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Effects of Alfalfa Crude Polysaccharides on Production Performance, Egg Quality, and Cecal Microbiota in Laying Hens (Postprint)

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Abstract

This study aimed to investigate the effects of dietary supplementation with different levels of alfalfa crude polysaccharides on production performance, egg quality, and cecal microbial counts in laying hens. A total of 540 Hy-Line Brown laying hens aged 30 weeks were randomly allocated into 6 groups with 6 replicates per group and 15 hens per replicate. The control group was fed a corn-soybean meal basal diet, while the experimental groups were fed the basal diet supplemented with 250, 500, 1,000, 2,000, and 4,000 mg/kg alfalfa crude polysaccharides, respectively. The experimental period lasted 144 days. The results showed: 1) During weeks 1-8, the 2,000 and 4,000 mg/kg alfalfa crude polysaccharide groups exhibited significantly higher laying rates compared with the control and 250 mg/kg groups ($P < 0.05$); during weeks 17-20, the 2,000 mg/kg group had significantly higher average daily feed intake than the control and 250 mg/kg groups ($P < 0.05$), while the 250 mg/kg group showed significantly lower feed/egg ratio than the control and 500 mg/kg groups ($P < 0.05$). 2) Compared with the control group, on day 144, all alfalfa crude polysaccharide groups demonstrated significantly deeper yolk color ($P < 0.05$); on day 90, the 4,000 mg/kg group had significantly lower Haugh unit ($P < 0.05$); on day 60, the 2,000 and 4,000 mg/kg groups exhibited significantly lower egg shape index ($P < 0.05$), and on day 120, the 1,000 mg/kg group also showed significantly lower egg shape index ($P < 0.05$); during days 61-90, the 250 and 1,000 mg/kg groups had significantly reduced soft-shell and broken egg rate ($P < 0.05$). 3) Compared with the control group, on day 80, the 500 and 2,000 mg/kg groups showed significantly decreased cecal Escherichia coli counts ($P < 0.05$) and significantly increased cecal Lactobacillus counts ($P < 0.05$), while the 4,000 mg/kg group also exhibited significantly increased cecal Lactobacillus counts ($P < 0.05$); on day 144, the 1,000 mg/kg group had significantly reduced cecal Salmonella counts ($P < 0.05$); on days 40, 80, 120, and 144, the 500 and 1,000 mg/kg groups showed elevated cecal Bifidobacterium counts, but the

differences were not significant (P > 0.05). In conclusion, dietary supplementation with appropriate levels of alfalfa crude polysaccharides can significantly improve production performance, egg quality, and optimize cecal flora structure in laying hens during the laying period, with 500 mg/kg being the appropriate supplementation level.

Full Text

Effects of Alfalfa Crude Polysaccharides on Performance, Egg Quality and Cecal Microflora Numbers of Laying Hens

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Abstract

This experiment was conducted to investigate the effects of dietary supplementation with different levels of alfalfa crude polysaccharides (ACPS) on performance, egg quality, and cecal microflora numbers in laying hens. Five hundred and forty 30-week-old Hy-Line Brown laying hens were randomly allocated to 6 groups with 6 replicates per group and 15 hens per replicate. The control group received a corn-soybean meal basal diet, while the treatment groups received the basal diet supplemented with 250, 500, 1,000, 2,000, or 4,000 mg/kg ACPS. The experiment lasted for 144 days. The results showed: 1) During weeks 1–8, the laying rate in the 2,000 and 4,000 mg/kg ACPS groups was significantly higher than in the control and 250 mg/kg ACPS groups (P < 0.05). During weeks 17–20, the average daily feed intake (ADFI) in the 2,000 mg/kg ACPS group was significantly higher than in the control and 250 mg/kg ACPS groups (P < 0.05), while the feed-to-egg ratio in the 250 mg/kg ACPS group was significantly lower than in the control and 500 mg/kg ACPS groups (P < 0.05). 2) Compared with the control group, all ACPS groups significantly improved yolk color on day 144 (P < 0.05); the 4,000 mg/kg ACPS group significantly reduced Haugh unit on day 90 (P < 0.05); the 2,000 and 4,000 mg/kg ACPS groups significantly reduced egg shape index on day 60 (P < 0.05), and the 1,000 mg/kg ACPS group also significantly reduced egg shape index on day 120 (P < 0.05); the 250 and 1,000 mg/kg ACPS groups significantly reduced soft-shelled or cracked egg rate during days 61-90 (P < 0.05). 3) Compared with the control group, on day 80, the 500 and 2,000 mg/kg ACPS groups significantly reduced cecal *Escherichia coli* numbers and increased cecal *Lactobacillus* numbers (P < 0.05), while the 4,000 mg/kg ACPS group also significantly increased cecal *Lactobacillus* numbers (P < 0.05). On day 144, the 1,000 mg/kg ACPS group significantly reduced cecal *Salmonella* numbers (P < 0.05). On days 40, 80, 120, and 144, the 500 and 1,000 mg/kg ACPS groups showed increased cecal *Bifidobacterium* numbers, but the differences were not significant (P > 0.05). In conclusion, dietary supplementation with appropriate levels of ACPS can significantly improve laying performance, enhance egg quality, and optimize cecal microflora structure in laying hens, with 500 mg/kg being the optimal supplementation level.

Keywords: alfalfa crude polysaccharides; laying hens; performance; egg quality; cecal microflora

Introduction

Alfalfa (*Medicago sativa*), also known as lucerne, is a high-quality perennial legume forage widely valued in animal husbandry for its high yield, balanced nutrition, digestibility, and palatability. Alfalfa polysaccharides (APS) are water-soluble non-starch acidic polysaccharides extracted from alfalfa stems and leaves, composed primarily of xylose, arabinose, glucose, rhamnose, galactose, glucuronic acid, and galacturonic acid. APS exhibit various biological activities, including promoting animal growth, improving nutrient utilization, enhancing protein synthesis, boosting immunity, and increasing antioxidant capacity. The animal digestive tract harbors a vast and diverse microbial community that participates in nutrient synthesis, digestion, and absorption while providing immunomodulatory functions and preventing pathogen invasion. Therefore, a healthy and stable intestinal microflora is crucial for promoting animal growth, improving production performance, and enhancing product quality. Previous studies have shown that alfalfa polysaccharides can promote the proliferation of beneficial bacteria in the hindgut, regulate intestinal microflora, and improve gut health. Like other plant polysaccharides, APS are natural and non-toxic, making them promising candidates for green feed additives to replace antibiotics. However, research on the effects of alfalfa polysaccharides on laying hens remains scarce. This study investigated the effects of dietary supplementation with different levels of alfalfa crude polysaccharides on production performance, egg quality, and cecal microflora numbers in Hy-Line Brown laying hens, providing a theoretical foundation and experimental basis for the application of alfalfa polysaccharides as a green feed additive in the egg industry.

1.1 Experimental Materials

First-cut alfalfa at the budding stage was purchased from Tumd Zuoqi, Inner Mongolia, cut into 60 cm segments, and air-dried. Alfalfa crude polysaccharides were extracted using water extraction, alcohol precipitation, and deproteinization methods. The ACPS content was determined to be 22.71% using the phenol-sulfuric acid method.

1.2 Experimental Animals and Basal Diet

Five hundred and forty 30-week-old Hy-Line Brown laying hens were used as experimental animals. The basal diet was formulated with corn and soybean meal as main ingredients, with nutrient levels based on NRC (1994) recommendations for laying hens. The composition and nutrient levels of the basal diet are shown in Table 1 .

Table 1 Composition and nutrient levels of the basal diet (air-dry basis), %

Notes: 1) The premix provided the following per kg of diet: Mn 63.6 mg, Zn 69 mg, Fe 30 mg, Cu 6.25 mg, I 0.4 mg, Se 0.2 mg, VA 8,000 IU, VD_3 3,000 IU, VE 15 IU, VK_3 2 mg, VB_1 2 mg, VB_2 4 mg, VB_6 4 mg, VB_{12} 0.01 mg, calcium pantothenate 12 mg, nicotinic acid 40 mg, folic acid 1 mg, biotin 0.1 mg, choline 212.5 mg. 2) Available phosphorus (AP), metabolizable energy (ME), crude protein (CP), calcium (Ca), methionine (Met), lysine (Lys), and Met+Cys were calculated values according to *The Tables of Feed Composition and Nutritive Values in China* (2014).

1.3 Experimental Design and Management

A single-factor completely randomized design was employed. Five hundred and forty healthy 30-week-old Hy-Line Brown laying hens with similar body weight and laying rate ($P > 0.05$) were randomly divided into 6 groups with 6 replicates per group and 15 hens per replicate. The control group received the basal diet without ACPS, while treatment groups received the basal diet supplemented with 250, 500, 1,000, 2,000, or 4,000 mg/kg ACPS. A 2-week pre-trial period was conducted during which all groups received the basal diet. After confirming no significant differences in performance ($P > 0.05$), the formal 144-day trial began. The experiment was conducted at the Changping Experimental Base of the Institute of Animal Science, Chinese Academy of Agricultural Sciences. Hens were housed in 3-tier battery cages (3 hens per cage) and fed three times daily with ad libitum access to feed and water. Lighting consisted of 16 h per day (natural light supplemented with artificial light). House temperature and humidity were recorded daily, and mortality was recorded with causes.

1.4 Measurements

1.4.1 Production Performance During the experiment, daily egg number and egg weight were recorded per replicate, and feed consumption was measured weekly. Laying rate, average egg weight, average daily feed intake (ADFI), and feed-to-egg ratio were calculated from these records.

1.4.2 Egg Quality Soft-shelled or cracked eggs were recorded daily per replicate to calculate the incidence rate. On days 30, 60, 90, 120, and 144 of the experiment, all eggs laid were collected and egg quality parameters were measured within 12 h. Haugh unit and yolk color were measured using an egg quality analyzer (EMT-2500, Robotmation, Japan). Egg shape index was determined using an egg shape coefficient meter (NFN384, FHK, Japan) to measure longitudinal and transverse diameters. Egg shape index = (longitudinal diameter/transverse diameter) $\times$ 100.

1.4.3 Cecal Microflora Numbers On days 40, 80, 120, and 144, one hen near the average body weight was selected from each replicate, euthanized, and the abdominal cavity was opened. The right cecum was ligated, disinfected with alcohol, and immediately transported to the laboratory. In a sterile environment, 0.5 g of cecal content was weighed into a sterile 10 mL centrifuge tube containing 4.5 mL sterile saline, vortexed for 4-5 min to prepare a 10^{-1} dilution. After standing, 0.5 mL supernatant was transferred to 4.5 mL sterile saline for 10^{-2} dilution, vortexed for 4-5 s, and serially diluted to 10^{-7}. Three appropriate dilutions were selected, and 50 L of each was inoculated onto eosin methylene blue (EMB) agar for *Escherichia coli*, Salmonella-Shigella (SS) agar for *Salmonella*, de Man, Rogosa and Sharpe (MRS) agar for *Lactobacillus*, and TPY agar for *Bifidobacterium*. *E. coli* and *Salmonella* were aerobically cultured at 37 °C for 24 h; *Lactobacillus* and *Bifidobacterium* were anaerobically cultured at 37 °C for 48-72 h. Bacterial numbers were expressed as $\log_{10}$ CFU/g using the formula: $\log_{10}$ CFU/g = $\log_{10}$[(colony count × dilution factor × sample volume per dilution)/(inoculation volume × sample weight)].

1.5 Statistical Analysis

Data were analyzed using the ANOVA procedure of SAS 9.2 software. Duncan's multiple range test was used for inter-group comparisons, with $P < 0.05$ considered statistically significant. Results are expressed as means ± SD. Laying rate and soft-shelled/cracked egg rate were arcsine-transformed before analysis.

Results

2.1 Effects of Alfalfa Crude Polysaccharides on Production Performance of Laying Hens

As shown in Table 2 , during weeks 1-8, the 2,000 and 4,000 mg/kg ACPS groups significantly increased laying rate compared with the control group ($P < 0.05$). During weeks 1-20, laying rates in the 250, 500, 1,000, 2,000, and 4,000 mg/kg ACPS groups increased by 0.07%, 1.21%, 2.19%, 1.59%, and 1.12%, respectively ($P > 0.05$). Among ACPS groups during weeks 1-8, the 2,000 and 4,000 mg/kg groups had significantly higher laying rates than the 250 mg/kg group ($P < 0.05$), with no significant differences observed in other periods ($P > 0.05$). During weeks 17-20, ADFI in the 500, 1,000, 2,000, and 4,000 mg/kg ACPS groups was significantly higher than in the control group ($P < 0.05$). Although ADFI was higher in all ACPS groups than in the control group during weeks 1-20, the differences were not significant ($P > 0.05$). The 2,000 mg/kg ACPS group had significantly higher ADFI than the 250 mg/kg group during weeks 17-20 ($P < 0.05$). Regarding feed-to-egg ratio, the 250 mg/kg ACPS group showed a significant reduction during weeks 17-20 compared with the control group ($P < 0.05$), and both the 250 and 1,000 mg/kg groups had significantly lower ratios than the 500 mg/kg group ($P < 0.05$). Dietary ACPS supplementation had no significant effect on average egg weight ($P > 0.05$).

Table 2 Effects of alfalfa crude polysaccharides on performance of laying hens (n = 6)

Note: In the same row, values with the same or no letter superscripts indicate no significant difference ($P > 0.05$), while different lowercase letters indicate significant difference ($P < 0.05$). The same applies below.

2.2 Effects of Alfalfa Crude Polysaccharides on Egg Quality of Laying Hens

As shown in Table 3 , compared with the control group, yolk color was significantly reduced in the 1,000, 2,000, and 4,000 mg/kg ACPS groups on day 30 ($P < 0.05$), and in the 500, 1,000, and 4,000 mg/kg groups on day 90 ($P < 0.05$). However, on day 144, all ACPS groups significantly improved yolk color ($P < 0.05$). Among ACPS groups, the 250 and 500 mg/kg groups had significantly higher yolk color than the 2,000 mg/kg group on days 30 and 144 ($P < 0.05$), while on day 90, the 2,000 mg/kg group had significantly higher yolk color than the 500, 1,000, and 4,000 mg/kg groups ($P < 0.05$). The 4,000 mg/kg ACPS group showed significantly lower Haugh unit than the control and 500 mg/kg groups on day 90 ($P < 0.05$). Egg shape index was significantly reduced in the 2,000 and 4,000 mg/kg ACPS groups on day 60 ($P < 0.05$) and in the 1,000 mg/kg group on day 120 ($P < 0.05$) compared with the control group. The 2,000 mg/kg group had significantly lower egg shape index than the 250, 500, and 1,000 mg/kg groups on day 60 ($P < 0.05$), while the 1,000 mg/kg group had significantly lower index than the 250, 500, and 4,000 mg/kg groups on day 120 ($P < 0.05$). Regarding soft-shelled or cracked egg rate, the 250 and 1,000 mg/kg ACPS groups were significantly lower than the control group during days 61–90 ($P < 0.05$). Across the entire experimental period (days 1–144), all ACPS groups had lower soft-shelled or cracked egg rates than the control group, though the differences were not significant ($P > 0.05$).

Table 3 Effects of alfalfa crude polysaccharides on egg quality of laying hens (n = 6)

2.3 Effects of Alfalfa Crude Polysaccharides on Cecal Microflora Numbers of Laying Hens

As shown in Table 4 , dietary ACPS supplementation significantly affected cecal *E. coli*, *Salmonella*, and *Lactobacillus* numbers on day 80 ($P < 0.05$). Compared with the control group, the 500, 1,000, and 2,000 mg/kg ACPS groups significantly reduced cecal *E. coli* numbers on day 80 ($P < 0.05$), with the 500 and 2,000 mg/kg groups showing significantly lower counts than the 250 mg/kg group ($P < 0.05$). On day 80, the 500, 1,000, 2,000, and 4,000 mg/kg ACPS groups had lower cecal *Salmonella* numbers than the control group, though not significantly ($P > 0.05$); however, the 1,000, 2,000, and 4,000 mg/kg groups had significantly lower *Salmonella* counts than the 250 mg/kg group ($P < 0.05$). On day 144, the 1,000 mg/kg ACPS group significantly reduced cecal *Salmonella* numbers compared with the control and 250 and 2,000 mg/kg groups ($P < 0.05$). Cecal *Lactobacillus* numbers were significantly higher in the 500, 2,000, and 4,000 mg/kg ACPS groups than in the control group on day 80 ($P < 0.05$). On day 120, all ACPS groups had higher *Lactobacillus* numbers than the control group, though not significantly ($P > 0.05$), with the 500 and 2,000 mg/kg groups being significantly higher than the 250 mg/kg group ($P < 0.05$). Dietary ACPS supplementation promoted *Bifidobacterium* proliferation to some extent, but the effect on cecal *Bifidobacterium* numbers was not significant ($P > 0.05$).

Table 4 Effects of alfalfa crude polysaccharides on cecal microflora numbers of laying hens (n = 6), $\log_{10}$(CFU/g)

Discussion

3.1 Effects of Alfalfa Crude Polysaccharides on Production Performance of Laying Hens

To date, no studies have reported the effects of alfalfa polysaccharides as a single feed additive on laying hen performance. Our results showed that during weeks 1–8, dietary supplementation with 2,000 and 4,000 mg/kg ACPS significantly improved laying rate, and all ACPS groups had higher laying rates than the control group throughout the entire experimental period. High-producing hens experience continuous heavy ovulation during peak production, leading to accumulated oxidative damage and functional decline in ovarian tissue, which reduces laying rate. The improved laying rate observed with ACPS supplementation may be attributed to its ability to inhibit 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radical generation, enhance superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities, and alleviate ovarian oxidative damage, thereby promoting laying rate. Additionally, the ACPS used in this study contained small amounts of flavonoids with estrogen-like biological activity that may regulate reproductive hormone levels, increase follicle number, and improve laying rate. During weeks 17–20, the 500, 1,000, 2,000, and 4,000 mg/kg ACPS groups significantly increased ADFI, while the 250 mg/kg group significantly reduced feed-to-egg ratio. The increased feed intake and improved feed efficiency may result from enhanced metabolism of crude protein, crude fat, and other nutrients by ACPS. Our results also demonstrated that ACPS increased beneficial gut bacteria (*Lactobacillus* and *Bifidobacterium*) while inhibiting *E. coli* and *Salmonella* proliferation, promoting intestinal health and facilitating nutrient digestion and absorption, thereby improving production performance.

3.2 Effects of Alfalfa Crude Polysaccharides on Egg Quality of Laying Hens

Yolk color primarily depends on the quantity and type of carotenoids consumed by poultry, with lutein being the main pigment. Previous studies have shown that dietary supplementation with *Rehmannia glutinosa* polysaccharide extract significantly increased yolk color with increasing dosage, and that 100–

250 mg/kg *Astragalus* polysaccharide improved yolk color by enhancing pigment deposition. In contrast, our study found that ACPS had limited effects on yolk color during the early period (days 30–90), with the 1,000 and 4,000 mg/kg groups even significantly reducing yolk color. This may be attributed to high ambient temperatures during the early period, causing severe oxidative stress in hens, and the unsaturated fatty acids in soybean oil being prone to oxidation. Since lutein has antioxidant properties, its structure may have been destroyed, compromising its pigmenting function. During the later period (days 120–144), ACPS groups showed improved yolk color, possibly because ACPS enhanced the stability of lutein and other carotenoids, increasing pigment deposition in the yolk.

Haugh unit is an important indicator of egg protein quality and freshness, with higher values indicating thicker, better-quality protein. Our results showed that 4,000 mg/kg ACPS significantly reduced Haugh unit, possibly because galacturonic acid and other components in ACPS are non-starch polysaccharides with anti-nutritional effects that, at high concentrations, may hinder intestinal absorption of dietary protein, adversely affecting protein quality.

Studies have found that dietary natural antioxidants reduce egg shape index (length/width ratio). Reynard and Savory reported that stress delays ovulation and prolongs egg retention in the uterus, resulting in more elongated eggs with higher shape indices. On day 60, the 2,000 and 4,000 mg/kg ACPS groups significantly reduced egg shape index compared with the control, likely due to ACPS alleviating stress responses, though the specific mechanisms require further investigation.

Calcium is the primary component of eggshells, and impaired calcium secretion or inadequate supply results in thin, fragile shells. Across the entire experimental period, all ACPS groups had lower soft-shelled or cracked egg rates than the control, with the 250 and 1,000 mg/kg groups showing significant reductions during days 61–90, indicating that ACPS improves shell quality. Research has shown that plant polysaccharides affect intracellular calcium concentration through extracellular calcium influx and intracellular calcium release. Deng et al. reported that water-soluble plant polysaccharides enhance intestinal function and promote calcium absorption. We hypothesize that ACPS may regulate intra- and extracellular calcium concentrations, promoting calcium deposition in eggshells and improving shell quality. Additionally, beneficial gut bacteria produce short-chain fatty acids and lactic acid that lower gastrointestinal pH, increasing mineral solubility and promoting calcium and phosphorus absorption to enhance shell quality. Thus, the reduced soft-shelled egg rate in ACPS groups may also result from increased *Lactobacillus* and *Bifidobacterium* numbers.

3.3 Effects of Alfalfa Crude Polysaccharides on Cecal Microflora Numbers of Laying Hens

Poultry possess paired ceca that provide ideal conditions for microbial colonization and proliferation due to stable temperature, suitable pH, and prolonged retention time. Studies indicate that total bacterial counts in chicken ceca reach approximately 10^{11} cells/g (wet weight), with anaerobic bacteria numbering 10^8–10^9 cells/g and comprising at least 640 species from 140 genera. Given the short length of the chicken large intestine, the ceca represent the primary site of microbial activity. Under normal conditions, intestinal microflora maintain dynamic equilibrium, with their structure and function influenced by gastrointestinal environment (redox potential, pH), external conditions (temperature, humidity), and dietary nutrient composition and chemical structure.

Previous research demonstrated that dietary APS supplementation at 1% and 8% levels significantly promoted beneficial bacteria (*Bifidobacterium* and *Lactobacillus*) proliferation while inhibiting *E. coli* in broiler ceca. Numerous studies have confirmed that natural plant polysaccharides positively regulate intestinal microflora. Chen et al. found that *Achyranthes bidentata* polysaccharide inhibited *E. coli* while promoting *Bifidobacterium* and *Lactobacillus* growth in piglets, with effects superior to antibiotics. Dong et al. reported that *Glycyrrhiza* polysaccharide significantly promoted *Lactobacillus* and *Bifidobacterium* proliferation while reducing *E. coli* and *Salmonella* numbers in broiler ceca. Shen et al. showed that *Hericium erinaceus* fermentation extract polysaccharide significantly increased *Lactobacillus* and *Bifidobacterium* numbers while reducing *E. coli*, *Salmonella*, and enterococci in broiler intestines, with the 0.5% supplementation level being most effective. Our results are consistent with these findings, showing that ACPS significantly increased cecal *Lactobacillus* numbers, promoted *Bifidobacterium* proliferation, and inhibited *E. coli* and *Salmonella*.

The regulatory mechanisms of alfalfa polysaccharides on intestinal microflora remain unclear but may involve several pathways. Some plant polysaccharides exhibit prebiotic effects, as their unique chemical structures resist enzymatic hydrolysis and absorption in the foregut, reaching the hindgut as fermentation substrates that selectively stimulate beneficial bacteria growth and activity, enhancing resistance to pathogens and improving host health. Additionally, active polysaccharide components may compete with pathogenic bacteria such as *E. coli* and *Salmonella* for attachment sites on the intestinal mucosa through a "occupancy" effect, preventing colonization and reducing their numbers. Furthermore, organic acids (acetic acid, lactic acid) produced during *Bifidobacterium* and *Lactobacillus* fermentation lower intestinal pH and redox potential, inhibiting pathogen growth. The improved laying rate, increased ADFI, and reduced feed-to-egg ratio and soft-shelled egg rate observed with ACPS supplementation corresponded with increased beneficial bacteria, indicating that optimal production performance and egg quality are closely associated with maintaining *Lactobacillus* and *Bifidobacterium* proliferation and dominance in the ceca.

Conclusion

Dietary supplementation with appropriate levels of alfalfa crude polysaccharides can significantly improve laying performance, enhance egg quality, and optimize cecal microflora structure in laying hens. Based on comprehensive consideration, 500 mg/kg ACPS is recommended as the optimal supplementation level in laying hen diets.

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