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Original research article

Isolation and identification of sinapine-degrading bacteria from the intestinal tract of laying hens

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ABSTRACT

This study was aimed to isolate sinapine-degrading bacteria from the intestinal tract of laying hens and to identify the predominant bacteria. Thirty-week old healthy laying hens were killed, and the chyme in the digestive tract was inoculated into modified Czapek medium containing sinapin and cultivated at 37 °C for 10 days. The optical density (OD) values of the bacterial solutions at different cultivating times were detected by a spectrophotometric method. The predominant strains were identified by 16S rRNA gene analysis. We extracted the extracellular products of the predominant strains to determine the total protein using the Coomassie brilliant blue method, and to determine the activities of some extracellular enzymes using the agar plate diffusion method. Nine strains were isolated from the lower intestinal tract of laying hens. Among the 9 strains, 5 were from the ileum, 2 were from the ceca and 2 were from the jejunum. We could not isolate any strains from the upper intestinal tract, such as the stomach and duodenum. Eight of those 9 isolated strains were gram negative and one was gram positive. Strains YD-1 and YD-2 were better than other strains in their abilities to degrade sinapine. Strains YD-1 and YD-2 were identified as *Escherichia coli* and *Klebsiella* spp., respectively, by the 16S rRNA sequence analysis. The total protein level of the extracellular products was 1.213 g/L for YD-1 and 1.990 g/L for YD-2. Both extracellular products of YD-1 and YD-2 had the activities of protease, amylase and urease. This study confirmed that the primary site of sinapine degradation is in the lower intestinal tract of laying hens. The sinapine-degrading strains are mainly gram negative. Strains YD-1 and YD-2 are predominant in degrading sinapine and they belong to *E. coli* and *Klebsiella* spp., respectively. Both extracellular products of YD-1 and YD-2 contain protease, amylase and urease. Strain YD-2 is better than strain YD-1 in its ability to degrade sinapine.

1. Introduction

Sinapine is one of the main anti-nutritional factors in the feed resources of Brassicaceae such as rapeseed meal. Sinapine tastes bad and can reduce the digestibility of protein. The most adverse effect of sinapine is to induce fishy taint in some animals (Niu et al., 2014), thus making meat, eggs, milk less acceptable to consumers (Wang et al., 2011). Fishy taint will seriously affect the quality of livestock products (Bao et al., 2013). The fishy factor is an irritant and can make eyes, nose, pharynx and respiratory tract discomfort. Therefore, understanding the metabolism mechanism by which sinapine induces fishy taint will help the use of Brassicaceae species as a feed resource in a variety of different feed formulations. Sinapine is one of the most important simple polyphenols and accounts for 70% to 85% of all phenols in rapeseed meals (Naczk and Shahidi, 1989). Despite a number of studies exploring the use of fungi or bacteria to degrade sinapine, there still lacks definite answers as to how it can be effectively de-activated. Thus, we isolated

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and characterized a number of sinapine degrading bacteria in the intestinal tract of laying hens and elucidated their ability to degrade sinapine in this study.

2. Materials and methods

2.1. Experimental materials

2.1.1. Experimental animals

Thirty-week old healthy laying hens were bought from Sichuan Dahen Poultry Breeding Co., LTD. The experimental protocol involved in the present study was approved by the Animal Care and Use Committee of Sichuan Animal Science Academy.

2.1.2. Reagents and instruments

Reagents mainly included sinapine thiocyanate standard substance (bought from Chengdu Inspection Institute of Food and Drug, Chengdu), sinapine thiocyanate extract (made in our laboratory), $NaNO_3$, $FeSO_4$, yeast extract, tryptone, NaCl, gelatin, skim milk, Tween 80, soluble starch, urea, phenol red, ammonium sulfate, Tris, etc. The genomic DNA extraction kit was bought from Qiangen company, Germany; HS Taq DNA Polymerase was bought from Takara Biotechnology (Dalian) CO., LTD; primer was synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Instruments mainly included electron balance, autoclave, water bath oscillator, clean bench, constant temperature oven, high speed refrigerated centrifuge, ultraviolet spectrophotometer.

2.2. Testing method

2.2.1. Isolation and culture of sinapine-degrading bacteria

1) Culture medium

Improved Czapek medium: $NaNO_3$, $FeSO_4$, KCl, sinapine thiocyanate were diluted with double-distilled water to 1,000 mL. Improved Czapek agar plate medium: Agar (2% to 3%) was added into the improved Czapek medium. The medium was autoclaved at 121°C for 30 min.

2) Inoculation and culture

Thirty-week old healthy Dahen laying hens that had been fed 8% rapeseed meal ration for 30 days and at peak production were killed by cervical dislocation. The chyme in the gizzard, glandular stomach, duodenum, jejunum, ileum and cecum was gathered and cultured for 10 days in the improved Czapek medium at 37 °C, then isolated bacteria were cultured in the same medium. The bacteria that grew the best were defined as the predominant bacteria. The common physical and chemical characteristics of the predominant bacteria were analyzed by referring to the method detailed in *Bergey's Manual of Determinative Bacteriology* and *Common bacteria identification manual.*

2.2.2. Study on the sinapine-degrading efficiency of the predominant bacteria

Bacterial solutions on the 1st, 2nd, 4th, 6th, 8th and 10th enrichment-culture-day were collected and filtered through microfiltration membrane (0.45 μm) (He, 2010). The optical density (OD) of sinapine in the filtrate was measured by spectrophotometry at 326 nm.

2.2.3. Gene sequencing and phylogenetic analysis of the predominant bacteria by 16S rRNA

The DNA of the predominant bacteria was extracted. Upstream primer: 5′-AGAGTTTGATCCTGGCTCAG-3′, and downstream primers: 5′-TACGGCTACCTTGTTACGAC-3'. As template, the extractive DNA was amplified the 16S rRNA gene sequence of the isolated bacteria. The PCR reaction systems: Add 12.5 μL 2 × PCR Premix, 1 μL upstream primer (10 μmol/μL), 1 μL downstream primer (10 μmol/μL), and 1.0 μL template into a 0.2-mL reaction tube, then added 25 μL double-distilled water. The PCR reaction parameters were 94 °C 5 min, 94 °C 1 min, 55 °C 1 min, 72 °C 1.5 min, 30 cycles, extending 10 min at 72 °C. The PCR products were sequenced. The similarity of the sequences was analyzed by the Blast of National Center of Biotechnology Information (NCBI). The multi alignments of the sequences were analyzed by the program of Multiple Sequence Alignment of DNA star software. A phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis 5.0 (MEGA 5.0).

2.2.4. Study on the extracellular products of YD-1 and YD-2

Preparation of extracellular products: We inoculated two 1-L Lysogeny Broth (LB) broth media with two 10-mL bacterial suspensions of YD-1 and YD-2, respectively, shook 120 g culture for 24 h at 28 °C, and then centrifuged the bacteria solution 12,000 × *g* at 4 °C for 30 min. The supernate was filtered by vacuum, and then filtered again by 0.22 μm millipore. The filtered sediment was re-suspended and dialyzed in a buffer solution of 0.02 mol/L Tris-HCl (pH 7.5), then concentrated by polyethylene glycol 20,000. The concentrate was purified by 0.22 μm millipore filters to gain pure extracellular products.

Determination of the total protein of the extracellular products: the total proteins of the extracellular products in YD-1 and YD-2 were determined by Coomassie Brilliant Blue. The application solution of Coomassie Brilliant Blue was comprised of Coomassie Brilliant Blue stock solution and distilled water at the ratio of 1:4.3. The test tubes were labeled as blank tube, standard tube and testing tube. They were added 50 μL distilled water, 50 μL protein markers (0.563 g/L) and 50 μL extracellular products, respectively. Then 3 mL application solution of Coomassie Brilliant Blue was added in each tube, mixed and stood for 10 min at room temperature. The OD value of the reaction solutions was determined by the spectrophotometer at 595 nm. The total protein was calculated by the following formula.

$$\begin{aligned}\text{Total protein}(\text{g/L}) = {} & (\text{OD value of testing tube} \\ & - \text{OD value of blank tube})/ \\ & \times (\text{OD value of standard tube} \\ & - \text{OD value of blank tube}) \\ & \times \text{Concentration of standard tube}.\end{aligned}$$

We analyzed the activity of some enzymes in the extracellular products. The activity of the extracellular enzymes was analyzed by the agar diffusion method (Li et al., 1999). We drilled 2 holes in each of the 4 agar mediums containing 1% Tween 80, 8% skim milk, 2% urea and 4% soluble starch, respectively. Then, we injected 50 μL extracellular products of YD-1 or YD-2 into one of the two holes for each medium respectively, cultivated 24 h at 28 °C and judged whether they had the activities of protease, lipase, amylase and urease in extracellular products of YD-1 and YD-2 by the color reaction of the medium.

3. Results

3.1. Isolation and culture of sinapine-degrading bacteria

We successfully isolated 9 strains from laying hens' intestinal tracts and successfully cultivated them by an improved sinapine medium in this study (Table 1). The 9 strains were from the ileum,

Table 1
Sources and properties of 9 isolated strains.

Bacteria No.	Separated part	Colony morphology	Gram staining[1]
H-1	Ileum	Coccus	G−
H-2	Ileum	Coccus	G−
YD-1	Ileum	Coccus	G−
H-3	Ileum	Coccus	G−
H-4	Ileum	Coccus	G+
M-1	Cecum	Coccobacillus	G−
M-2	Cecum	Coccobacillus	G−
K-1	Jejunum	Bacillus	G−
YD-2	Jejunum	Bacillus	G−

[1] G− = gram negative; G+ = gram positive.

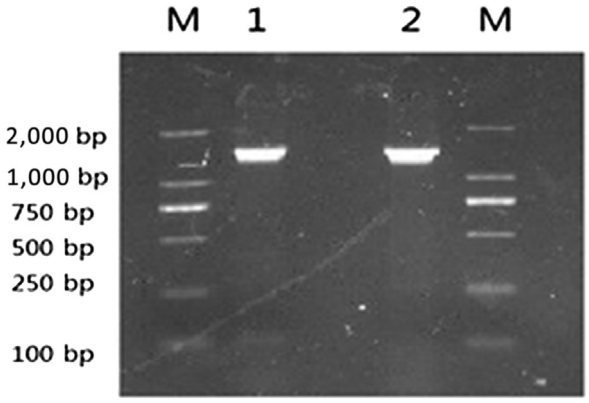

Fig. 1. PCR products of 16S rRNA gene from YD-1 and YD-2. M = Marker, DL2000; 1 = YD-1; 2 = YD-2.

ceca and jejunum, among which 8 strains were gram-positive and one gram-negative. We could not isolate or cultivate any bacteria from the muscular stomach, gland stomach and duodenum.

3.2. Study on the sinapine-degrading efficiency of the predominant bacteria

The sinapine OD values of the culture solutions at different incubation times were determined (Table 2). The OD values of YD-1 and YD-2 significantly decreased ($P < 0.05$) or extremely increased ($P < 0.01$) as the incubation time increased. The OD values of YD-1 and YD-2 on the 8th day were the lowest and decreased 29.89% ($P < 0.01$) and 33.83% ($P < 0.01$) compared with those before fermentation, respectively. The OD values of other strains were fluctuated and had no clear regularity at different incubation times.

3.3. Gene identification of YD-1 and YD-2 by 16S rRNA

The genes of YD-1 and YD-2 were amplified in PCR (Fig. 1). A gene fragment of 1,449 and 1,454 bp was obtained and the GenBank accession numbers were KT372239.1 and KT372240.1, respectively. Phylogenetic analysis showed a 97% sequence similarity between YD-1 and *Escherichia coli* (Fig. 2), and a 95% sequence similarity between YD-2 and *Klebsiella pneumonia* (Fig. 3).

3.4. Study on the extracellular products of YD-1 and YD-2

3.4.1. Content of the total protein in the extracellular products

The concentration of the standard substance as known was 0.563 g/L. The OD value was determined as 0.025. The OD values of extracellular products in YD-1 and YD-2 were determined as 0.025 and 0.041, respectively. According to the formula: Total protein (g/L) = (OD value of testing tube−OD value of blank tube)/(OD value of standard tube−OD value of blank tube) × Concentration of standard tube, we calculated that the total proteins of extracellular products in YD-1 and YD-2 were 1.213 and 1.990 g/L, respectively (Table 3).

3.4.2. Activity of some enzymes in the extracellular products

The capital letters of A, B, C and D indicated Tween 80, skim milk, urea and soluble starch plate media, respectively (Fig. 4). The area and color of transparent halo represented the activity of lipase, protease, urease and amylase respectively. Numbers 1 and 2 represented the result of YD-1 and YD-2, respectively. There was no transparent halo around the two holes in Tween 80 plate medium. There were transparent halo, red halo and yellow halo around the two holes in skim milk, urea and soluble starch plate media, respectively. Therefore, both extracellular products of YD-1 and YD-2 had activities of protease, urease and amylase but did not have lipase activity. For the halo area, the protease activity of the extracellular products in YD-2 was higher than that of YD-1. There were no significant differences for urease and amylase activities between the extracellular products of YD-1 and YD-2.

4. Discussion

Despite the importance of sinapine metabolites in animal products, very few reports have investigated sinapine degradation in situ and the mechanism by which it occurs. Some studies examined the degradation of phenolic substances by exogenous

Table 2
Optical density (OD) values of sinapine in the culture solutions of 9 isolation strains at different fermentation times.

Item	Day 1	Day 2	Day 4	Day 6	Day 8	Day 10
H-1	3.072 ± 0.001^{Cb}	3.089 ± 0.006^{Cb}	2.987 ± 0.100^{Ca}	3.049 ± 0.045^{Ca}	3.495 ± 0.030^{B}	3.621 ± 0.084^{A}
H-2	3.076 ± 0.003^{D}	3.378 ± 0.119^{C}	3.526 ± 0.096^{C}	3.932 ± 0.105^{Ba}	4.287 ± 0.151^{A}	4.138 ± 0.085^{Ab}
YD-1	3.045 ± 0.002^{A}	3.045 ± 0.002^{A}	2.853 ± 0.045^{B}	2.544 ± 0.087^{C}	2.135 ± 0.059^{D}	2.836 ± 0.031^{B}
H-3	3.076 ± 0.003^{C}	3.058 ± 0.062^{C}	3.188 ± 0.057^{BC}	3.386 ± 0.190^{B}	3.701 ± 0.152^{A}	3.842 ± 0.110^{A}
H-4	3.045 ± 0.003^{bA}	3.059 ± 0.031^{b}	2.421 ± 0.577^{B}	3.064 ± 0.087^{b}	3.098 ± 0.019^{b}	3.590 ± 0.089^{a}
M-1	3.075 ± 0.002^{C}	3.063 ± 0.035^{C}	3.604 ± 0.344^{Bb}	3.961 ± 0.023^{Ba}	4.685 ± 0.042^{A}	4.765 ± 0.236^{A}
M-2	3.072 ± 0.002^{Da}	3.013 ± 0.014^{Da}	3.296 ± 0.191^{Cb}	3.285 ± 0.076^{Cb}	3.788 ± 0.079^{B}	4.187 ± 0.074^{A}
K-1	3.064 ± 0.005^{bD}	2.939 ± 0.033^{bD}	3.294 ± 0.027^{Ca}	3.417 ± 0.025^{C}	4.116 ± 0.083^{B}	4.562 ± 0.234^{A}
YD-2	3.062 ± 0.002^{A}	2.771 ± 0.006^{Ba}	2.748 ± 0.017^{Bb}	2.050 ± 0.020^{Cd}	2.026 ± 0.011^{Cc}	2.038 ± 0.011^{Cc}

[a–d] Within a same row, means with common small letter superscripts differ not significantly ($P > 0.05$), with different small letter superscripts differs significantly ($P < 0.05$).
[A–D] Within a same row, means without a common capital letter superscripts differ extremely ($P < 0.01$).

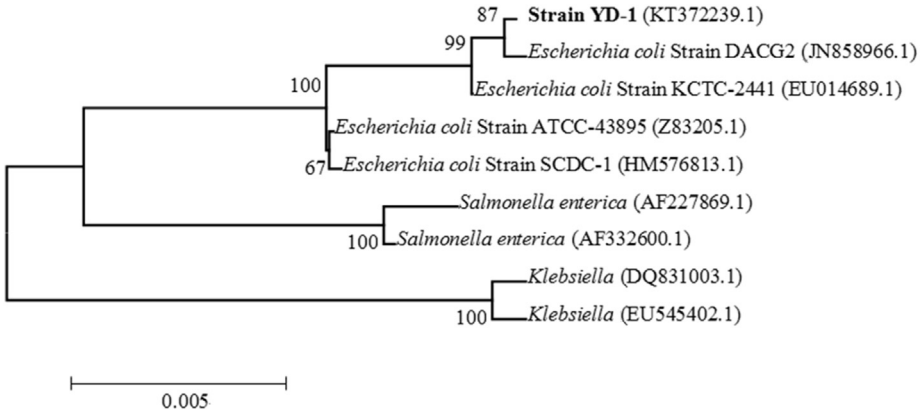

Fig. 2. Phylogenetic tree on the partial 16S rRNA gene sequences of YD-1.

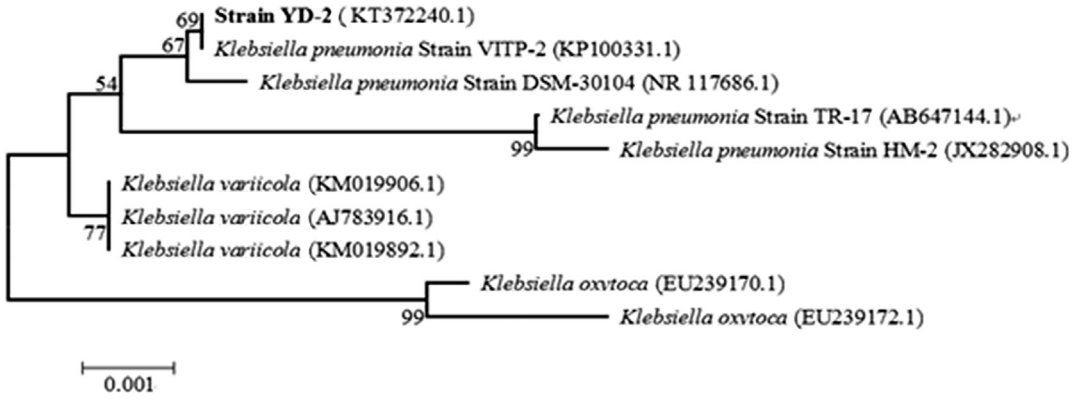

Fig. 3. Phylogenetic tree on the partial 16S rRNA gene sequences of YD-2.

fungi, such as aspergillus, white-rot fungi and mushroom (Rozan et al., 1996; Thurston, 1994). We successfully isolated and cultured 9 sinapine-degrading strains from the lower intestinal tract of laying hens, but not from the duodenum and the upper tract. These results demonstrated that the primary site of the sinapine degradation was in the lower, rather than the upper intestinal tract. We could also further explain the reason why sinapine was easier to induce fishy taint than choline chloride as they were both the precursor of choline. Choline chloride can be directly absorbed into the blood circulation in the duodenum and had a rare opportunity to enter the lower digestive tract, but sinapine can hardly be absorbed in the duodenum because of its larger molecular weight, thus it would enter into the lower digestive tract for microbial degradation (Ward, 2008). Pearson and March also found that the metabolites of sinapine in the ceca of laying hens were more than those in the small intestine (Pearson et al., 1983). Almost all of the strains that we had isolated and cultured in this study were gram-negative bacteria and the reason warrants further study.

Because the molecule of sinapine has a longer conjugate structure, it has an obvious ultraviolet absorption peak at 326 nm, thus we could directly determine it using an ultraviolet spectrophotometer. From the OD values of the 9 strains, YD-1 and YD-2 had the best sinapine-degrading effects compared with the other 7 strains, and the optimum incubation time for these 2 strains were 6 to 8 days under our experimental condition. Compared with YD-1, YD-2 had a better sinapine-degrading effect. The reason why the OD values of other strains increased along with the culture time is still

Table 3
Total protein concentrations of extracellular products of YD-1 and YD-2.[1]

Item	OD value			Concentration of the standard tube, g/L	Total protein concentration of the extracellular products, g/L
	Blank tube	Test tube	Standard tube		
YD-1	0	0.025	0.116	0.563	1.213
YD-2	0	0.041	0.116	0.563	1.990

[1] Strains YD-1 and YD-2 were identified as *Escherichia coli* and *Klebsiella* spp.

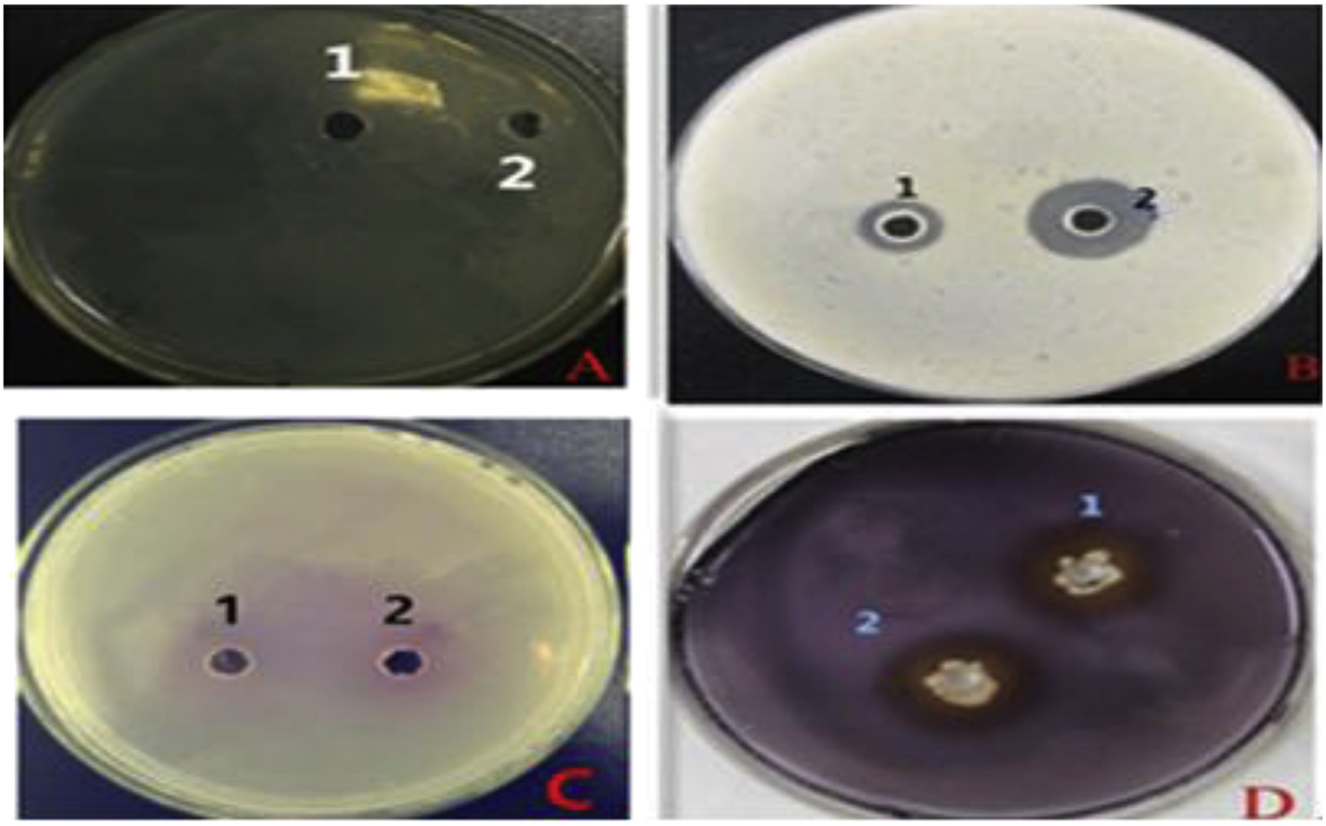

Fig. 4. Extracellular enzyme activity of the YD-1 and YD-2. Capital letters of A, B, C and D indicated Tween 80, skim milk, urea and soluble starch plate media, respectively. Numbers 1 and 2 represented the results of YD-1and YD-2, respectively.

not known. We speculated that some metabolites that could interfere with the determination of sinapine were produced during the degradation of sinapine, but it needs to be further confirmed.

Strains YD-1 and YD-2 not only can effectively degrade sinapine, but also have the activities of protease, urease and amylase. If YD-1 and YD-2 were used to ferment feedstuffs, they could not only decrease the anti-nutritional effect of sinapine, but also further improve the quality and digestibility of feedstuffs by their extracellular enzymes. Thus the application prospect would be quite broad.

Investigations on sinapine-degrading enzymes are rare. Enzymes, such as laccase, polyphenol oxidase, β-glycosidase and lipase, could involve in the degradation of sinapine. For instance, the degradation rate of sinapine by laccase was as high as 81.93% (He, 2010). Some researchers have isolated proteins from *E. coli* and *K. pneumonia*, which have laccase activity (Brown et al., 1995). Furthermore, Kim et al. (2001) detected phenol oxidase activity in genetically modified *E. coli*. The strains of YD-1 and YD-2 isolated in this study belonged to *E. coli* or *K. pneumonia*, respectively. However, this experiment could not determine whether laccase and phenol oxidase were involved in sinapine degradation. This is something that should be evaluated in future studies.

5. Conclusions

This study confirmed that the site of sinapine degradation in laying hens is in the lower digestive tract, especially the ileum. The strains and the bacteria that can degrade sinapine are mostly gram negative. Strains of YD-1 and YD-2, as sinapine-degrading bacteria, which we isolated and cultured first time from the digestive tract of laying hens, belong to *E. coli* and *K. pneumonia*, respectively, which have protease, urease and amylase activities. In general, YD-2 was more efficacious than YD-1 in degrading sinapine.

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