for quantitative real-time PCR

Name	GenBank	Primer (5'→3')		Length (bp)
		Forward	Reverse	
NP-3A	NM_001082298.1	aggttctagaccaacagcagc	tagcgggctccattgactct	175
DEFB1	XM_008274178.2	gccaccatgcgaatccacta	ttggaaaagagcgggcaaga	167
REG3G	XM_002709697.2	gcctcagaccgaggttactg	cccttcctgggtgagtcttc	190
LGALS4	NM_001082713.1	cctcttcgactacgcccatc	acacctgtatcagggtcgga	166
ACTB	X60733.1	tgtggccgaggactttgatt	ttacacaaatgcgatgctgcc	172

ACTB - actin beta; DEFB1 - defensin beta 1; LGALS4 - galectin 4; NP-3A - neutrophil peptide defensin 3a; REG3G - regenerating family member 3 gamma.

95 °C for 15 s, and 60 °C for 1 min). No amplification signal was detected in water or no-RT RNA samples. Primer (Table 3) synthesis and qPCR reagents were provided by Dalian TaKaRa Co., Ltd. (Liaoning, China).

Data were subjected to ANOVA and means (n = 6) were separated by Tukey's b-test at $P<0.05$ using IBM SPSS (version 23). The average of five rabbits per replicate was the statistical unit for growth performance, gut bacteria (Log_{10}cfu), and mRNA expression of genes.

Variables were analyzed according to the following mathematical model:

$$Y_{ij} = \mu + \beta_i + \varepsilon_{ij},$$

in which Y_{ij} = observation j of experimental unit subjected to treatments i, μ = general constant, β_i = effects of rations with *A. apiacea* meal at different levels, and ε_{ij} = random error associated to each observation.

Linear and quadratic equation of polynomial contrasts were used for the analysis of dose responses of *A. apiacea* meal at 25, 50, and 75 g kg^{-1}.

Results

Mortality of rabbits throughout the experiment was <5%, not statistically significant between treatments. The diets supplemented with *A. apiacea* Hance at 25, 50, and 75 g kg^{-1} improved ($P<0.05$) ADFI by 1.7 to 5.1% and ADG by 5.0 to 9.4% during 35-70 and 70-105 days of age (Table 4), but FCR was decreased ($P<0.05$) by 2.8 to 4.4% during 70-105 day of age. A quadratic response ($P = 0.013$) of herb doses was found on ADFI at 35-70 days, and a linear response ($P = 0.009$) on ADFI at 70-105 days.

During the whole period, diets supplemented with *A. apiacea* Hance at 25, 50, and 75 g kg^{-1} improved ($P<0.05$) ADFI by 3.4, 4.1, and 4.1% and ADG by 7.4, 7.2, and 7.5%, respectively. The effects of 50 and 75 g kg^{-1} doses on ADFI were more pronounced ($P<0.05$) than that of the 25 g kg^{-1} dose. Likewise, the three herb doses decreased FCR by 3.0 to 3.7%. The ADFI responded linearly ($P = 0.001$) and quadratically ($P = 0.039$) to the herb doses, but there were no dose effects on ADG and FCR.

As to cecal opportunistic pathogenic bacteria, compared with the control treatment, the three tested doses of *A. apiacea* Hance did not affect the population of *E. coli* (Table 5). The herb doses at 50 and 75 g kg^{-1} decreased ($P<0.05$) *C. perfringens* by 40.5 and 56.8%, respectively, and also decreased ($P<0.05$) $Gram^-$ by 9.5 and 23.5%, respectively. The herb doses decreased ($P<0.05$) *Salmonella* spp. by 16.9 to 23.2%, and the effect of high dose was more pronounced ($P<0.05$) than the low and middle doses. Significant linear responses ($P\leq0.009$) of herb doses were found on the four opportunistic bacteria, and a quadratic response ($P = 0.009$) was found on *Salmonella* spp.

Compared with the control treatment, the three doses of *A. apiacea* Hance increased ($P<0.05$) mRNA levels of gut defensive peptides, including neutrophil peptide defensin 3a (NP-3A) by 22.4 to 38.3%, defensin beta 1 (DEFB1) by 17.1 to 28.8%, and galectin 4 (LGALS4) by 35.7 to 57.8%. The herb at 50 and 75 g kg^{-1} increased ($P<0.05$) the transcript levels of regenerating family member 3 gamma (REG3G)

Table 4 - Effect of *Artemisia apiacea* Hance meal on the growth performance of rabbits from 35 to 105 days of age

Item	Tukey's b-test				SEM	Polynomial contrast	
	A. apiacea Hance meal (g kg^{-1} as fed)					P-value	
	0	25	50	75		Linear	Quadratic
Initial BW (g rabbit^{-1})	751.3	751.7	751.5	751.0	1.560	0.786	0.938
Final BW (g rabbit^{-1})	2262b	2374a	2371a	2375a	5.856	0.924	0.666
Days 35-70							
ADFI (g day^{-1})	63.06c	64.13b	64.46ab	64.98a	0.182	0.274	0.013
ADG (g day^{-1})	19.10b	20.05a	20.33a	20.36a	0.169	0.268	0.600
FCR	3.30a	3.20ab	3.20ab	3.17b	0.029	0.493	0.691
Days 70-105							
ADFI (g day^{-1})	110.0c	114.8b	115.1ab	115.6a	0.176	0.009	0.627
ADG (g day^{-1})	24.04b	26.29a	25.94a	26.03a	0.207	0.427	0.442
FCR	4.57a	4.37b	4.44b	4.44b	0.034	0.143	0.470
Days 35-105							
ADFI (g day^{-1})	86.51c	89.47b	90.03a	90.03a	0.102	0.001	0.039
ADG (g day^{-1})	21.58b	23.17a	23.14a	23.20a	0.086	0.832	0.668
FCR	4.01a	3.86b	3.89b	3.88b	0.015	0.365	0.277

ADFI - average daily feed intake; ADG - average daily body weight gain; FCR - feed conversion ratio (ADFI/ADG).
a-c - Means within a row with different letters are significantly different (P<0.05).

Table 5 - Effect of *Artemisia apiacea* Hance meal on the cecal opportunistic bacteria and antimicrobial proteins of rabbits

Item	Tukey's b-test				SEM	Polynomial contrast	
	A. apiacea Hance meal (g kg^{-1} as fed)					P-value	
	0	25	50	75		Linear	Quadratic
Opportunistic bacteria (Log_{10}cfu g^{-1})							
Escherichia coli	4.89	4.61	4.43	4.44	0.117	0.009	0.225
Clostridium perfringens	1.48a	1.27ab	0.88b	0.64b	0.111	<0.001	0.906
Salmonella	1.42a	1.18b	1.12b	1.09b	0.033	<0.001	0.009
Gram–	7.14a	6.75ab	6.46b	5.46c	0.154	<0.001	0.064
Antimicrobial peptides (mRNA, $2^{-\Delta\Delta Ct}$)							
NP-3A	2.90b	3.55a	4.01a	3.80a	0.134	<0.001	0.005
DEFB1	1.46b	1.71a	1.85a	1.88a	0.064	<0.001	0.140
REG3G	2.06b	2.02b	2.32a	2.32a	0.057	<0.001	0.764
LGALS4	2.94d	3.99c	4.35b	4.64a	0.074	<0.001	<0.001

a-d - Means within a row with different letters are significantly different (P<0.05).
DEFB1 - defensin beta 1; LGALS4 - galectin 4; NP-3A - neutrophil peptide defensin 3a; REG3G - regenerating family member 3 gamma.

by 12.6%. Furthermore, the effects on the four defensive peptides linearly responded (P<0.001) to the herb doses, and quadratic effects (P≤0.005) were observed on NP-3A and REG3G.

Discussion

In the present study, the addition of *A. apiacea* Hance at 25, 50, and 75 g kg^{-1} improved ADFI, ADG, and feed efficiency. Most *Artemisia* species have a wide distribution and high yield, are edible, and of medicinal character for humans (Lee et al., 2002, 2003). In theory, these *Artemisia* species meals can be alternatives for forages or as natural growth-promoters for farm animals, especially herbivores. Indeed, *Artemisia vulgaris* meal added at 30, 60, or 90 g kg^{-1} improved feed intake and body weight gain of rabbits (Wang et al., 2019). However, for non-herbivores, such as broilers, *Artemisia annua* added at 5 g kg^{-1} led to a lower ADG, but its zymolyte added at 1 g kg^{-1} increased ADG (Wan et al., 2017). In contrast, *Artemisia*

argyi aqueous extract prevented reductions in ADG and ADFI of broilers induced by lipopolysaccharide (Zhang et al., 2017). In laying hens, *Artemisia annua* leaves added at 50 and 75 g kg^{-1} had no influence on egg production but increased yolk color and eggshell thickness (Baghban-Kanani et al., 2019).

The improved growth performance by *A. apiacea* Hance was further demonstrated by the modified gut opportunistic bacteria in the present study. However, information about the antibacterial activity of *A. apiacea* Hance is very limited. Only an *in vitro* study showed that *A. apiacea* Hance suppressed the growth of pathogenic bacterial and fungal strains, including *A. niger*, *C. albicans*, *B. subtilis*, and *S. aureus* (Trinh et al., 2018). Wang et al. (2019) found that *Artemisia vulgaris* meal increased gut beneficial bacteria and decreased opportunistic bacteria of rabbits. *Artemisia* extracts (PMI 5011, Santa or Scopa) increased the Bacteroidetes to Firmicutes ratio in mucosal samples in diet-induced obese mice (Wicks et al., 2014). *Artemisia princeps* inhibited growth, biofilm formation, and virulence factor expression of *S. mutans* (Yang et al., 2019). The antimicrobial mechanism of *Artemisia species* may include damage of bacterial membrane and inhibition of macromolecular synthesis (Gutiérrez-Del-Río et al., 2018), which needs further study.

Gut epithelial tissue of the body is in direct contact with the external environment and is thus continuously exposed to large numbers of microorganisms. To cope with the substantial microbial exposure, epithelial surfaces produce a diverse arsenal of antimicrobial peptides that directly kill or inhibit the growth of microorganisms. The activity of epithelial antimicrobial peptides varies with the endogenous and exogenous factor of the host (Gallo and Hooper, 2012; Sierra et al., 2017). This was consistent with the finding in the present study, in which the mRNA levels of gut defensive peptides were upregulated by the addition of *A. apiacea* Hance, and linear increasing responses were found on DEFB1, LGALS4, NP-3A, and REG3G, as well as quadratic effects on NP-3A and REG3G.

The NP-3A and DEFB1, as important members of antimicrobial peptides defensins, have direct antibacterial, antiviral, and antifungal activity (Holly et al., 2017; Kudryashova et al., 2017). Besides, they are natural components and effectors of body innate immunity and have a potent immunomodulatory activity to infections (Jarczak et al., 2013; Hazlett and Wu, 2011). The effect of *A. apiacea* Hance on the immunity of animals was not detected in the present study. The meal of its genus member, *Artemisia vulgaris*, increased blood levels of IgA, IgM, IgG, and lymphocytes in rabbits (Wang et al., 2019). Therefore, *A. apiacea* Hance may have a similar effect on the body immunity by the defensin activity, which deserves further study.

The regenerating family is a group of small secretory proteins. Its member REG3G is a C-type antimicrobial lectin with the activity against Gram-positive bacteria, mainly expressed in epithelial cells of the airways and intestine (Matsumoto et al., 2012). The information on REG3G gene in farm animals is very limited. A study showed that increased mRNA level of REG3G improved intestinal function of pigs by supplementing oleum cinnamomi in the diet (Yi et al., 2018). Interestingly, REG3G can be a trait indicator associated with weight gain from weaning to yearling of Nelore cattle (Terakado et al., 2018). Similar results were found in the present study, in which dietary *A. apiacea* Hance positively influenced ADFI, ADG, and REG3G gene. However, Xia et al. (2016) argued that REG3G overexpression promoted β cell regeneration and induced immune tolerance in a nonobese-diabetic mouse model.

Galectins, including LGALS4, can mediate effective antimicrobial and immune-regulatory activities by recognizing self-like antigens on blood group positive microbes, and this form of immunity fundamentally differs from defensins and other cationic antimicrobial peptides that engage features unique to an intact microbial membrane (Zasloff, 2002; Ganz, 2003; Stowell et al., 2010). In the present study, the addition of *A. apiacea* Hance linearly and quadratically upregulated the mRNA levels of LGALS4 in the cecum of rabbits, indicating that *A. apiacea* Hance can influence the activity of this antimicrobial peptide. Thus, it will be interesting to further investigate whether the LGALS4 activity regulated by *A. apiacea* Hance leads to immune responses associated with disease.

Conclusions

Diets supplemented with *A. apiacea* Hance at 25, 50, and 75 g kg^{-1} improved growth performance, decreased gut opportunistic bacteria, and increased mRNA levels of microbicidal peptides in rabbits. These results suggest that *A. apiacea* Hance can be a promising additive to promote animal growth and gut health.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Data curation: K. Ding. Formal analysis: K. Ding. Project administration: K. Ding, J. Wang and F. Zhang. Resources: F. Zhang. Software: K. Ding and J. Wang. Supervision: K. Ding. Validation: F. Zhang. Writing-review & editing: K. Ding and N. Liu.

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