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Dietary *Lactobacillus plantarum* alleviated *Salmonella* Typhimurium infection and suppressed Jak/Stat pathway in rabbits

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Abstract: This study aimed to investigate the effect of a *Lactobacillus plantarum* DPP8 supplementation on the growth performance, pathogenic invasion, inflammation and pathogen resistance signal pathway in rabbits infected with *Salmonella* Typhimurium (*S.* Typhimurium). The treatments included a negative control, a positive control with an *S.* Typhimurium infection and a positive control plus DPP8 at 10^6, 10^8 or 10^{10} cfu/kg of diet using 300 weaned rabbits. The results showed that supplementing with DPP8 improved ($P < 0.05$) the feed intake, body weight gain and feed efficiency compared to the positive control. Also, DPP8 decreased ($P < 0.05$) the *S.* Typhimurium colonisation and translocation, serum IL-1β, IL-6 and TNF-α, and intestinal mucosa mRNA expressions of the inflammatory mediators Janus kinase (Jak) 2, the signal transducer and activator of transcription (Stat) 1 and 3 at 7 and 14 d post administration. The dose analysis of DPP8 showed linear increases ($P \leq 0.007$) in the feed intake and body weight gain, but linear decreases ($P \leq 0.022$) in the *S.* Typhimurium loads, IL-1β, IL-6 and Jak2. It is concluded that *Lactobacillus plantarum* DPP8 can be used as a supportive probiotic against an *S.* Typhimurium infection and it possibly plays a direct or indirect role in the downregulation of the Jak/Stat pathway in rabbits.

Keywords: growth performance; inflammatory cytokines

Salmonella Typhimurium (*S.* Typhimurium) is the main serovar responsible for salmonellosis in animal production and the consequent economic losses and food safety concerns (Sisak et al. 2006; Liu et al. 2019). With the ban of food animal growth-promoting antibiotics, the use of probiotics is considered an important strategy for the on-farm control of *Salmonella* sources. Probiotics have long been used as health-promoting agents in the aspects of prevention or alleviation of enteric infections, allergic diseases and chronic inflammatory diseases, thereby gaining increasing attention in replacing antibiotics or anti-inflammatory drugs (Liu et al. 2017; Liu et al. 2018a; Liu et al. 2018b; Zhao et al. 2020a; Zhao et al. 2020b).

Probiotics including *Lactobacillus plantarum* (*L. plantarum*) against *S.* Typhimurium infections have been well documented. The most commonly known action mechanisms of probiotics are associated with the modulation of the gut microbiota, epi-

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thelial barrier, inflammatory interactions among the probiotics, host and pathogens (Wang et al. 2019a; Deng et al. 2020). The Janus kinase/signal transduction and activator of transcription (Jak/Stat) signal pathway is generally considered a prototypical proinflammatory pivot by which many cytokines transduce intracellular signals in the inflammatory process. Probiotic yeast exopolysaccharides (EPSs) hindered the Jak1 pathway and induced apoptosis in a colorectal cancer model (Saadat et al. 2020). The *L. plantarum* supernatant protected against the transmissible gastroenteritis virus based on the Jak/Stat1 pathway *in vitro* (Wang et al. 2019c).

As known, probiotics are effective in several ways, but they are highly strain-specific. It is necessary to confirm a specific probiotic strain that has some specific features through extensive and long-term clinical studies. In a previous study, the *L. plantarum* DPP8 strain has been reported to be capable of attenuating an *S.* Typhimurium infection by inhibiting Jak2/3 and Stat3/4/5/6 in chickens (Shi et al. 2021); however, further studies are needed to confirm its efficacy and action mechanism in farm animals. Therefore, with a different experimental design than the previous study by Shi et al. (2021), the present study aimed to further test the efficacy of probiotic DPP8 on an *S.* Typhimurium infection, Jak2, and Stat1/3 in rabbits.

MATERIAL AND METHODS

Statement of animal rights

The trial protocol was approved by the Institutional Committee for Animal Use and Ethics of the College of Animal Science in Henan University of Science and Technology (HAUST, No. 2018016).

Treatments, bacterial strains and diets

The treatments included a negative control (NC) without an *S.* Typhimurium infection, a positive control (PC) with an *S.* Typhimurium infection, and based on the PC, *L. plantarum* DPP8 was incrementally added at 10^6 (T1), 10^8 (T2) and 10^{10} (T3) colony forming units (cfu)/kg in the corn of the basal diet. The DPP8 (CCTCC M2016136) was obtained from the China Center for Type Culture Collection (Being, P.R. China). The lyophilised DPP8 was recovered and aerobically enriched in a de Man, Rogosa and Sharpe (MRS) broth (HB0384-1; Qingdao Hopebio Co. Ltd., Shandong, P.R. China) at 37 °C for 48 hours. *S.* Typhimurium SL1344 from the Animal Biotechnology Lab in HAUST was grown overnight at 37 °C in a Rappaport-Vassiliadis medium (HB4092; Qingdao Hopebio, Shandong, P.R. China). After the bacterial enumeration, the broth with the DPP8 was sprayed onto a corn powder (40-mesh) and mixed into the basal diet (Table 1) using a step-by-step method, while the broth with *S.* Typhimurium was used to establish an animal model.

Feeding trial, animal model and sample collection

A total of 300 weaned female Hyla rabbits (body weight ± SD of 724 ± 7.0 g; negative for *S.* Typhimurium by rectal swab detection) were randomly assigned into 5 groups with 6 replicates of 10 rabbits each with respect to the 5 treatments. All the rabbits were individually raised in stainless steel cages (length × width × height of 35 × 45 × 40 cm) and had free access to their respective experimental diets and water for 14 days (Stastnik et al. 2019; Wang et al. 2019b). On the first day of the feeding trial, the rabbits in PC, T1–T3 were orally gavaged

Table 1. Ingredients and nutrition levels in the basal diet (as fed basis)

Ingredient	Content (%)	Calculated composition[1]	Content (%)
Corn	36.0	crude protein	17.04
Soybean meal	16.5	digestible energy (MJ/kg)	11.33
Brewers dried grain	5.0	crude fibre	12.99
Alfalfa meal	35.0	lysine	0.80
Wheat bran	5.0	methionine + cysteine	0.50
Dicalcium phosphate	1.5	Ca	0.92
Premix[2]	1.0	P	0.50

[1]Calculated by Chinese Feed Database, v25, 2014; [2]The premix provided the following per kg of diet: 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
IU = international unit

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with 1 ml of 10^3 cfu of *S.* Typhimurium to establish a subclinical salmonellosis model.

At 7 d post-administration, 5 rabbits per replicate were randomly collected, euthanised by CO_2 and subjected to post-mortem sampling. At 14 d post-administration, samples were collected again using the remainder of the rabbits. Blood samples were collected from the heart and the sera were prepared by centrifuging at 1 000 × *g* for 10 min (Liu et al. 2019) and stored at −20 °C till further use. Approximately 5 g of the caecal content was collected and stored at −40 °C for the enumeration of *S.* Typhimurium. The caecal mucosa was collected and stored in RNA later for the mRNA assay. The liver (approximately 5 g from the left lateral lobe) and the whole spleen were collected and stored at −40 °C for the bacterial enumeration.

Bacterial enumeration and biochemical assay

For the DPP8 and SL1344 enumeration, each sample was homogenised, weighed, and diluted at 1 : 10 (w/v) with phosphate buffered saline (pH 7.2) and mixed thoroughly according to the methods by Liu et al. (2019). Briefly, commercial media were used to detect the DPP8 and SL1344 using MRS agar (HB0384; Qingdao Hopebio, Shandong, P.R. China) and *Salmonella* deoxycholate hydrogen sulfide lactose agar (HB4087; Qingdao Hopebio, Shandong, P.R. China), respectively, under aerobic condition at 37 °C for 48 hours. The cfu number was expressed as a logarithmic (log_{10}) transformation per gram of intestinal digesta. Rabbit enzyme-linked immunosorbent assay kits from Cusabio Technology LLC (Distributor in Wuhan, P.R. China) were used for the detection of the cytokines according to the manual, including tumour necrosis factor α (TNF-α), interleukin 6 (IL-6; detection range: 15.6 ng/l to 1 000 ng/l) and interferon γ (IFN-γ).

The total mRNA isolation and cDNA synthesis for the samples were carried out as described by Liu et al. (2018c). The mRNA concentration was determined by the optical density (OD) reading at 260 nm, and the purity was checked using an A260/A280 ratio (1.8 to 2.0) and A260/A230 ratio (> 1.5) on a NanoDrop™ 2000 Spectrophotometer (Liu et al. 2018c). The primer information is listed in Table 2. The qPCR reactions were set at 10 µl with 5 µl of a SYBR Green Master Mix, 1 µl of primer, 4 µl of 10 × diluted cDNA using an ABI Prism 7900HT Fast Real-Time (RT) polymerase chain reaction (PCR) System by the same thermal cycles (50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 minute). No amplification signal was detected in the water or in the non-RT RNA samples. The transcript levels were expressed as the relative expression to a housekeeping gene according to the $2^{-\Delta\Delta Ct}$ method by Schmittgen and Livak (2008). The primers synthesis and qPCR reagents were provided by Dalian TaKaRa Co., Ltd. (Liaoning, P.R. China). The samples were detected in duplicate.

Statistical analysis

The data are represented as the mean and standard error of mean (SEM) using SPSS software (v23; IBM, Armonk, NY, USA). The differences between the mean values of the normally distributed data were assessed by a one-way analysis of variance (ANOVA; Tukey's b-test) at a $P < 0.05$ significance

Table 2. Information about the primers for the quantitative real-time PCR

Names	GenBank	Primers (5'→3')	Length (bp)	Efficiency (%)	Reference
Jak2	XM_002708002.3	F: atcttcccaacgggggagta R: gcacctcgagatactccgtg	254	97	Lowe and Eddy (1997)
Stat1	XM_002712346.3	F: cccgcaaatctcggatgtct R: cgtgctcccagtcctgattt	150	98	Lowe and Eddy (1997)
Stat3	XM_008271503.2	F: ctgccccatacctgaagacc R: tcctcacatggggaggtag	212	96	Lowe and Eddy (1997)
GAPDH	NM_001082253.1	F: ggcgtgaaccacgagaagta R: atgccagtgagtttcccgtt	293	98	Kurganov (2018)

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; Jak = Janus kinase 2; Stat = signal transducer and activator of transcription

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level, and Tamhane's T2 test for the parameters with variance in the heterogeneity. The mean of the replicate is a statistical unit (n = 6). The trend of the probiotic doses at 10^6, 10^8 and 10^{10} cfu/kg was analysed using contrasts of linear and quadratic polynomials.

RESULTS

Effect of DPP8 on the growth performance and *S.* Typhimurium load

At 7 and 14 d post-administration, the rabbits in the PC treatment showed a worsened ($P < 0.05$) feed intake, body weight gain and feed efficiency, compared to the NC treatment, whereas the DPP8 addition in T1 to T3 improved ($P < 0.05$) the growth performance data (Table 3). Linear responses ($P \leq 0.007$) to the probiotic doses were found on the feed intake and body weight gain. During the period of 1–7 d post-administration, there was no fatality. During the period of 8–14 d post-administration, the mortality in the PC group was greater than the other treatments, but there were no statistical differences among the treatments.

The present study further investigated the colonisation and translocation of *S.* Typhimurium. DPP8 decreased ($P < 0.05$) the pathogen population in the caeca, liver and spleen, where linear and quadratic decreases ($P \leq 0.045$) were found on the pathogen in the caeca and liver at 7 and 14 d post-administration (Table 4). These findings

Table 3. Effect of *Lactobacillus plantarum* (*L. plantarum*) on the growth performance of the rabbits

Item	*Salmonella* Typhimurium infection					SEM	*P*-values	
	NC	PC	T1	T2	T3		linear	quadratic
L. plantarum (cfu/kg)	0	0	10^6	10^8	10^{10}	–	–	–
7 d post-administration								
FI (g/rabbit)	245.4^a	153.4^c	200.2^b	206.5^b	229.9^a	5.422	0.007	0.316
BWG (g/rabbit)	153.7^a	71.8^d	104.5^c	110.4^c	121.2^b	2.791	0.003	0.564
FCR	1.598^c	2.136^a	1.923^b	1.873^b	1.894^b	0.036	0.662	0.529
14 d post-administration								
FI (g/rabbit)	647.9^a	366.1^d	466.6^c	483.2^c	517.6^b	7.438	< 0.001	0.379
BWG (g/rabbit)	343.2^a	160.1^e	233.0^d	250.1^c	265.9^b	3.951	< 0.001	0.882
FCR (FI/BWG)	1.890^b	2.289^a	2.005^b	1.934^b	1.947^b	0.033	0.266	0.349
Mortality (%)	0	3.33	1.67	0	1.67	0.755	< 0.001	0.081

$^{a–e}$Means within a row without the same superscript are significantly different ($P < 0.05$)

BWG = body weight gain; FCR = feed conversion ratio; FI = feed intake, FI/BWG; NC = negative control; PC = positive control

Table 4. Effect of *Lactobacillus plantarum* on the *Salmonella* Typhimurium colonisation and translocation of the rabbits

Item	*Salmonella* Typhimurium infection					SEM	*P*-value	
	NC	PC	T1	T2	T3		linear	quadratic
L. plantarum (cfu/kg)	–	–	10^6	10^8	10^{10}	–	–	–
S. Typhimurium count on 7 d post-administration (Log_{10} cfu/g)								
Caecum	-	4.18^a	3.56^b	3.18^c	3.15^c	0.062	< 0.001	0.033
Liver	-	0.31^a	0.24^b	0.18^c	0.19^c	0.010	0.002	0.011
Spleen	-	0.44^a	0.30^b	0.29^b	0.29^b	0.010	0.482	0.559
S. Typhimurium count on 14 d post-administration (Log_{10} cfu/g)								
Caecum	-	4.68^a	3.96^b	3.56^c	3.50^c	0.069	< 0.001	0.045
Liver	-	0.37^a	0.27^b	0.20^c	0.21^c	0.011	< 0.001	0.010
Spleen	-	0.50^a	0.34^b	0.33^b	0.32^b	0.009	0.069	0.727

$^{a–c}$Means within a row without the same superscript were significantly different ($P < 0.05$)

(-) = undetectable; NC = negative control; PC = positive control

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indicate that DPP8 can attenuate the negative effect of an *S.* Typhimurium infection on the growth performance and inhibit the colonisation and translocation in the rabbits.

Effect of DPP8 on the inflammatory cytokines induced by *S.* Typhimurium

At 7 d post-administration, rabbits in the PC group had greater ($P < 0.05$) serum levels of IL-1β, IL-6 and TNF-α, whereas these parameters were decreased ($P < 0.05$) with the DPP8 addition in T1 to T3 (Table 5). Similar results were found at 14 d post-administration. For the dose effect analysis, IL-1β and IL-6 linearly decreased ($P \leq 0.022$) with the probiotic dose changing from T1 to T3 at 7 and 14 d post-administration; TNF-α showed linear and quadratic decreases ($P \leq 0.036$) at 14 d post-administration. These findings demonstrate that the probiotic attenuated the inflammatory response induced by *S.* Typhimurium in the rabbits.

Effect of DPP8 on the Jak/Stat pathway induced by *S.* Typhimurium

As for the gene regulation, at 7 d post-administration, the rabbits in the PC group had the highest ($P < 0.05$) mRNA levels of Jak2, Stat1 and Stat3, whereas the addition of DPP8 at the three doses down-regulated ($P < 0.05$) these genes (Table 6); the

Table 5. Effect of *Lactobacillus plantarum* on the inflammatory cytokines of the rabbits

Item	*Salmonella* Typhimurium infection					SEM	*P*-value	
	NC	PC	T1	T2	T3		linear	quadratic
L. plantarum (cfu/kg)	–	–	10^6	10^8	10^{10}	–	–	–
7 d post-administration (ng/l)								
IL-1β	25.03^{d}	205.6^{a}	122.0^{b}	101.5^{bc}	87.6^{c}	4.831	< 0.001	0.617
IL-6	59.45^{c}	267.7^{a}	155.7^{b}	133.4^{b}	123.0^{b}	8.124	0.012	0.557
TNF-α	37.82^{c}	143.1^{a}	88.9^{b}	87.0^{b}	83.2^{b}	5.316	0.488	0.893
14 d post-administration (ng/l)								
IL-1β	62.17^{d}	257.5^{a}	175.6^{b}	161.5^{bc}	142.9^{c}	5.367	0.003	0.785
IL-6	80.12^{c}	317.0^{a}	222.2^{b}	206.7^{b}	195.2^{b}	6.361	0.022	0.832
TNF-α	79.45^{d}	210.1^{a}	184.7^{b}	179.0^{b}	140.5^{c}	6.518	< 0.001	0.036

$^{a–d}$Means within a row without the same superscript were significantly different ($P < 0.05$)

IL = interleukin; NC = negative control; PC = positive control; TNF = tumour necrosis factor

Table 6. Effect of *Lactobacillus plantarum* on the Jak/Stat pathway in the rabbits

Item	*Salmonella* Typhimurium infection					SEM	*P*-value	
	NC	PC	T1	T2	T3		linear	quadratic
L. plantarum (cfu/kg)	–	–	10^6	10^8	10^{10}	–	–	–
7 d post-administration (mRNA, $2^{-\Delta\Delta Ct}$)								
Jak2	0.107^{d}	0.577^{a}	0.307^{b}	0.255^{bc}	0.232^{c}	0.009	0.001	0.375
Stat1	0.255^{d}	0.487^{a}	0.356^{bc}	0.382^{b}	0.295^{cd}	0.017	0.011	0.007
Stat3	0.335^{c}	0.765^{a}	0.559^{b}	0.511^{b}	0.537^{b}	0.012	0.199	0.015
14 d post-administration (mRNA, $2^{-\Delta\Delta Ct}$)								
Jak2	0.231^{c}	0.692^{a}	0.491^{b}	0.453^{b}	0.421^{bc}	0.022	0.016	0.889
Stat1	0.299^{d}	0.674^{a}	0.465^{c}	0.484^{bc}	0.438^{c}	0.016	0.128	0.040
Stat3	0.310^{c}	0.670^{a}	0.414^{bc}	0.429^{ab}	0.399^{bc}	0.025	0.629	0.391

$^{a–d}$Means within a row without the same superscript were significantly different ($P < 0.05$)

Jak = Janus kinase; NC = negative control; PC = positive control; Stat = signal transducer and activator of transcription

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T3 effect on Stat1 reached ($P > 0.05$) the level of the NC group; linear decrease effects ($P \leq 0.011$) were found on Jak2 and Stat1, quadratic decrease effects ($P \leq 0.015$) were exhibited on Stat1 and Stat3. Also, at 14 d post-administration, DPP8 down-regulated ($P < 0.05$) the three genes; the T1 to T3 effect on Stat3 reached ($P > 0.05$) the level of the NC group; and there was a linear response ($P = 0.016$) on Jak2 and a quadratic response ($P = 0.040$) on Stat1.

DISCUSSION

Rabbit meat is increasingly becoming popular due to its low fat and high protein while *Salmonella* infections often occur in feeding rabbits and processing the carcasses, thereby threatening the economic profits and food safety (Ding et al. 2019). In the present study, the rabbits in the PC group showed the greatest load of *S.* Typhimurium, which also primarily indicated the successful establishment of the animal model. Importantly, the pathogen loads in the organs were declined with the addition of the probiotic DPP8. This was supported by the literature that probiotics lowered the intestinal and faecal *Salmonella* counts in chickens or mice (Upadhaya et al. 2016; Yin et al. 2018; Adhikari et al. 2019; Shi et al. 2021). Inconsistently though, the spleen exhibited the greatest *Salmonella* load, but the probiotics did not decrease the pathogen load in the chicken viscera (Adhikari et al. 2019). However, the literature about this is scarce in rabbits. Linear and quadratic decreases in the pathogen loads in the caeca and liver by the probiotic in the present study demonstrate the more efficient dosages of the T2 and T3 groups. Additionally, the probiotics reduced the *S.* Typhimurium colonisation in the intestine by competing for iron (Deriu et al. 2013), whether there are interactions among the DPP8, *S.* Typhimurium and nutrients is an open question.

In the present study, the decreased *S.* Typhimurium loads with the DPP8 also contributed to the attenuation of the inflammatory response, namely the lower the pathogen count, the lower the oxidative stress and inflammatory responses. Similar results were found in studies where *L. acidophilus* and *L. reuteri* decreased *S.* Typhimurium induced IL-1β, TNF-α and IFN-γ of mice (Yin et al. 2018); *L. brevis* decreased *Salmonella* induced IL-6, IL-8 and IL-1β levels in human intestinal epithelial cells (Kanmani and Kim 2020); *L. plantarum* B7 produced a clear zone on *S.* Typhimurium with lower serum levels of TNF-α and IL-6 in mice (Wongsen et al. 2019); *L. plantarum* LTC-113 prevented an increase in the inflammatory meditators myeloperoxidase, IL-1β, IL-6 and in the inflammation scores induced by *Salmonella* in chicks (Wang et al. 2018).

Jaks are essential signal mediators downstream of many pro-inflammatory cytokines, and Jak inhibitors have gained attraction in the treatment of inflammation-driven pathologies (Schwartz et al. 2017). In the present study, DPP8 down-regulated the Jak2 transcriptional levels, indicating its inhibition in Jak2 and the consequent anti-inflammatory effect. This is supported by studies in which the probiotic Kimchi inhibited IL-6 and Jak2 in mice (An et al. 2019); probiotics hindered the Jak1 pathway and induced cell apoptosis (Saadat et al. 2020); probiotics down-regulated Jak2/3 of chickens (Shi et al. 2021). Conversely, probiotics enhanced the hypothalamic Jak2 phosphorylation in a diet induced mouse model (Bagarolli et al. 2017). The different Jak signal events caused by the pathogen and the diet deserve further studies.

The Stat family members are a set of transcription factors, which operate the downstream of the cytokine and hormone receptors to convert extracellular stimuli into biochemical signals that instruct the gene expression. Stat1/3, important upstream regulators of cytokines, are phosphorylated by the cytokine receptor-associated Jaks (Morris et al. 2018). The Jak/Stat pathway is a chain of interactions between the proteins in a cell responsible for apoptosis and inflammation. In a mouse model with inflammatory induction, Jak2 and Stat1/3 were down-regulated by probiotics (Bagarolli et al. 2017). In chickens, probiotic strains attenuated an *S.* Typhimurium infection by inhibiting Jak2/3 and Stat3/4/5/6 (Shi et al. 2021). Similar results were found in the present study where the transcriptional levels of Jak2 and Stat1/3 were deregulated by DPP8, and thus of IL-1β, IL-6, and TNF-α. Additionally, in chickens, the literature and present study showed the increased expressions of Jak/Stat induced by *Clostridium perfringens* or *S.* Typhimurium (Truong et al. 2017; Shi et al. 2021); whereas the signals were weakened by *S. enteritidis* (Coble et al. 2013).

Therefore, the inconsistence of the Jak/Stat signal activity induced by different *Salmonella* strains needs more studies.

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In conclusion, it could be stated that supplementing with DPP8 supported the growth performance of rabbits with an *S.* Typhimurium challenge, and decreased the organ loads of the pathogen, inflammatory cytokines and mediators. Linear or quadratic responses were found to be significant on the feed intake, body weight gain, caecal and hepatic pathogen loads, and Stat1. Furthermore, with an increase in the DPP8 from 10^6 cfu/kg to 10^8 cfu/kg, there was an increasing effect on the growth performance and a decreasing effect on the pathogen load, cytokines and Jak2. It is concluded that *Lactobacillus plantarum* DPP8 can be used as a supportive probiotic against *S.* Typhimurium infections and it possibly plays a direct or indirect role in the downregulation of the Jak/Stat pathway in rabbits.

Conflict of interest

The authors declare no conflict of interest.

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