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Evaluation of Sprouted Barley as a Nutritive Feed Additive for *Protaetia brevitarsis* and Its Antibacterial Action against *Serratia marcescens*

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Interest in edible insects such as *Protaetia brevitarsis* has increased rapidly, and several insect producers use these insects in industrialized mass production. However, mass rearing of insects can cause insect diseases. Sprouted barley is a valuable source of nutrients and has antioxidant, antimicrobial, anti-inflammatory, and anti-cancer effects. This study was conducted to investigate the effect of sprouted barley as a feed additive for producing healthy *P. brevitarsis* larvae. *P. brevitarsis* larvae were fed feeds with or without sprouted barley, and their body weight and larval period wewe checked weekly. To confirm the antibacterial effects of sprouted barley, *in vitro* bioassays were performed by counting *Serratia marcescens* colonies, and *in vivo* bioassays were performed by determining the survival rate and body weights of the *S. marcescens*-infected larvae. Larvae fed different feeds were analyzed for their nutrient compositions (i.e., such as proximate composition, minerals, amino acids, and heavy metals). Larvae fed 5% and 10% sprouted barley had maximum weight increases of 19.2% and 23.1%, respectively. Both treatment groups had significantly shorter larval periods than those of the control group. Sprouted barley markedly inhibited the growth of entomopathogenic *S. marcescens*. Furthermore, larvae fed sprouted barley exhibited higher Cu, Zn, and K levels. Seventeen amino acids were present in larvae fed sprouted barley, of which, tyrosine and glutamic acid were predominant. No heavy metals were detected in any of the investigated groups. Therefore, sprouted barley may be a suitable feed additive for producing high-quality *P. brevitarsis* larvae.

Key words : Edible insect, growth, *Protaetia brevitarsis*, *Serratia marcescens*, spouted barley

Introduction

The white-spotted flower chafer, *Protaetia brevitarsis* (Coleoptera; Scarabaeidae), is used as a traditional medicine for treating diseases such as hepatic cancer, breast cancer, inflammatory disease, liver cirrhosis, and hepatitis [20]. In 2016, the Ministry of Food and Drug Safety of Korea registered *P. brevitarsis* larvae as a general food ingredient. Owing to increased interest, producers have launched businesses for mass production of these larvae for food and feed; however, this has led to the emergence of several bacterial, viral, and fungal diseases among these insects. Insect diseases can be lethal and cause serious economic damage to farms rearing the insects [8, 33]. Thus, these insect diseases must be prevented and controlled.

Serratia marcescens is an opportunistic gram-negative bacterium in the Enterobacteriaceae family. *S. marcescens* was originally thought to be a non-pathogenic saprophytic water organism that formed red colonies [13]. Animal and insect infections caused by *S. marcescens* have been increasingly reported since 1960 [7, 11]. Importantly, *S. marcescens* was shown to be lethal to insects [15, 23, 32]; thus, production of consistently safe and high-quality insects is necessary.

Sprouted grain has been suggested as a method of producing fresh forage grains with little water. Sprouted barley ([SB], *Hordeum vulgare* L.) is a young barley leaf grown to approximately 20 cm and harvested at 10-15 days after sowing. Barley is one of the most important crops worldwide and is the second most consumed grain after rice in Korea [17]. Several reports have shown that SB is a valuable source of nutrients, such as amino acids, minerals, vitamins, dietary fiber, and other bioactive substances such as superoxide dismutase, catalase, carotenoids, and chlorophyll [5, 25]. Several researchers have investigated feeding sprouted grain to cattle, pigs, and poultry with a sustainable product

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quality [1, 12, 16, 22, 31]. These studies revealed an increased performance in both lambs and cattle fed barley grain. Additionally, the barley fodder helped reduce operating costs and improve product quality. However, SB is rarely used as a feed supplement in insect farming. Therefore, the present study was designed to evaluate the effects of SB as an insect feed supplement using 5% and 10% w/w of feed.

Materials and Methods

Insects

P. brevitarsis eggs were purchased from a private seller (Wanju-gun, Jeollabuk-do, Korea). After hatching, the larvae were reared in a laboratory regulated at 26±1℃ and 40%~60% relative humidity. The larvae were individually reared and checked once weekly to measure their body weight and larval period. Twenty larvae were tested per group, and each treatment was repeated three times.

Feed with different SB contents

To test the effects of SB on *P. brevitarsis* larval growth, different amounts of SB were mixed with oak sawdust and fermented for at least 1 month (Table 1). The Korea Feed Ingredients Association recommended that the content of feed additives be added to less than 15% of the total feed. In this study, SB was added at the levels of 5% and 10% as an additive for insect feeds. SB was kindly provided by the National Institute of Crop Science.

S. marcescens and colony counting

The bacterial strain *S. marcescens* (Korean Agricultural Culture Collection [KACC] No. 10502) was obtained from the KACC (Wanju-gun, Jeollabuk-do, Korea). The strains were incubated on nutrient agar medium (Becton, Dickinson and Co., Inc., Franklin Lakes, NJ, USA). For the antibacterial assays, *S. marcescens* cultures treated with 1%, 5%, and 10% SB were incubated on an orbital shaker at 200 rpm and serially diluted to 10-fold. Ten microliters of the 3-fold dilution was spread on nutrient agar. The plates were incubated overnight at 30℃ in the dark. The colony-forming numbers were determined, and the bacterial concentration in each original sample was calculated. The tests were performed in triplicate. Each data point was composed of the average of three independent samples.

Table 1. Formulation of experimental feed concentrate composition (%)

Treatment	Feed source (%)	
	Fermented oak sawdust	Sprouted barley
Control	100	-
5% SB	95	5
10% SB	90	10

S. marcescens infection of *Protaetia brevitarsis*

Bioassays were performed by injecting healthy third-instar *P. brevitarsis* larvae with 10 µl of bacterial suspension containing 10^5 bacteria per larva, directly into the hemocoel. The negative- and positive-control larvae were injected with 10 µl of nutrient broth and bacterial suspension, respectively, and fed control feed. Survival rates and larval weights were checked weekly. Each treatment included ten larvae and was repeated four times.

Proximate analysis

Proximate analysis was performed according to the standard methods of the Association of Official Analytical Chemists [2] for determining moisture, crude fiber, protein, and fat content in the samples. The proximate values were reported as percentages. Moisture contents of the samples were determined by oven-drying at 105℃ to a constant weight. The Kjeldahl method was used to determine the protein content with a conversion factor of 6.25. Crude fat was extracted with hexane via the Soxhlet method. Crude fiber content was determined via the digestion method, and the ash content was estimated by ashing at 550℃ for 3 hr.

Mineral, amino acid, and heavy metal compositions

The mineral elements in the *P. brevitarsis* larvae, i.e., copper, zinc, potassium, magnesium, and phosphorus were determined using an atomic absorption spectrophotometer as per the AOAC methods. The mineral values were reported in milligrams per kilogram (mg/kg). Amino acids were determined using an automated Amino Acid Analyzer (Beckman 6300, Brea, CA, USA). The amino acid compositions were expressed as percentage. Heavy metals were determined using an atomic absorption spectrophotometer as per the AOAC methods. The results were expressed as mg/kg dry weight.

Statistical analysis

Values from each experiment are expressed as the mean ± standard deviation (SD) and compared with the controls.

Table 2. Growth parameters of *P. brevitarsis* after feeding feeds treated with different concentrations of sprouted barley

Feed	Mean ± SD				
	Maximum larval weight (g)	Percentage weight gain (%)	Time to reach maximum weight (days)	Survival rate (%)	Pupation rate (%)
Control	2.90±0.34[a]		82.21±21.2[a]	90.00±11.55[a]	87.50±14.43[a]
5% SB	3.45±0.12[b]	119.17	35.73±4.92[b]	95.00±10.00[a]	95.00±10.00[a]
10% SB	3.56±0.14[c]	123.07	33.67±2.31[b]	90.00±8.16[a]	90.00±8.16[a]

Columns with different letters differed significantly. Means followed by the same letters within a column do not significantly differ (p>0.05); based on Tukey's multiple comparisons test.

Comparisons between means were performed using one-way analysis of variance, followed by Tukey's multiple comparisons test. The significance level was set at p<0.05.

Results and Discussion

Growth performance of larvae fed SB-added feed

Table 2 shows the effect of SB as a feed additive on *P. brevitarsis* larval growth. Maximum larval weights of the 5% and 10% SB-fed groups were 3.45 g and 3.56 g, respectively. The 5% and 10% SB-fed groups reached their maximum weights markedly earlier than did the control group. Maximum larval weights (p<0.001) and the days at which the maximum larval weight (p<0.001) differed significantly. For commercial use in animal feed and human food, each third-instar larva should weigh >2.0 g. Thus, insects fed SB were more efficient and cost-effective to produce. Feeds containing 5% and 10% SB yielded larvae that could be used in feed and food at 6 and 5 weeks of feeding, respectively. SB improved the larval growth and weight gain compared with those of the control larvae. Thus, using SB could reduce rearing costs and labor supplies for insect farms.

At the end of the trial, the average survival rate for all three groups was >90% (Table 2). The pupation rates of larvae fed both 5% and 10% SB were higher than that of the control (Table 2); all larvae fed SB pupated 9 or 10 weeks after hatching. In the control group, pupation was delayed by 17 weeks. Survival and pupation rates did not significantly differ among the groups.

The improvements in *P. brevitarsis* growth performance after SB treatment were attributed to the nutrients in the SB. Researchers have reported that SB can be used to enhance growth performance in lambs [1, 12]. Additionally, feeding SB to growing goats increased nutrient digestibility, body weight gain and feed conversion efficiency [16]. The improved performance of SB-supplemented livestock could be due to the ability of the supplements to supply necessary nutrients. The higher performance of *P. brevitarsis* in the present study was consistent with these studies, although differences existed among the species. SB is a rich source of nutrients and contains enzymes that promote growth performance [25, 34].

Table 3. Larval periods for each *P. brevitarsis* instar treated with three formulations

Feed	Larval period (days, mean ± SD)		
	1st instar	2nd instar	3rd instar
Control	15.53±2.58[a]	16.47±2.59[a]	92.87±5.53[a]
5% SB	10.13±1.89[b]	13.75±1.65[b]	23.13±3.05[b]
10% SB	10.80±2.04[b]	12.60±2.80[b]	21.60±9.53[b]

Table 3 showed the larval periods for each *P. brevitarsis* larval instar. Larvae fed 5% and 10% SB had shorter larval periods than did the control larvae at each stage. Interestingly, the average larval periods of the third instar larvae fed 5% and 10% SB were 75.1% and 76.7% shorter, respectively, than those of the control. Therefore, larvae fed SB grew faster, and nutrients in the SB contributed to this growth. Thus, SB could be used as a nutritive additive for *P. brevitarsis* to promote larval growth and reduce the labor and costs to rear the larvae.

Growth-inhibitory effect of SB against entomophagous *S. marcescens*

To evaluate the *in vitro* antibacterial activity of SB, bioassays were performed by counting colonies on nutrient agar plates. Significantly fewer *S. marcescens* colonies grew in 5% and 10% SB agar than in the control agar (p<0.01; Fig. 1), possibly because of the antibacterial effect of policosanol in the SB. Policosanol is a natural mixture of higher aliphatic primary alcohols (C24-C36). Many studies have reported that policosanols substantially inhibit bacterial growth [28-30].

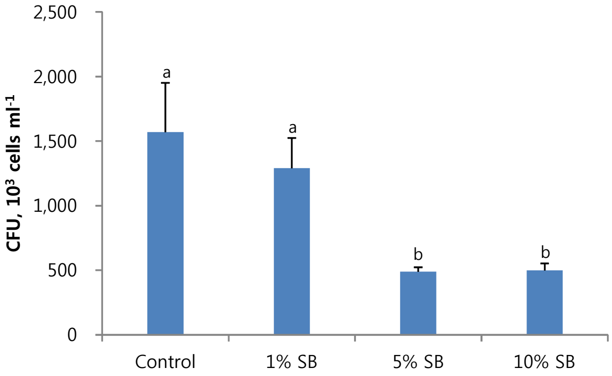

Fig. 1. Effect of sprouted barley on the antibacterial activity against *S. marcescens*. Columns with different letters differed significantly (p<0.01). Error bars represent the standard deviation of the mean (n=3).

S. marcescens inhabits soil and exhibits entomopathogenic characteristics [15, 23, 32]. In Korea, *S. marcescens* is the most common bacterium to cause bacterial diseases in *P. brevitarsis* on domestic insect farms. To confirm the *in vivo* antibacterial activity of SB, *S. marcescens* was injected into the hemocoels of third-instar *P. brevitarsis* larvae fed feeds supplemented with or without SB (Table 4). Approximately 50% of the positive-control larvae, which were infected with *S. marcescens* and fed control feed, died 4 weeks postinoculation, revealing that inoculation of entomopathogenic *S. marcescens* could kill *P. brevitarsis* larvae. *S. marcescens* may secrete toxins and damage the larvae during the inoculation period. Despite the bacterial infection, larvae fed SB exhibited a survival rate of >70%. Components in the SB seemed to confer the nutrition and immunity required for the infected larvae to survive. Weight gained by the larvae fed control feed was 54%-57%. The final body weights of the infected larvae fed 5% and 10% SB increased by 68% and 75%, respectively. Arabinoxylan polysaccharides and ɣ-aminobutyric acid (GABA) in the SB causes the immunomodulatory activity [17, 18]. Immunomodulatory compounds can interact with the immune system and enhance specific host response mechanisms [9, 18, 34]. Thus, the immune enhancement associated with these functional ingredients might occur in infected larvae fed SB. Further studies on the mechanisms of immunomodulatory molecules would help determine their functional roles or involvement in insect immune responses.

Nutrient composition of *P. brevitarsis* with different feeds

The Korean Ministry of Food and Drug Safety registered *P. brevitarsis* larvae as a general food ingredient in 2016. To guarantee the safety of their use in foods and feeds, we analyzed larval nutrient compositions in different feeds. Table 5 presented the proximate larvla compositions. Crude protein contents in the 5% and 10% SB (59.29% and 55.03%) were significantly higher than those in the control feed (43.68%; 5% SB, p<0.01; 10% SB, p<0.05). Crude protein in meat is ~22% and varies according to source (e.g., beef and pork) [26]. Edible insects are considered a good protein source [4, 6]. Larvae fed SB-added feeds had much higher protein concentrations than did other meats. Crude fat was high in the larvae fed 5% SB (3.49%) and 10% SB (3.03%). Crude fiber percentages differed significantly at 5.13% in the control larvae and 6.83% in the larvae fed 10% SB (p<0.001). Therefore, SB might be a nutritional feed source for *P. brevitarsis*.

Table 5 showed the mineral compositions. Larvae fed SB had higher copper levels than did the controls. Zinc and potassium levels varied significantly (p<0.001) in larvae fed 5% and 10% SB compared with those of the controls. Animals need copper for growth and nerve fiber health [14, 27]. Zinc is essential for animal nutrition and physiology [14, 24]. Potassium is a major mineral in intracellular fluid and is important for body health and cellular functions in humans and animals [21]. The high levels of copper, zinc, and potassium in the 5% and 10% SB may enhance *P. brevitarsis* larval growth.

Table 6 showed the larval amino acid profiles. The highest essential amino acid concentrations were for tyrosine at 1.48% (5% SB) and 1.66% (10% SB). The lowest were for methionine (0.27% for 5% SB; 0.28% for 10% SB); however, the

Table 4. Survival rates and body weights of *S. marcescens*-infected *P. brevitarsis* larvae fed different feeds

Feed	Survival rate (%)	Larval body weight (g)		Larval body weight gain	
		Start of trial	End of trial	g/larva	%
(-) Control	95.0±5.0^{a}	1.52±0.04	2.39±0.07	0.87±0.04	57.1±1.8
(+) Control	51.7±12.6^{c}	1.51±0.08	2.33±0.37	0.82±0.34	54.1±21.7
5% SB	85.0±5.0^{ab}	1.46±0.12	2.43±0.19	0.98±0.20	67.7±16.5
10% SB	75.0±15.0^{a}	1.54±0.04	2.68±0.17	1.14±0.20	74.8±14.3

larvae fed SB-added feeds showed higher methionine contents than those of the controls. Methionine is important for animal growth [10]. For non-essential amino acids, the highest concentrations were for glutamic acid at 1.71% (5% SB) and 1.79% (10% SB). Cysteine had the lowest concentrations (0.22% for both 5% and 10% SB), but the rate of increase in its contents in both groups was the highest of all amino acids compared with those of the controls. Cysteine can be synthesized from methionine, and increased cysteine contents were likely due to the increased methionine contents. Adding SB would improve the nutritional value of the larvae as food and feed sources, because these amino acids can support larval growth performance.

However, as the use of insects as food sources has increased, concerns have been raised regarding food insecurities such as chemical hazards. Heavy metals can pose serious health hazards to humans and contribute to mortality. No heavy metals, such as arsenic, cadmium, or lead, were detected in any tested samples (Table 5), thus indicating that larvae fed SB are safe and immediately available as food and feed sources.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 흰점박이꽃무지 사료첨가제로서 새싹보리의 곤충병원성 세균에 대한 항균 효과에 관한 연구

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국내에서 2016년 흰점박이꽃무지 등 일부 곤충이 식품원료로 등록되면서 곤충사육 농가가 폭발적으로 증가하고 있다. 하지만 곤충사육 농가가 보유하고 있는 사육기술, 병해충 관리기술이 농가별로 차이가 있고, 대량으로 곤충을 사육하면서 높은 사육밀도, 병충해 감염 등으로 인해 곤충이 대량 폐사하게 됨으로써 곤충사육 농가에 심각한 경제적 손실을 입고 있다. 이에 본 연구에서는 다양한 영양성분을 함유할 뿐만 아니라 항균, 항염증 등의 효과를 가진 새싹보리를 흰점박이꽃무지 유충의 주 먹이원인 발효톱밥에 첨가하여 주 먹이원 첨가제로써 적용 가능성 및 곤충병원성 세균에 대한 항균 효과를 확인하고자 하였다. 흰점박이꽃무지 유충의 주 먹이원에 5%와 10% 새싹보리를 첨가했을 때 유충의 증체율이 19.2%와 23.1%로 높았고, 사육 기간이 대조군 대비 단축된 것으로 조사되었다. 또한 새싹보리는 곤충병원성 세균 *Serratia marcescens*의 성장을 저해하고, 세균에 감염된 흰점박이꽃무지 유충의 생존율과 증체율이 건강한 유충의 수준으로 회복되는 경향을 보였다. 뿐만 아니라 새싹보리를 첨가한 먹이원으로 사육한 유충의 영양성분 분석 결과, 조단백질, 조지방, 구리, 아연, 칼륨 등 미네랄 및 아미노산 함량이 대조군보다 높게 나타났다. 결론적으로 흰점박이꽃무지 유충의 주 먹이원에 새싹보리를 첨가한다면 단기간에 대량생산이 가능하고, 곤충병원성 세균 감염에 의한 폐사를 효율적으로 예방할 수 있어 안정적인 곤충 생산이 가능할 것으로 판단된다.