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Recombinant *Bacillus subtilis* CM65-P_{att}' is a potential feed attractant of *Megalobrama amblycephala*: evaluation based on appetite regulatory system, growth performance, and intestinal functions

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This study was conducted to investigate the feed attractant properties of recombinant *Bacillus subtilis* CM65-P_{att}' (secreting P_{att}, a functional peptide screened after enzymatic hydrolysis of the cotton meal) on the appetite regulatory system, growth performance, intestinal structure integrity, and digestive and absorptive functions of blunt snout bream (*Megalobrama amblycephala*). A total of 240 blunt snout breams were selected (10.85 ± 0.25 g) and divided into four groups (three replicates per group), including control group (C, basic diet), original bacteria group (OB, 1 × 10⁸ CFU/kg *Bacillus subtilis* CM65) and two levels of recombinant bacteria groups (RB1, 1 × 10⁸ CFU/kg *Bacillus subtilis* CM65-P_{att}'; RB2, 1 × 10⁹ CFU/kg *Bacillus subtilis* CM65-P_{att}'). After a 10-week feeding trial, the results showed that the appropriate dietary *Bacillus subtilis* CM65-P_{att}' supplementation (1 × 10⁹ CFU/kg) enhanced appetite and increased feed intake while improving growth performance via the activation of growth hormone–insulin-like growth factor (GH–IGF) axis, enhanced intestinal absorptive capability, and maintained the integrity of intestinal structure by increasing the relative mRNA expression of tight junction proteins. Overall, those indices in the OB group had no significant change. In conclusion, this study confirmed that *Bacillus subtilis* CM65-P_{att}' (or P_{att}) has great potential as a feed attractant, which can be recommended for use as a long-lasting effective feed attractant for fish.

KEYWORDS

Bacillus subtilis CM65-P_{att}', appetite regulatory, feed attractant, intestinal functions, *Megalobrama amblycephala*

1 Introduction

Aquaculture, as an important source of high-quality animal protein, has proven to play a vital role in contributing to food security, alleviating global hunger and promoting economic growth (Yan et al., 2023). With the rapid development of aquaculture, the demand and production of aquafeed have increased rapidly, while the gap between the supply and demand of fishmeal (a high-quality protein source for aquafeeds) is becoming increasingly serious, forcing experts to search for new cheaper alternative protein sources to partly or even completely substitute fishmeal (Mugwanya et al., 2022). However, many studies have shown that fishmeal substitution may lead to poor palatability, low feed intake, and poor digestibility (Wang A. et al., 2024; Xu et al., 2022; Yan et al., 2024), which makes the development of feed attractants a hot topic (Rao et al., 2018) because a high-quality feed attractant can not only alleviate the adverse effects mentioned above (Li et al., 2025; Pang et al., 2024) but also reduce feed losses and wastes in water, thereby lessening environmental pressure and reducing the cost of aquaculture (Ballester-Moltó et al., 2017; Llagostera et al., 2019). Overall, feed attractants are important for sustainable aquaculture development.

Screening methods for aquafeed attractants can be briefly divided into two categories based on the duration of the screening experiment: one is short-term screening, which is mainly based on the intuitive feeding behavior of aquatic animals, including the maze method, touch ball method, and so on (Cheng et al., 2019). By this way, the attractant being screened itself possesses a strong odor and can stimulate feeding by activating the sensory organ of the fish (Yacoub et al., 2002). Another category relies on long-term culture experiment to evaluate the quality of feed attractants by using the actual feed intake as an index, which was considered as a more accurate and practical way (Soengas et al., 2018). Moreover, the feed attractants selected by this method are considered able to act on the feeding regulatory system of fish, thereby exerting a long-term feed-promoting effect (Bertucci et al., 2019). Peptides have a variety of flavor properties that stimulate the nervous system of aquatic animals, increasing their appetite and improving feed taste and palatability (Ball et al., 2004). Small peptides are potent attractants currently used as alternatives to traditional feed attractants in aquaculture (Conti et al., 2024; Peng et al., 2022; Tusche et al., 2011).

Previous laboratory studies have shown the promotion of feeding behavior using enzyme-digested cotton meal products on blunt snout bream (Yuan et al., 2019), which might be related to the increase in the content of small peptides (180–1,500 Da) before and after the enzyme digestion (Yuan et al., 2020). Based on this, a functional small peptide, P_{att} (FRQGDVIALP), was preliminarily screened from the digested products of its content. Furthermore, its appetite-stimulating potential was evaluated *in vitro* by using primary intestinal epithelial cells isolated from blunt snout bream (*Megalobrama amblycephala*). P_{att} might have the potential to promote feeding, which needed to be verified by a long-term growth experiment. However, the high price of adding peptides directly to aquatic feed is detached from production realities, which leads to peptides usually being added to feed in the form of

enzymatic digests of protein products (Chen et al., 2023; He et al., 2023; Yuan et al., 2019). However, insufficient investigation of the functional mechanisms of such peptides has prompted a shift toward probiotics and genetically engineered-driven peptide expression.

Bacillus subtilis (*B. subtilis*), a promising probiotic, has several advantages for aquaculture. It can form spores under unfavorable conditions and is non-pathogenic to aquatic animals, making it a better choice to be added as a vector for administering an aquafeed supplement while also serving as a probiotic (Ghosh, 2025; Kuebutornye et al., 2020). It has been reported that *B. subtilis* can enhance feed intake, promote growth, and improve antioxidant defense as well as the intestinal functions of aquatic animals (Kuebutornye et al., 2019). In addition, *B. subtilis* is a good vector for heterologous protein expression. According to previous studies (He et al., 2024; Xiong et al., 2024), a peptide with antioxidant and immune-enhancing properties was successfully inserted into *B. subtilis* CM65 and enhanced the antioxidant capacity of Chinese mitten crab (*Eriocheir sinensis*) and blunt snout bream, which provides a theoretical basis for this experiment. Combining *B. subtilis* CM65 with P_{att} and adding P_{att} in the form of added recombinant *B. subtilis* CM65-P_{att}' can save costs and enable the evaluation of the actual feed attractant effect of the P_{att}.

Blunt snout bream, one of the popular freshwater species in China, is famous between fish farmers and consumers for its high growth rate and tasty flesh, thus having a significant economic value (Jiang et al., 2024; Wang X. et al., 2024). As an herbivorous species used in traditional culture, developing a suitable aquafeed attractant for it can not only promote the development of the farming industry but also provide new ideas for the preparation and selection of feed attractants. Obviously, evaluating the function of the aquafeed attractant only from the perspective of feed intake is biased. Although some feed attractants can increase feed intake, they have no significant growth-promoting effect on aquatic animals (Calo et al., 2024; Pulido-Rodriguez et al., 2021; Zarantoniello et al., 2022). The activation of the appetite regulatory system contributes to the increasing feed intake, while the digestive and absorptive system determines growth performance (Zhao et al., 2019). The intestine is an important part of the peripheral appetite regulatory system as well as the largest digestive and absorptive organ of blunt snout bream, and the integrity of the intestinal structure is fundamental to its functions (Zhou et al., 2023). Overall, this study aimed to construct recombinant *B. subtilis* CM65 expressing P_{att} peptide and investigated its appropriate supplementation level and effects on the growth, appetite regulation, and the structure and functions of the intestine to assess its potential effectiveness as a feed attractant of blunt snout bream. These results can offer new theoretical insight into the development of aquatic feed additives.

2 Materials and methods

In this study, the feeding schemes and all experimental operations were handed by The Animal Care and Use Committee

at Nanjing Agriculture University which granted permission (license number: SYXK (Su)2021-0086).

2.1 P_{att}, *B. subtilis* CM65, and recombinant *B. subtilis* CM65-P_{att}'

Peptide sequences of enzyme-digested cotton meal products were determined according to the previous laboratory study of Yuan et al. (2020), and P_{att} (FRQGDVIALP) was selected from the products. An *Escherichia coli*–*Bacillus subtilis* shuttle expression vector pBE plasmid, which consist of an ampicillin-resistant gene originated from *Escherichia coli* as well as a kanamycin-resistant gene originated from *B. subtilis*, was stored in our lab. As P_{att} itself has a very low molecular weight (1,100 Da), it is difficult to be recognized and expressed by direct introduction of the pBE. According to previous laboratory studies (He et al., 2024; Xiong et al., 2024), five P_{att} segments were tandemly attached to His-tag to constitute the pBE-P_{att}'. To clone the plasmid, *Escherichia coli* was cultured in Luria–Bertani (LB) medium at 37°C with 200 r/min shaking.

A plasmid-free strain of *B. subtilis* CM65, whose NCBI accession number is “SAMN37990”, has shown that the survival rate of its spore approached to 100% under reaction conditions of 80°C for 15 min, suggesting that it can be used as a feed additive to be added prior to pelleting (Xiong et al., 2024). The pBE-P_{att}' plasmid obtained was transformed into competent *B. subtilis* CM65 cell to constitute the recombinant *B. subtilis* CM65-P_{att}' and was then cultured in LB medium containing 100 µg/mL kanamycin (CAS#25389-94-0, Merck KGaA, Darmstadt, Germany) at 37°C with 200 r/min shaking for 48 h to obtain spore and harvested by centrifugation at 10,000 × g at 4°C for 10 min (TGL16M, YINGTAI Instrument, China). The same protocols (without kanamycin) above were observed to harvest the spore of *B. subtilis* CM65. After that, the spread plate method was adopted to calculate the colony-forming units (CFU) of the spores obtained, wherein *B. subtilis* CM65-P_{att}' was 1 × 10¹² CFU/kg and *B. subtilis* CM65 was 2 × 10¹³ CFU/kg. Additionally, the content of P_{att} in *B. subtilis* CM65-P_{att}' (1 × 10¹² CFU/kg) was about 25.96% (of total protein), which was measured by using liquid chromatography–mass spectrometry (LC-MS) (Orbitrap Astral and Vanquish Neo UHPLC, Thermo Scientific, American).

2.2 Experimental design and feeding trial

Four isonitrogenous (27% crude protein) and isolipidic (7% crude lipid) experimental diets were formulated as follows: the control group (C, basic diet), the original bacteria group (OB, 1 × 10⁸ CFU/kg *B. subtilis* CM65), and two levels of recombinant bacteria groups (RB1, 1 × 10⁸ CFU/kg *B. subtilis* CM65-P_{att}'; RB2, 1 × 10⁹ CFU/kg *B. subtilis* CM65-P_{att}'). The composition of the experiment diet is shown in Table 1, with fish meal, soybean meal, cottonseed meal, and rapeseed meal served as protein sources, and soybean oil was adopted as the lipid source. In addition, the bacteria were

separately added by dissolving in 50 mL sterile water and then spraying into the well-mixed diet. After that, the diet was prepared into to particles with a particle size of 2 mm and stored at -20°C.

The juvenile blunt snout bream of this study was provided by a fish hatchery at Ezhou (Hubei, China). Before the formal feeding trial, a 2-week acclimation culture was performed to establish the feeding reflexes of fish. After that, a total of 240 healthy blunt snout bream (10.85 ± 0.25 g) were randomly and evenly divided into 12 net cages (three replicates per diet at 20 fish per cage), each with a capacity of 2 m³ (length/width/height = 2:1:1) in an outdoor pond, with each net cage providing ample space for the fish to move freely (Liu et al., 2024). During the feeding trial, the fish were fed to saturation three times a day (8:00, 12:00, and 16:00) for 10 weeks. On each day before the morning feeding, 50 g feed for each cage was weighed. After feeding, the remaining feed would be reweighed to calculate the feed intake. Throughout the feeding trial, the water conditions were measured using a YSI ProPlus (YSI Water Quality Testing Instrument Co., Ltd., OH, USA) within a temperature range from 23°C to 28°C, pH was 7.1 to 7.3, dissolved oxygen levels were maintained at 5.0 to 6.0 mg/L, and ammonia nitrogen concentrations were below 0.04 mg/L.

TABLE 1 Formulation and proximate composition of diets.

Ingredients (%)	C	OB	RB1	RB2
Fish meal (g kg ⁻¹)	5.0	5.0	5.0	5.0
Soybean meal (g kg ⁻¹)	25.0	25.0	25.0	25.0
Cottonseed meal (g kg ⁻¹)	10.7	10.7	10.7	10.7
Rapeseed meal (g kg ⁻¹)	13.0	13.0	13.0	13.0
Soybean oil (g kg ⁻¹)	2.6	2.6	2.6	2.6
Corn starch (g kg ⁻¹)	28.0	28.0	28.0	28.0
Rice bran (g kg ⁻¹)	12.0	12.0	12.0	12.0
Calcium biposphate (g kg ⁻¹)	1.8	1.8	1.8	1.8
Ethoxyquin (g kg ⁻¹)	0.5	0.5	0.5	0.5
Salt (g kg ⁻¹)	0.5	0.5	0.5	0.5
Premix ^a (g kg ⁻¹)	1.0	1.0	1.0	1.0
<i>B. subtilis</i> supplement				
<i>B. subtilis</i> CM66, CFU kg ⁻¹	0	1 × 10 ⁸	0	0
<i>B. subtilis</i> CM66-P _{att} ', CFU kg ⁻¹	0	0	1 × 10 ⁸	1 × 10 ⁹
Proximate composition (dry matter basis)				
Crude protein (%)	27.81	27.71	26.94	27.51
Crude lipid (%)	7.96	7.35	7.88	7.65
Crude ash (%)	8.09	8.10	7.83	8.15
Moisture (%)	13.48	13.18	12.70	12.93

^aPremix supplied the following minerals (g/kg of diet) and vitamins (IU or mg/kg of diet): CuSO₄·5H₂O, 2.0 g; FeSO₄·7H₂O, 25 g; ZnSO₄·7H₂O, 22 g; MnSO₄·4H₂O, 7 g; Na₂SeO₃, 0.04 g; KI, 0.026 g; CoCl₂·6H₂O, 0.1 g; vitamin A, 900,000 IU; vitamin D, 200,000 IU; vitamin E, 4,500 mg; vitamin K₃, 220 mg; vitamin B₁, 320 mg; vitamin B₂, 1,090 mg; vitamin B₅, 2,000 mg; vitamin B₆, 500 mg; vitamin B₁₂, 1.6 mg; vitamin C, 5,000 mg; pantothenate, 1,000 mg; folic acid, 165 mg; and choline, 60,000 mg.

2.3 Proximate composition analysis

According to the standard method of AOAC (2005), the contents of moisture, crude protein, crude lipid, and crude ash of the diets (nine samples per group) were measured. The moisture contents were determined by drying samples at 105°C to a constant weight according to (AOAC, 2005) method no. 925.10. Crude protein content (N factor = 6.25) was quantified by using an automated Kjeldahl nitrogen analyzer (FOSS KT260, Switzerland) by Kjeldahl (AOAC, 2005) method no. 990.03. The crude lipid content was quantified by ether extraction using a Soxhlet System (Soxtec System HT6, Sweden) according to AOAC (2005) method no. 2003.05. The crude ash contents were determined by burning samples at 550°C in a muffle furnace to a constant weight according to AOAC (2005) method no. 923.03. The proximate composition of each diet is shown in Table 1.

2.4 Sample collection

At the end of the feeding trial, the fish were starved for 24 h for sampling. All of the fish in each cage were numbered and weighed. Four fish were randomly selected from each cage (four individual samples per replicate; 12 samples per diet in total) and anesthetized with MS-222 (100 mg/L) (#E10521, Sigma, USA) to measure the body weight, total length, body length, and body width. Based on a study by Chen et al. (2022), blood was collected from the tail vein and stored in anticoagulant tubes, which were moistened with sodium heparin (0.2 g dissolved in 5 mL distilled water) (CAS#9041-08, Yuanye Bio-Technology Co., Ltd, China) and oven-dried at 65°C in advance. Blood was later rapidly sampled and centrifuged ($3,000 \times g$, 4°C, 10 min) in a centrifuge (TGL16M, Yingtai Instrument, China) to collect plasma, and those samples were then stored at -20°C for subsequent experiments. Then, samples of brain, liver, intestinal, and intraperitoneal fat were collected and weighed to calculate the biometric parameters. After that, the whole intestines were carefully rinsed with sterile deionized water, and the mid-intestine was dissected with sterile scissors following the method described by Huang et al. (2025). One portion of the mid-intestine was preserved in 4% paraformaldehyde for histological observation, and the remaining samples were stored at -80°C for further analysis.

2.5 Analytical procedures

2.5.1 Plasma appetite hormone analysis

An ELISA method was adopted to quantify plasma neuropeptide Y (NPY) and peptide YY (PYY) level (two individual samples per replicate; six samples per diet in total). The kits (cat. no. XFH14038 and cat. no. XFH14015) used were from Nanjing Xin Fan Biology Co., Ltd. (Nanjing, China), and the method of determination was performed according to the protocols.

2.5.2 Intestinal digestive analyses

Intestinal samples (three individual samples per replicate; nine samples per diet in total) were homogenized with sterile saline at a ratio of 1:9 by mass volume and then centrifuged. The supernatant was collected, and 10% tissue homogenate was prepared for the determination of digestive enzyme activities. A colorimetric assay was adopted to determine the α -amylase (AMS) activity, trypsin (TPS) activity, and lipase (LPS) activity by using the kits (cat. no. C016-1-1, cat. no. A080-2-2, and cat. no. A054-2-1) from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), following the manufacturer's instructions.

2.5.3 Intestine hematoxylin and eosin

Hematoxylin and eosin (HE) staining was performed at Shandong SparkJade Biotechnology Co., Ltd. (Shandong, China). Intestine tissues (two individual samples per replicate; six samples per diet in total) were fixed in 4% paraformaldehyde for over 24 h, subsequently dehydrated gradually with alcohol, and then embedded in paraffin after being transferred to a xylene solution. A 6- μ m-thick paraffin section was prepared and stained with conventional HE, followed by fixation with neutral gum. Subsequently, images (one slide per sample) were captured using an upright microscope (BX51, Olympus, Japan). The field of view with complete and straight villi was selected on the tissue sections of each part. Villus height (VH), villus width (VW), and muscle layer thickness (MLT) were measured by using Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) for further structural analysis with each three fields analyzed per slide.

2.5.4 Real-time quantitative PCR

Intestinal and brain samples (two individual samples per replicate; six samples per diet in total) were lysed with trizol (Invitrogen, CA, USA) at a ratio of 1/10 to extract RNA. The absorbance at 260 and 280 nm of each sample was measured by using NanoDrop 2000 (Thermo Fisher Scientific, USA) to assess the content and purity of the extracted RNA. Next, the extracted RNA was then reverse-transcribed into cDNA by using Evo M-MLV RT Mix Kit with gDNA Clean for qPCR Ver.2 (Accurate Biology, Hunan, China). The cDNA was then used for qRT-PCR with the polymerase chain reaction performed on CFX96TM Real-Time PCR Detection System (Bio-Rad, USA). The qPCR reaction protocols were as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 30 s. Melting curve analysis was performed by heating from 60°C to 95°C with a ramp rate of 0.1°C/s according to the instructions for SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology, Hunan, China).

The expression levels of the target genes were normalized to elongation factor 1 alpha (*ef1 α*) using the comparative Ct method ($2^{-\Delta\Delta C_t}$) (Liu et al., 2024). The primers designed are shown in Table 2. It should be mentioned that all of the PCR were highly specific and reproducible ($0.998 > R^2 > 0.983$), where all primer pairs had equivalent PCR efficiencies (from 0.91 to 1.01).

2.6 Calculations of growth performance parameters

The following formula was used to calculate the growth performance indicators:

$$\begin{aligned}\text{Weight gain rate (WGR, \%)} &= (W_t - W_0)/W_0 \times 100 \\ \text{Specific growth rate (SGR, \% /d)} &= (\ln W_t - \ln W_0)/t \times 100 \\ \text{Feed conversion rate (FCR)} &= F/(W_t - W_0) \\ \text{Feed intake (FI, g per fish)} &= F/N \\ \text{Visceral somatic index (VSI, \%)} &= (G_1/W_b) \times 100 \\ \text{Hepatosomatic index (HSI, \%)} &= (G_2/W_b) \times 100 \\ \text{Intraperitoneal fat ratio (IPE, \%)} &= (G_3/W_b) \times 100 \\ \text{Intestinesomatic index (ISI, \%)} &= (G_4/W_b) \times 100 \\ \text{Intestine length index (ILI, \%)} &= (L_1/L) \times 100 \\ \text{Condition factor (CF, g/cm}^3\text{)} &= W_b/L^3 \times 100\end{aligned}$$

In the formula, W_0 and W_t are the initial and final weights (g); t represents the trial days; F is the total feed intake of each cage (g); N represents the total number of fish in each cage; G_1 , G_2 , G_3 , and G_4 are the visceral weight (g), liver weight (g), intraperitoneal fat weight (g), and intestine weight (g), respectively; W_b represents the final weight of each fish (g); L_1 and L represents the intestine length (cm) and body length (cm); and L^3 represents for cube of body length (cm^3).

2.7 Data statistics and analysis

All data are presented as means \pm SEM. The data between the control group and the OB group were analyzed by independent-samples t -test with SPSS 22.0 (Chicago, IL, USA). When analyzing the data among OB, RB1, and RB2 groups, Levene's test was firstly performed to check if the data conform to normality and homogeneity. For data conforming to the assumptions ($P > 0.05$), one-way ANOVA followed by Tukey's multiple-range test was used to compare significant differences among those groups. Otherwise, nonparametric test was performed.

3 Results

3.1 Effect of recombinant *B. subtilis* CM65- $P_{att'}$ on feed intake

In terms of the entire feeding trial, no statistical difference on the cumulative FI was observed between the control group and the OB group ($P > 0.05$) (Figure 1A). However, from the 14th day, RB supplementation significantly promoted cumulative FI ($P < 0.05$), and this influence lasted even until the end of the trial ($P < 0.05$). As shown in Figure 1B, an analysis of weekly total FI revealed no significant difference between the OB and control groups ($P > 0.05$). Over the 10-week period, the FI of the RB groups exhibited a gradually increasing trend. Specifically, the FI of the RB2 group was significantly higher than that of the OB group in the first, second, third, fourth, fifth, eighth, and ninth weeks ($P < 0.05$).

3.2 Effect of recombinant *B. subtilis* CM65- $P_{att'}$ on growth performance and organ coefficient

As shown in Figure 2, there was no statistical difference on WGR and SGR between the control group and the OB group ($P > 0.05$). Additionally, WGR and SGR showed an increased trend in the OB, RB1, and RB2 groups, and this trend was significant ($P < 0.05$) in the RB2 group. However, FCR showed no significance among all treatments ($P > 0.05$).

No statistical differences were observed in CF, ISI, and ILI among all of the groups ($P > 0.05$). However, OB could significantly increase VSI ($P < 0.05$), while the opposite was found for HSI ($P < 0.05$). However, with the addition of RB, VSI was lowered first and then raised and was lowest in the RB1 group ($P < 0.05$). Furthermore, RB could increase IPE, with the highest value observed at a dosage of 1×10^9 CFU/kg ($P < 0.05$).

3.3 Effect of recombinant *B. subtilis* CM65- $P_{att'}$ on plasma NPY and PYY levels

No significant differences were observed between the control and the OB group in plasma NPY and PYY ($P > 0.05$), whereas RB addition significantly increased the levels of NPY and decreased PYY ($P < 0.05$), with RB2 showing the most significant effect ($P < 0.05$) (Figure 3).

3.4 Effect of recombinant *B. subtilis* CM65- $P_{att'}$ on intestinal digestive enzyme activity

There were no statistical differences on LPS and TPS among all treatments (Figure 4). However, it was found that *B. subtilis* addition can significantly decrease the activity of AMS ($P < 0.05$). Supplying RB can mitigate this situation ($P < 0.05$) but cannot reach the same level as the control group.

3.5 Effect of recombinant *B. subtilis* CM65- $P_{att'}$ on the transcription of brain appetite-regulating and growth-related genes

As shown in Figure 5, there was little difference observed in the expression of *npv*, *igf*, *cck*, and *npv 1r* among all treatments ($P > 0.05$). However, the mRNA transcriptions of *gh* and *pyy* were both significantly higher in the OB group compared with the control group ($P < 0.05$), but the opposite was presented in *ghr*, *pomc*, and *lep* ($P < 0.05$). The expression of *gh* showed a significant dose-dependent increase with the graded addition of RB ($P < 0.05$). The transcription of *ghr* and *npv 2r* both tended to be upregulating while respectively significant in the RB1 group and the RB2 group ($P < 0.05$). However, an opposite result was noted in *agrp*, and its transcription was lowest in the RB1 group ($P < 0.05$).

TABLE 2 Nucleotide sequences of the primers used to assay gene expression by RT-PCR.

Genes	Forward primer (5'–3')	Reverse primer (5'–3')	Accession numbers or references	Efficiency
Appetite regulation				
<i>npv</i>	CGCCTGCTTGGGAACCTCTTA	TGGACCTTTTGCCGTACCTC	JQ301475.1	0.97
<i>pyy</i>	AGTCTACTTTCAACAATAAAGCAGG	GCGTCATGTTCCCTAACCCA	AF233875.1	0.95
<i>npv 1r</i>	GTGTGAGGTAACGCAGCTCT	CAACCACCGAACGAACACAC	XM_048197605.1	0.92
<i>npv 2r</i>	GCAGACAATTCAAGGTTGCG	GCCACAAGCATTTTCGTCGT	XM_048197241.1	0.91
<i>agrp</i>	GGAGTCTTGTGCAGACCCTC	CAGCCTTACCCAGATCCTCATC	MK165096.1	0.94
<i>pomc</i>	GTGGGTTTCCTACCTGCACA	CCCCTCACCATCTCTTTTGC	AY125332.2	0.91
<i>lep</i>	CACATCACTGCGTAACTGG	TGACACCCTAACTACCTTCC	KJ193853.1	0.96
<i>cck</i>	CGCAGAAGTTCCTCTCCGAA	TCATACTCTTCTGCGCTCCG	AY125332.2	0.96
Growth promotion				
<i>igf1</i>	CGGTTTTCACAGGTGCACAG	ACAGGGTGCCCCAAATCCTT	JQ398496.1	0.99
<i>Gh</i>	GAGCCATCTCAAACAGCC	AGCAAGCCAGAAGACGAA	AY170124.1	1.01
<i>Ghr</i>	TGGCAGAGATACCAAGCA	GGGAGAAGATGAGCAGGA	JN896373.1	0.98
Intestine structure				
<i>claudin 3c</i>	GGGCATTATCGGCTGGATCA	TGTCCGGTGCTCTGTACAAC	XM_048167600.1	1.02
<i>claudin 4l</i>	TTGTGATTGGGATCCTGGGC	TGGTTTGGAGCTCTCGTCC	XM_048167602.1	0.97
<i>occludin</i>	GCGCGTTCTGTGGTATGATG	GTCAGAACCACTACTTGAAGGT	XM_048187597.1	0.96
Intestine absorption				
<i>slc1a3</i>	GGAATGCTTTCGTATCCTCAC	CAGAGCGGCCATACCTGTAATT	KY200978	0.90
<i>slc6a19b</i>	ACCGCATCTTGCTCACAGTTATT	ACCACTGGAAAGGCTGTTTATGT	KX823960	0.91
<i>slc7a7</i>	GTCCCATACTTCACATAGCA	GATAGCCATCACTTTCTCCAAC	JX013494	0.93
<i>slc7a8</i>	TGGTGAGAAGCTGTTGGGAGTGA	GCAAGTGAAGAGTAGGGCTGGAA	GU474428	0.90
<i>slc7a9</i>	TTCTACAGTCTTCTGCCGTTGC	AGAGCTGGAGAAGGCGTGTAAC	KX823958	0.92
<i>slc7a1</i>	TGCACAGGCTGAACAGGACACT	CGACATCACTGAACCCCTCCAAC	KY200979	0.95
<i>slc38a2</i>	AGAAGAGTCTGCAAAACCAA	CACAAACATTTCCAGAAACGA	KY200981	0.92
<i>slc7a5</i>	CAACATGAGCCGACCAGGAGAC	CCAGCGACAACCCGACTGAACC	KY200980	0.96
<i>slc6a6</i>	GCAGAGTTGGGACGACAATCAC	GGAAGAGCGGCTGAACCAAGTAG	KX682391	0.93
<i>slc6a14</i>	TCATTGCGTACCCTGATGCTCT	ACTTCAGAACTTTGGGATAGGCA	KX823959	0.93
Reference gene				
<i>ef1α</i>	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC	X77689.1	0.98

npv, neuropeptide Y; *pyy*, peptide YY; *npv 1r*, neuropeptide Y receptor Y1; *npv 2r*, neuropeptide Y receptor Y2; *agrp*, agouti related neuropeptide; *pomc*, proopiomelanocortin; *lep*, leptin; *cck*, cholecystokinin; *igf1*, insulin-like growth factors 1; *gh*, growth hormone; *ghr*, growth hormone receptor; *slc1a3*, solute carrier family 1 member 3; *slc6a19b*, solute carrier family 6 member 19b; *slc7a7*, solute carrier family 7 member 7; *slc7a8*, solute carrier family 7 member 8; *slc7a9*, solute carrier family 7 member 9; *slc7a1*, solute carrier family 7 member 1; *slc38a2*, solute carrier family 38 member 2; *slc7a5*, solute carrier family 7 member 5; *slc6a6*, solute carrier family 6 member 6; *slc6a14*, solute carrier family 6 member 14; *ef1α*, elongation factor 1 alpha.

3.6 Effect of recombinant *B. subtilis* CM65-P_{att}’ on the transcription of intestine appetite-regulating and growth-related genes

The transcription of appetite-regulating genes in the intestine was not exactly the same as observed in the brain. Comparing the

OB group with the control group, the transcription of *igf*, *npv*, *npv 2r*, *pyy*, and *lep* all had a significant upregulation ($P < 0.05$), while an opposite result was presented on *cck* ($P < 0.05$) (Figure 6). The expression of *npv 2r* was higher in the RB2 group than the OB group ($P < 0.05$), while the expression of *cck* in both the RB1 group and the RB2 group was significantly higher than that in the OB group ($P < 0.05$).

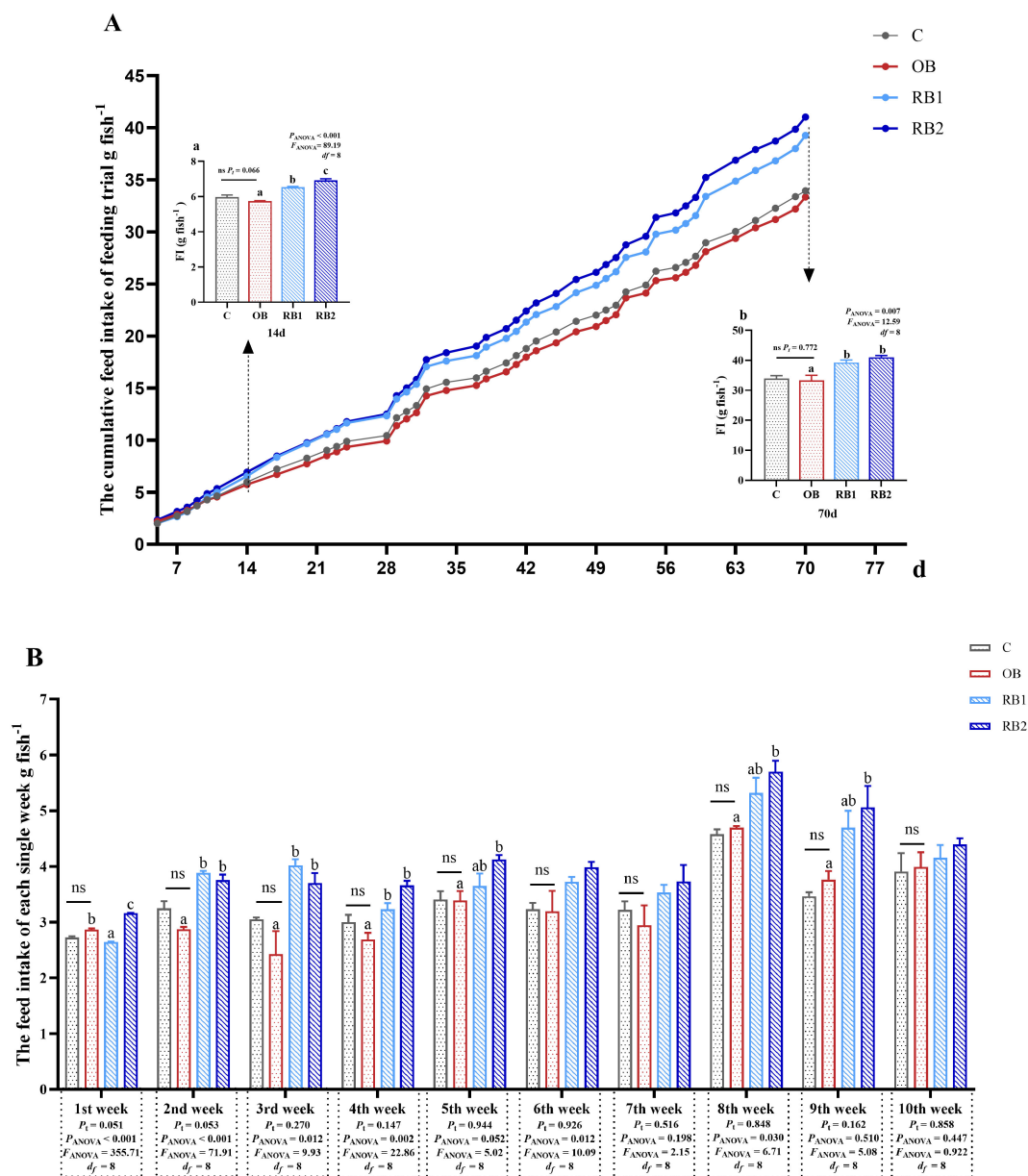


FIGURE 1

Feed intake of blunt snout bream fed different experiment diets. OB, original bacteria—*Bacillus subtilis* CM65; RB, recombinant bacteria—*Bacillus subtilis* CM65- $P_{att'}$. (A) Effect of different diets on cumulative feed intake of the feeding trial. (a) Cumulative feed intake of the first 14 days. (b) Cumulative feed intake of 70 days of feeding trial. (B) Effect of different diets on feed intake of each week. ^{ns} $P > 0.05$, compared between the control group and the OB group analyzed by independent-samples *t*-test. ^{abc} compared among the OB group, RB1 group, and RB2 group by using one-way ANOVA. Values in the same line with different superscripts are significantly different ($P < 0.05$).

3.7 Effect of recombinant *B. subtilis* CM65- $P_{att'}$ on the mRNA transcription of intestinal tight junction proteins and absorption-related genes

No significant difference was observed in the expression of *claudin-3c* and *occludin* between the control group and the OB group ($P > 0.05$), whereas the transcription of *claudin-4l* was significantly upregulated in the OB group ($P < 0.05$) (Figure 7A). Supplying *B. subtilis* CM65- $P_{att'}$ can significantly elevate the mRNA transcription of *occludin* ($P < 0.05$). The expression of *claudin-3c*

was significantly higher in the RB1 group, while the expression of *claudin-4l* was higher in the RB2 group compared with those in the OB group ($P < 0.05$).

As shown in Figure 7B, the mRNA expressions of *slc7a8* and *slc7a9* were significantly lower in the OB group compared with the control group ($P < 0.05$). The transcriptions of *slc7a7*, *slc7a5* as well as *slc6a14* were significantly upregulated when the dietary RB level was 1×10^9 CFU/kg ($P < 0.05$). With the dose up, the mRNA expressions of *slc6a19b* and *slc7a9* were significantly higher than the OB group, while no significance was observed between the RB1

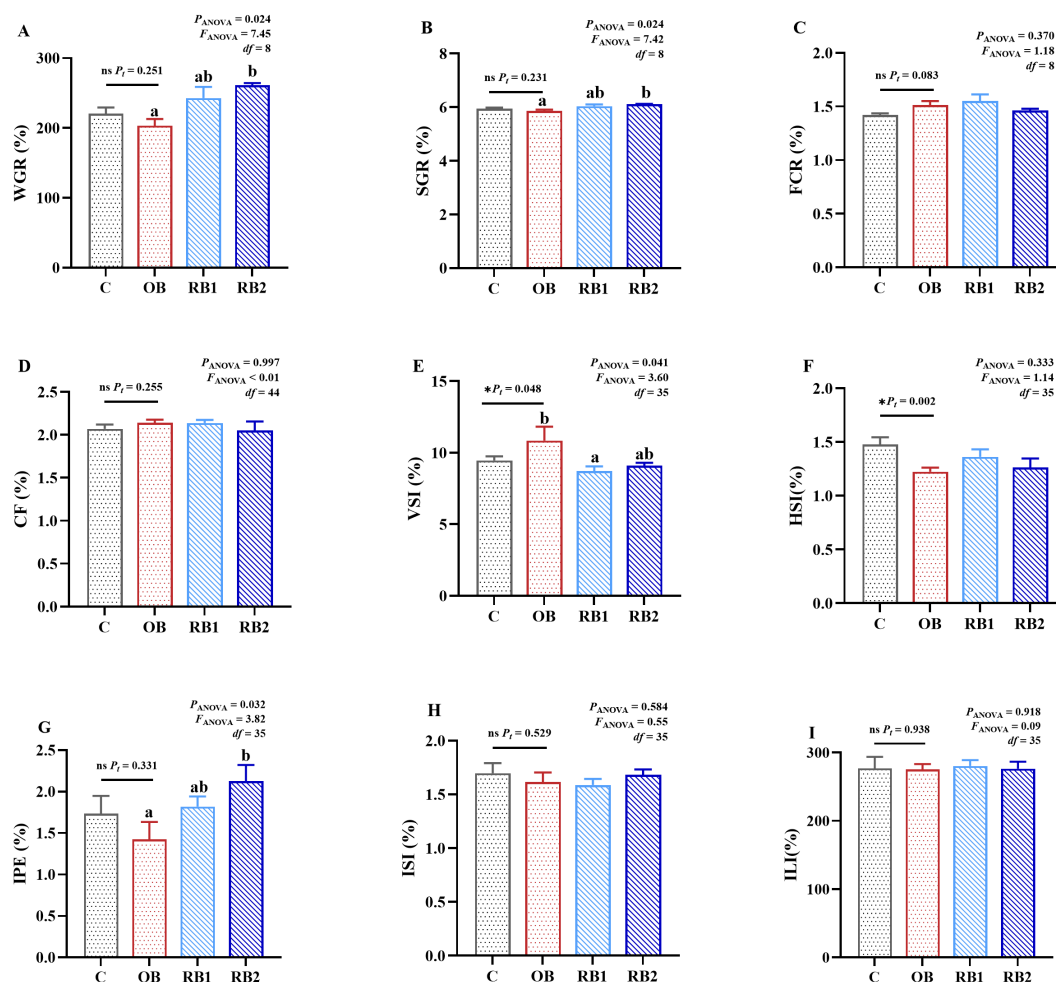


FIGURE 2

Growth performance and organ coefficient of blunt snout bream fed different experiment diets. OB, original bacteria—*Bacillus subtilis* CM65; RB, recombinant bacteria—*Bacillus subtilis* CM65- $P_{att'}$. (A) WGR, weight gain rate. (B) SGR, specific growth rate. (C) FCR, feed conversion ratio. (D) CF, condition factor. (E) VSI, visceral somatic index. (F) HSI, hepatosomatic index. (G) IPE, intraperitoneal fat ratio. (H) ISI, intestine somatic index. (I) ILI, intestine length index. * $P < 0.05$, $^{ns}P > 0.05$, compared between the control group and the OB group analyzed by independent-samples *t*-test. ab compared among the OB group, RB1 group, and RB2 group by using one-way ANOVA. Values in the same line with different superscripts are significantly different ($P < 0.05$).

group and the RB2 group ($P > 0.05$). However, the expression of *slc7a6* was significantly higher and higher ($P < 0.05$).

3.8 Effect of recombinant *B. subtilis* CM65- $P_{att'}$ on histological observation of the intestine

Little difference was observed on MLT among all treatments ($P > 0.05$) (Figure 8). Supplying OB can significantly increase VH and VW compared with the control group. However, with dietary supplied RB, there was a deep decrease on VH ($P < 0.05$). Upon reaching the dose of 1×10^9 CFU/kg (the RB2 group), VH significantly increased compared with the RB1 group but was still significantly lower than the OB group ($P < 0.05$).

4 Discussion

Changes in feed intake and growth performance are the key apparent indicators to assess the effectiveness of an aquafeed attractant. Previous research showed that dietary-supplied *B. subtilis* had a positive effect on growth in many aquatic animals such as *Oreochromis niloticus*, *Penaeus vannamei*, and *Rhamdia quelen* (Alves et al., 2024; Cao et al., 2022; Liao et al., 2023; Rodrigues et al., 2021). In this study, the addition of *B. subtilis* CM65 has no significant impact on the growth performance of blunt snout bream. The same occurred in the study of Xiong et al. (2024) using a dietary supplement of 2×10^7 – 2×10^9 CFU/kg *B. subtilis* CM65 for which no significant impacts on the growth performance of juvenile blunt snout bream were observed, while it was found that 1×10^8 – 1×10^9 CFU/kg *B. subtilis* CM65 supplied can significantly promote the

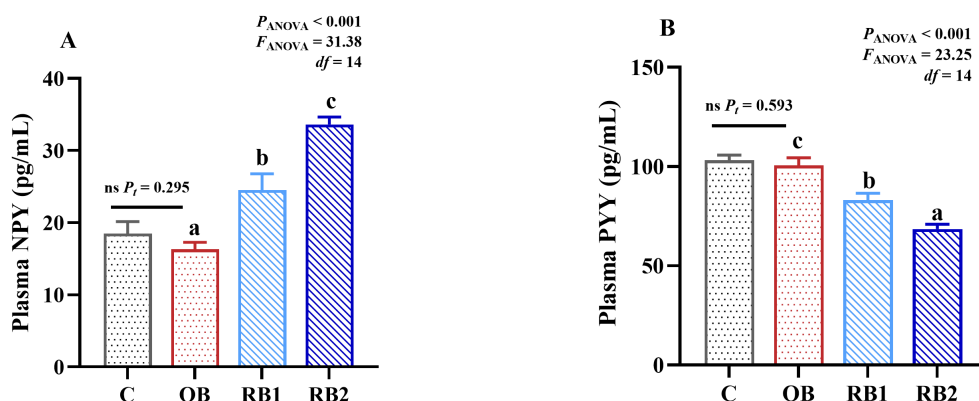


FIGURE 3

Plasma neuropeptide Y (NPY) and peptide YY (PYY) concentrations of blunt snout bream fed different experiment diets. OB, original bacteria—*Bacillus subtilis* CM65; RB, recombinant bacteria—*Bacillus subtilis* CM65-P_{att}'. (A) Plasma NPY (pg/mL). (B) Plasma PYY (pg/mL). ^{ns} $P > 0.05$, compared between the control group and the OB group analyzed by independent-samples *t*-test. ^{abc}compared among the OB group, RB1 group, and RB2 group by using one-way ANOVA. Values in the same line with different superscripts are significantly different ($P < 0.05$).

weight gain of Chinese mitten crab (He et al., 2024). However, the effect of *B. subtilis* CM65-P_{att}' on the FI of blunt snout bream was positive and significant. Firstly, the difference of cumulative FI among all treatments indicated that the superior feeding behavior promoted by the use of *B. subtilis* CM65-P_{att}' was completely apparent in the early stage of the feeding trial. Moreover, as shown by the changes in weekly FI, this beneficial effect did not diminish as the feeding trial progressed, which indicated that the feeding stimulation advantage of *B. subtilis* CM65-P_{att}' was long lasting. These data were also an indication that the P_{att} peptide successfully expressed from *B. subtilis* was functional. With increasing intake, there were bound to be effects on fish growth. Given that the relative molecular mass of P_{att} is only 1,100 Da, it has been proposed that short peptides (180–1,500 Da) could be absorbed directly by the intestine without consuming energy (Guo et al., 2009; Rashidian et al., 2021). Consequently, it is unsurprising that both SGR and WGR increased significantly when the addition of *B. subtilis* CM65-P_{att}' reached 1×10^9 CFU/kg. CF is a common index to represent the wellbeing of fish. However, not only *B. subtilis* CM65 but also the peptide added

had a significant impact on it. Additionally, dietary supplementation with 1×10^9 CFU/kg *B. subtilis* CM65-P_{att}' significantly increased IPE, suggesting that the lipogenic pathway might be activated due to the increase in feed intake (Liu et al., 2024).

In order to further assess the effect of OB and RB on appetite induction, we measured the hormone levels and gene expression of appetite-related factors in blunt snout bream. The results showed that the plasma levels of NPY and PYY had no significant differences between the control and OB groups, suggesting that the addition of OB in diet did not affect the appetite of fish. Notably, both NPY and PYY are widely expressed in the central nervous system and intestine of fish. While NPY is acting as an appetite stimulant, PYY does the opposite. This may also explain why the differences in FI were not significant. The increase in feed intake might be due to the appetite regulatory system activated, which mainly consists of the central nervous system (CNS) (brain) and peripheral organs (intestine). Appetite-stimulating hormones and anorexigenic hormones were transported *via* the blood to regulate the activity of the cells for appetite regulation (Bertucci et al., 2019;

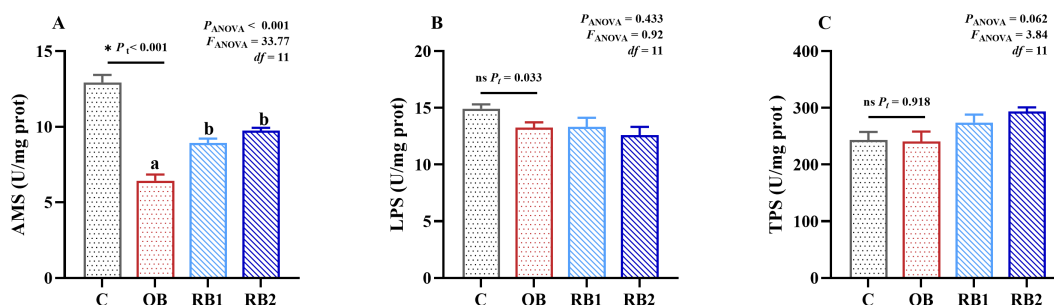


FIGURE 4

Intestinal digestive enzyme activity of blunt snout bream fed different experiment diets. OB, original bacteria—*Bacillus subtilis* CM65; RB, recombinant bacteria—*Bacillus subtilis* CM65-P_{att}'. (A) α-amylase (U/mg prot). (B) Lipase (U/mg prot). (C) total trypsin (U/mg prot). * $P < 0.05$; ^{ns} $P > 0.05$, compared between the control group and the OB group analyzed by independent-samples *t*-test. ^{ab}compared among the OB group, RB1 group, and RB2 group by using one-way ANOVA. Values in the same line with different superscripts are significantly different ($P < 0.05$).

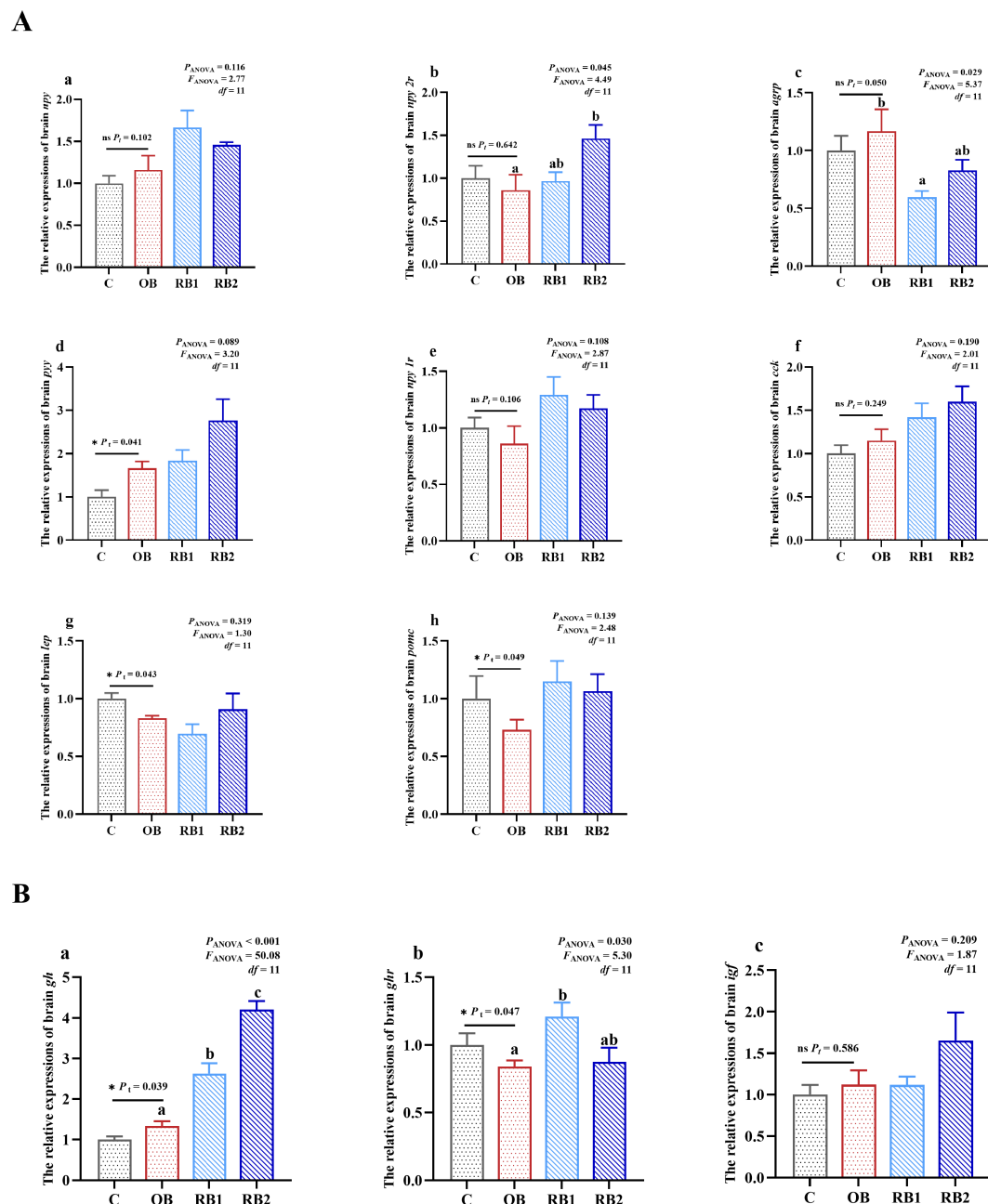


FIGURE 5

Relative mRNA transcriptions of brain appetite-regulated and growth-related genes of blunt snout bream fed different experiment diets. OB, original bacteria—*Bacillus subtilis* CM65; RB, recombinant bacteria—*Bacillus subtilis* CM65-P_{att}'. (A) Relative mRNA transcriptions of brain appetite-regulated genes. Relative transcriptions of *npv* (a), *npr* 2r (b), *agrp* (c), *pyy* (d), *npr* 1r (e), *cck* (f), *lep* (g), and *pomc* (h). (B) Relative mRNA transcriptions of brain GH-IGF axis. Relative transcriptions of *gh* (a), *ghr* (b), and *igf* (c). * $P < 0.05$; $ns P > 0.05$, compared between the control group and the OB group analyzed by independent-samples *t*-test. ^{ab}compared among the OB group, RB1 group, and RB2 group by using one-way ANOVA. Values in the same line with different superscripts are significantly different ($P < 0.05$).

Liang et al., 2019). In addition, the transcription of intestinal appetite stimulant factor *npv* and its receptor *npv* 2r as well as appetite suppressant factor *pyy* (also *pyy* in the brain), respectively, were all significantly increased while remarkably decreasing appetite suppressant factors *pomc* and *lep* of brain and *cck* in the intestine. Although similar transcription trends of *npv* and *pyy* in the intestine may lead to stable intestinal hormone levels, they exert specific action via different NPY Y receptors (Cox et al., 2010) and

NPY binding with NPY 2r, while PYY is binding its cognate receptor NPY 1r (Hyland et al., 2003; Tough et al., 2006). Increased intestinal mRNA expression of the *npv* 2r suggested that NPY played a more efficient and important role in the intestine. Though the transcription of appetite suppressant factors *pomc* and *lep* in the brain was significantly reduced, the expression of *lep* in the intestine was remarkably increased. This is explained by the following two reasons: Firstly, the CNS might not be as sensitive

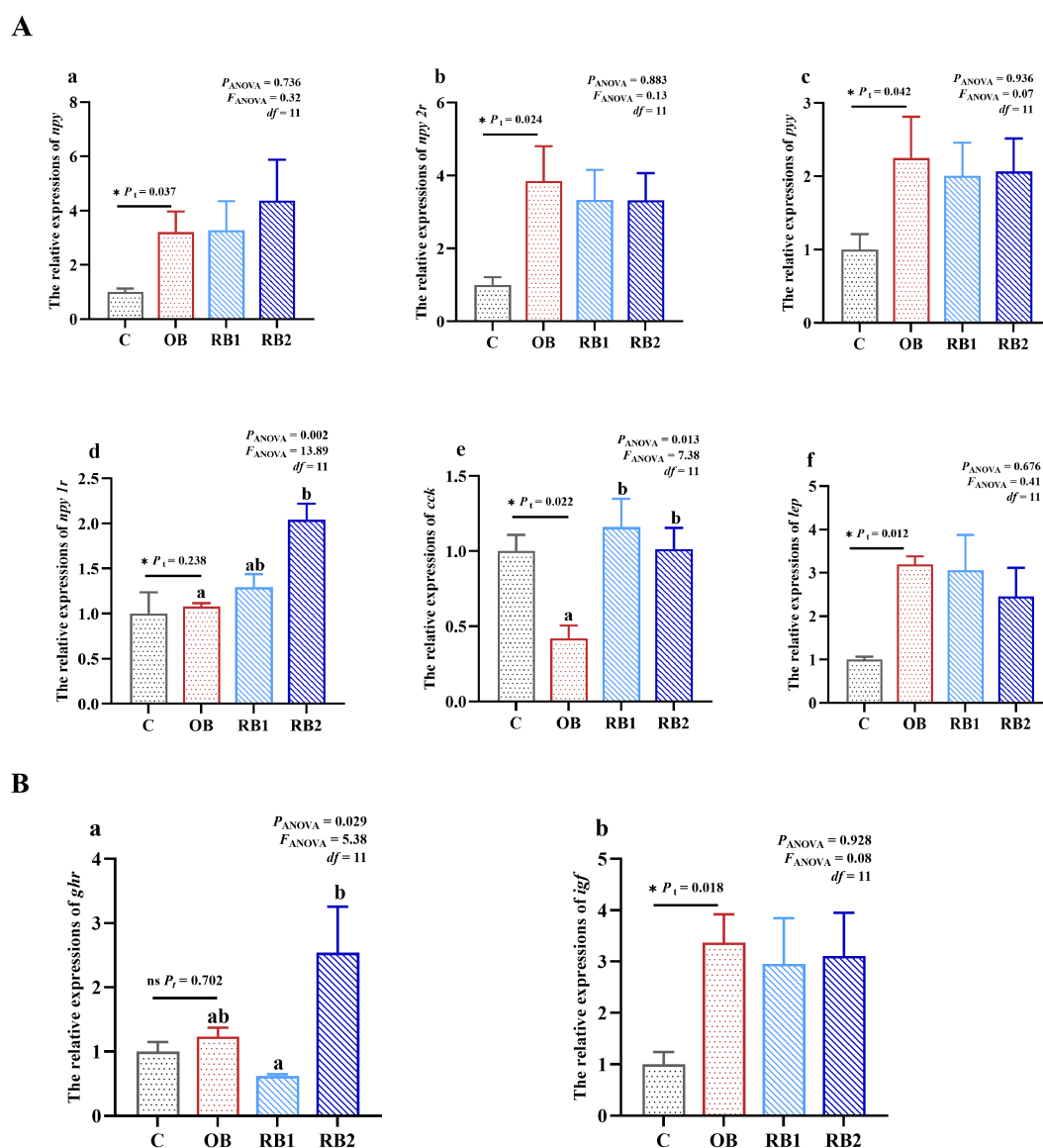
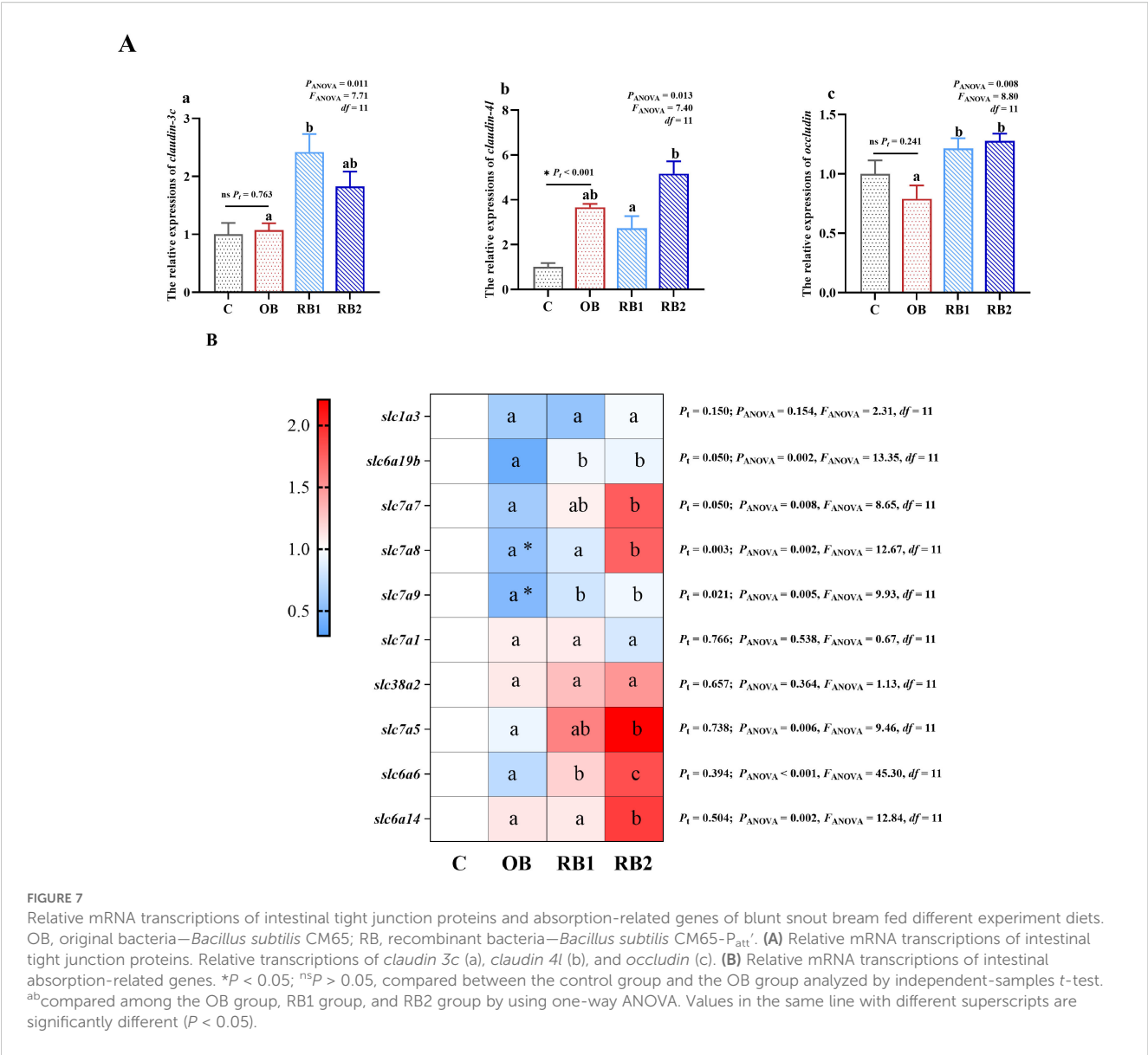


FIGURE 6

Relative mRNA transcriptions of intestinal appetite-regulated and growth-related genes of blunt snout bream fed different experiment diets. OB, original bacteria—*Bacillus subtilis* CM65; RB, recombinant bacteria—*Bacillus subtilis* CM65-P_{att}'. (A) Relative mRNA transcriptions of intestinal appetite-regulated genes. Relative transcriptions of *npv* (a), *npv 2r* (b), *pyy* (c), *npv 1r* (d), *cck* (e), and *lep* (f). (B) Relative mRNA transcriptions of intestinal GH-IGF axis. Relative transcriptions of *ghr* (a) and *igf* (b). $*P < 0.05$; $nsP > 0.05$, compared between the control group and the OB group analyzed by independent-samples t-test. ^{ab}compared among the OB group, RB1 group, and RB2 group by using one-way ANOVA. Values in the same line with different superscripts are significantly different ($P < 0.05$).

as the peripheral appetite regulatory system to the extinction of appetite (Sabioni et al., 2022). Secondly, the retention of incompletely digested food in the intestine may lead to a higher expression of *lep* in the intestine (Huang et al., 2019). However, the hormone levels and the expression of appetite-regulating genes were changed as *B. subtilis* CM65-P_{att}' was added. As the dose rose, the plasma NPY increased significantly while PYY decreased sharply. Plasma hormone levels can roughly reflect the overall levels in the organism, noting that the blunt snout bream of the RB1 and RB2 groups may have been in a chronic state of hyperphagia (Zhang et al., 2022), which led to higher FI compared to the OB group. When *B. subtilis* CM65-P_{att}' was

added at 1×10^8 CFU/kg, the expression of *agrp* in the brain was significantly downgraded during the transcriptional upregulation of *cck* in the intestine. As the supplement was increased to 1×10^9 CFU/kg, there was an upregulation trend of *agrp*, but the expression of *npv 1r* and *cck* were both remarkably higher than in the OB group. These data indicated that the hormone expression was regulated to maintain a stable internal environment. In addition, although the mRNA expression of *npv* (in the brain and intestine) and *igf* (in the intestine) was not significantly upregulated, they were maintained at a high level of expression. The expression of appetite-regulating genes was frequently used to assess the practical effects of novel feed ingredients and feed additives on aquatic animals. It has



been reported that dietary-supplied 6-g/kg cottonseed meal protein can upregulate the transcription of *npv* and *npv r* to promote Chinese mitten crab feeding (Cheng et al., 2019). However, the increased expression of hypothalamus *cck* indicated the negative effect on the consumption by *Salminus brasiliensis* fed vegetable-based diet supplied with 180 g/kg swine liver hydrolysate (Lorenz et al., 2022). In this study, the blunt snout bream dietary supplement of 1×10^9 CFU/kg *B. subtilis* CM65-P_{att}' had the best positive effect on appetite regulation. It might also be the P_{att} peptide functions that lead to homeostasis. Previous research had indicated that small peptides not only have specific flavors themselves but also strengthen the flavor by enhancing or masking the flavor of other substances (Agvei, 2015; Wang and De Mejia, 2005). Small polyglutamic acid peptides can mask the bitter taste present in protein hydrolysates and thus improve the palatability of protein hydrolysates (Ball et al., 2004). Research has shown that adding an appropriate amount of a small peptide to the

diet of Chinese mitten crab can have a better effect on feed attraction (Wang L. et al., 2024). In addition, the relative expression of growth-related genes (GH with its receptor GHR and IGF1) was investigated to further interpret the growth performance results. The GH-IGF1 axis has been demonstrated to play a key role in regulating blunt snout bream growth (Liu et al., 2024). As an important growth promoter, GH binds to its receptor GHR to stimulate the production of IGF1, which, in turn, increases the growth performance of fish (Zhong et al., 2012). In our research, diets supplemented with 1×10^8 CFU/kg *B. subtilis* CM65 remarkably increased the transcription of brain *gh* as well as intestine *igf1*, while the opposite *igf1* expression trends were observed in the brain, which might be caused by the negative feedback regulation that exists between IGF1 and GH (Liu et al., 2024). All of the results above indicated that *B. subtilis* CM65 can activate the GH-IGF1 axis of blunt snout bream, but no significant effect was found on the growth. Furthermore, with the additional

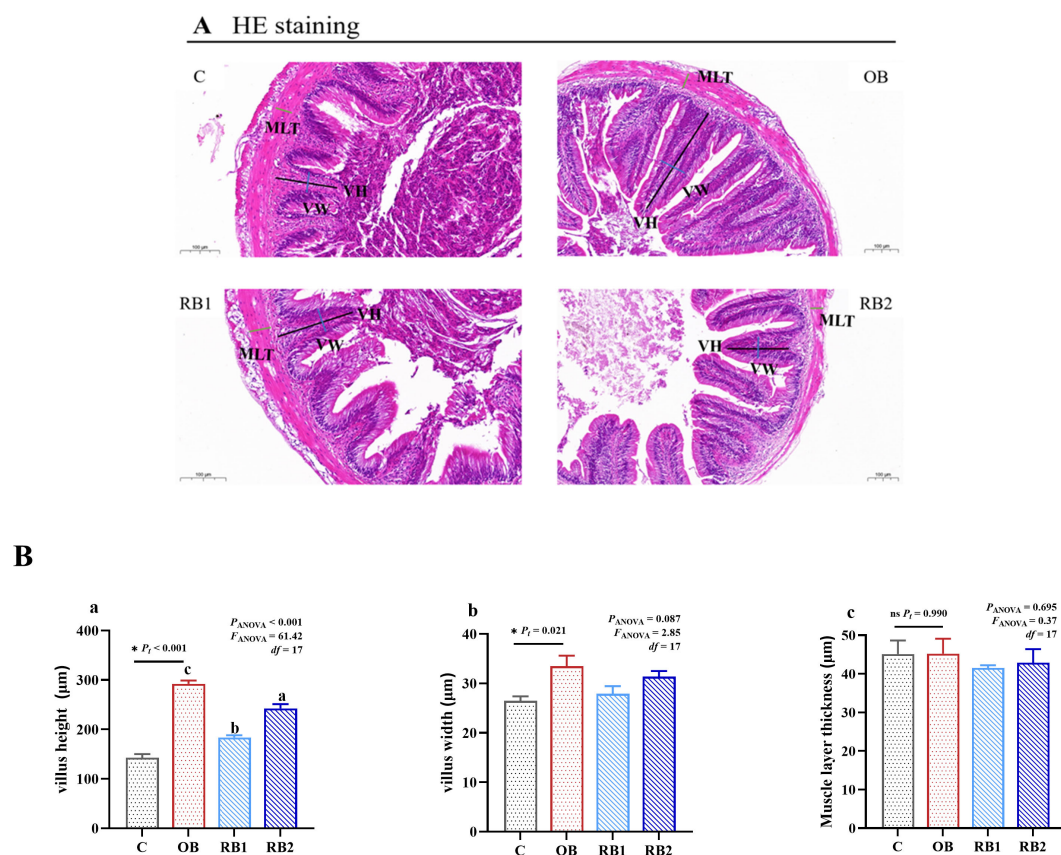


FIGURE 8

Intestinal structure of blunt snout bream fed different experiment diets. OB, original bacteria—*Bacillus subtilis* CM65; RB, recombinant bacteria—*Bacillus subtilis* CM65-P_{att'}. (A) Histological observations of the intestine stained with H&E (magnification $\times 100$; scale bars, 100 μm ; villus height, black line; villus width, blue line; muscular layer thickness, green line). (B) (a) Villus height, (b) villus width, and (c) muscular layer thickness. $*P < 0.05$; $^{ns}P > 0.05$, compared between the control group and the OB group analyzed by independent-samples t-test. ab compared among the OB group, RB1 group, and RB2 group by using one-way ANOVA. Values in the same line with different superscripts are significantly different ($P < 0.05$).

dose of *B. subtilis* CM65-P_{att'}, the expression of brain *gh* kept increasing significantly. It can also remarkably increase the expression of brain *ghr* with the addition of 1×10^8 CFU/kg *B. subtilis* CM65-P_{att'} and of intestinal *ghr* at the dose of 1×10^9 CFU/kg. These indicated that *B. subtilis* CM65-P_{att'} had a more conducive function to promote the growth performance of blunt snout bream than *B. subtilis* CM65, which could also explain the differences in SGR among all treatments.

The increase in feed intake might mainly be regulated by appetite, but it does not mean increased feed intake is positively correlated with growth performance in fish, which also depends on the structural and functional (digest and absorb) integrity of the intestine (Dawood et al., 2021). ISI and ILI, which are commonly presented as proxy for intestinal development (Wen et al., 2014), had no significant changes among OB or RB in this study. To further investigate the integrity of the structure, the transcription of tight junction proteins (Claudin 3c, Occludin, and Claudin 4l), which can maintain the integrity of the intestinal barrier (Li et al., 2023), was investigated. Research has revealed that *B. subtilis* can improve intestinal barrier function by upregulating the expression of *claudin* and *occludin* to promote defensins and mucin formation and regulate the immune function in the intestine (Chang et al.,

2021, 2023; Pothuraju et al., 2021). In this study, *B. subtilis* CM65 can remarkably upregulate the transcription of *claudin-4l*. In addition, with dietary supplement of *B. subtilis* CM65-P_{att'} raised from 1×10^8 CFU/kg to 1×10^9 CFU/kg, the transcription of *claudin-3c* significantly upregulated at first and then decreased, while the opposite was observed in *claudin-4l*. *B. subtilis* CM65 supplementation also had a positive effect on occluding. The results above indicated that *B. subtilis* CM65-P_{att'} (or P_{att}) is more conducive to use to maintain intestinal barrier health than *B. subtilis* CM65.

Digestive efficiency, which can be directly assessed by the activities of digestive enzymes, is a key determinant of growth performance (Javahery et al., 2019). *B. subtilis*, a class of probiotics widely used in aquaculture, has been substantiated to improve the digestive enzyme activities in many aquatic animals like *Catla*, *Pagrus major*, *Litopenaeus vannamei*, and *Oreochromis niloticus* (Bhatnagar and Raparia, 2020; Monier et al., 2023; Wu et al., 2021; Zaineldin et al., 2018). The observed increased digestibility of nutrients may be the result of probiotic supplementation, which increases the availability of nutrients in diets (Mohapatra et al., 2012). However, in this study, neither the addition of *B. subtilis* CM65 nor *B. subtilis* CM65-P_{att'} had an insignificant effect on the

activities of LPS and TBS in blunt snout bream. Moreover, *B. subtilis* CM65 can remarkably decrease AMS activity compared with the control group. Dietary supplied *B. subtilis* CM65-P_{att}' was effective in mitigating the decline in AMS but could not restore the same level of enzyme activity as in the control group. This result may be attributed to the amount of additive and strain specificity of *B. subtilis* (Ghosh, 2025). Moreover, this may explain the reason why there was no difference in WGR and SGR between the control group and OB groups. Diets supplemented with probiotics are easier to digest because probiotics can have the potential to synthesize digestive enzymes (Dawood et al., 2019; Ringo et al., 2020), which indicates that supplemented *B. subtilis* CM65 might act to augment AMS activity after settling in the intestinal tract of blunt snout bream, resulting in a reduction in enzyme activity by the host. Furthermore, the decrease in AMS is evidence of the development of pancreatic exocrine secretion in larval fish (Cai et al., 2015), which indicates that *B. subtilis* CM65 can promote the development of pancreatic exocrine secretion. However, a further in-depth study is needed to elucidate this assumption.

The structural integrity of the intestine is essential for the effective absorption of nutrients. Thus, the intestinal villi are not only closely involved in absorbing nutrients, but they also help to expel harmful microorganisms through their regular movement (Wang et al., 2022). In this study, compared with the control group, the VH and VW of the OB group increased significantly, which indicated that *B. subtilis* CM65 can promote intestinal development. Normally, the length of the intestinal villi indicates the metabolic rate of the intestinal epithelial cells and reflects the secretory function of the intestinal tract of an animal organism (Wang et al., 2018). VH and VW determine the area of contact between the intestinal villi and the chyme. However, when the addition of *B. subtilis* CM65-P_{att}' was 1×10^8 CFU/kg, there was a sharp decline in VH. The decline might be caused by the higher FI, which increased stress on the intestinal tract (always stays overloaded) and, in turn, had a negative effect on the growth of the intestinal villi (Xia et al., 2020). However, with a dose of up to 1×10^9 CFU/kg, this situation was improved somehow. The result was parallel with earlier studies (Chang et al., 2021; Du et al., 2021), indicating an appropriate dose of *B. subtilis* to induce favored intestinal development. In addition, *B. subtilis* CM65-P_{att}' can reduce MLT to a certain extent. As the thickness of the muscle layer decreased, intestinal permeability increased and, in turn, intestinal absorption was enhanced (Cadangin et al., 2024; Hu et al., 2024). This implied that *B. subtilis* CM65-P_{att}' can improve the absorption capacity for blunt snout bream.

The intestinal absorption of amino acids (AAs), which are crucial nutrients, is facilitated by their respective specific transporters (Verri et al., 2017; Wang et al., 2018). In this study, *B. subtilis* CM65 supplementation significantly downgraded the transcription of *slc7a8* (encoding LAT2 protein) and *slc7a9* (encoding a subunit of b^{0,+}AT1 amino acid transporter), which can mainly transform neutral AAs (Yuan et al., 2020). The decreased absorptions of neutral AAs might be a cause of the decrease in WGR. In addition, dietary *B. subtilis* CM65-P_{att}' supplementation was found to increase the transcription of amino

acid transporters to enhance the intestine absorption capacity. The y⁺L transport system, consisting of y⁺LAT1 (encoded by *slc7a7*) and y⁺LAT2 (encoded by *slc7a6*), which can transport cationic and neutral AAs, was activated with the dose of *B. subtilis* CM65-P_{att}' used in this study. In addition, other neutral AA transporters like B⁰AT1 (encoded by *slc6a19b*), LAT1 (encoded by *slc7a5*), and LAT2 as well as cationic AA transporter ATB^{0,+} (encoded by *slc7a6*) were significantly activated when the dose of *B. subtilis* CM65-P_{att}' was 1×10^9 CFU/kg. This also indicated that the bioavailability of nutrients from feed in the RB2 group was increased, thereby promoting fish growth. Although numerous studies indicated that diets supplemented with *B. subtilis* are easier to absorb (Liaquat et al., 2024; Mohammadi et al., 2020; Saravanan et al., 2021), up to now, the specific mechanism on how it promotes the expressions of AA transporters remained unknown. The possibilities are as follows: On the one hand, increased FI could lead to an upregulation of intestinal absorption and thus reach a healthy state for the organism (Sheng et al., 2023). On the other hand, probiotics colonizing the intestine can assist in digestion, leading to an increase in free amino acid contents that, in turn, leads to the increasing mRNA expression of respective transporters (Poncet and Taylor, 2013). Overall, the elevated gene expression observed is partly accounted for by the effective use of protein feed sources as well as the enhanced intestinal absorption capacity.

5 Conclusion

This current study indicated that *B. subtilis* CM65-P_{att}' has the potential to serve as an attractant added to the aquafeed of blunt snout bream. Dietary supplementation of *B. subtilis* CM65-P_{att}' at 1×10^9 CFU/kg was associated with stimulated appetite, increased feed intake, promoted growth, maintained intestinal integrity, and enhanced intestinal functions (digestive and absorptive). Furthermore, after the 10-week feeding trial, this supplementation resulted in a significant 26.59% increase in feed intake and a consequent 17.26% increase in the final body weight of the fish. Those improved capacities observed in blunt snout bream might be attributed to the secret ion of the P_{att} peptide by *B. subtilis* CM65-P_{att}'. P_{att} might activate the NPY-PYY brain–intestine appetite regulatory axis for feeding and the GH–IGF1 axis for growth (based on gene expression data). This study might hold significant importance for developing effective peptide feed attractants for modern intensive aquaculture. However, since the pBE plasmid used in this study carries antibiotic resistance genes (ARGs), the proactive mitigation of ARGs spread is a non-negotiable prerequisite for any consideration of its application in commercial feed.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The animal study was approved by The Animal Care and Use Committee at Nanjing Agriculture University granted permission (license number: SYXK (Su)2021-0086). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XYC: Data curation, Visualization, Investigation, Writing – original draft, Writing – review & editing, Methodology. JZ: Resources, Writing – review & editing, Investigation, Resources. SX: Writing – review & editing, Investigation, Resources. BY: Software, Resources, Writing – review & editing. ZL: Resources, Writing – review & editing, Software. XW: Resources, Software, Writing – review & editing. XT: Writing – review & editing, Resources. XL: Software, Writing – review & editing, Data curation. GJ: Conceptualization, Supervision, Writing – review & editing. WL: Conceptualization, Writing – review & editing, Supervision. XS: Software, Writing – review & editing. XX: Writing – review & editing, Software. XFC: Methodology, Investigation, Writing – review & editing, Visualization, Data curation.

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