

Valuation Bio-Impacts of Different Doses for *Bacillus Subtilis* on Growth Rates, Some Tissues and Genes of the Shrimp *Litopenaeus Vannamei*

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ABSTRACT

In the present study, five various treatment doses of *Bacillus subtilis* (*BS*) were examined associated with the growth rate, some tissues and genes for the *Litopenaeus vannamei* (*L. vannamei*). *BS* was secluded from the intestine of farmed shrimp (*L. vannamei*) and then added to the shrimp's diets in five doses of *BS*. Sixty shrimps were randomly divided into six groups as follows: group 1 ($T0_a$) as a control-farmed group, group 2 ($T1$), group 3 ($T2$), group 4 ($T3$), group 5 ($T4$) and Group 6 ($T5$). After two months of the experimental period, the $T0_b$ group (control-natural group), which contained ten shrimps brought from the Mediterranean Sea, was determined as group 7. At the end of the two months, the growth rates were calculated for the first six groups, and the histological status in hepatopancreas, intestine, muscle, and antenna in addition to the genetics studies were assessed for the seven groups. The group which was treated with $(4/5 \times 10^5$ CFU of *BS*/g) showed the best results in weight gain, survival rate, increase in body length, and enhancement in the tissues of the hepatopancreas, intestine, muscle, antenna and genes of the shrimp. Therefore, this dose is recommended to be used in culturing shrimp in order to increase productivity and contribute to solving the food problem as one of the goals of sustainable development concerning "Egypt Vision 2030".

INTRODUCTION

Litopenaeus vannamei (white legs shrimp) is the most important decapod crustacean for its economic significance regarded worldwide (Burgents *et al.*, 2005; Srinivas *et al.*, 2016). *L. vannamei* is distributed in ocean water where temperatures remain above 20°C such as those of the Pacific coast of Mexico and Central and South America (Phuoc, 2008). Aquaculture is a sustainable option for 11 billion people as it provides food, especially in rural area communities and improve food security, income, and employment rate in aquaculture which represents one of the main axes of Egypt's economic development since Egypt owns long coastlines, an abundance of lakes and the

Nile River. Shrimp aquaculture has become a major activity, accounting for 33% of the total world shrimp production (**Khademzade *et al.*, 2020**).

Some species of the beneficial bacteria are *Lactobacillus acidophilus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus coagulans*, and *Bacillus amyloliquefaciens*. *B. subtilis* is a Gram- positive bacterium with a high importance for bio-studies. It forms spores by sporulation to grow in difficult environments. Spores are metabolically inactive, and they can resist different extreme environments, including high temperatures, toxic chemicals, radiation, extreme pH, desiccation and the absence of nutrients. The bacteria have easy genetic manipulation and a great mass for protein production. Moreover, they can be cultured on a large-scale fermentation. In addition, this bacterium has a lot of industrial applications (**Zhang *et al.*, 2020**).

Bacillus subtilis (*B. subtilis*) used in this experiment has shown improvements in the gut. It enhanced the growth performance by stimulating the activity of the enzymes for digestion (**Mirbakhsh *et al.*, 2021; Cao *et al.*, 2022**). This was proved by studying the histological structures of most beneficial organs in shrimp as hepatopancreas, intestine, muscle and antenna. Hepatopancreas occupies the largest portion of the cephalothorax, it helps in the induction of enzymes for digestion and the intake of nutrients (**Ruiz *et al.*, 2020**). The intestine is located in the organism's abdomen and has a role in nutrition and immunity (**Duan *et al.*, 2019**).

In the current study, the structure of shrimp muscle was examined, being necessary for the support and movement of shrimp, and providing a good source of protein for human. Additionally, the antenna structure was analyzed since it helps in sensation and is considered the best indicator to examine shrimp's health condition. Following the precedures of **Chen *et al.* (2009)**, we confirmed this experiment by studying genetic variability and relationships between seven groups of shrimps by using a start codon targeting (SCoT) and gel electrophoresis technique.

The present study aimed to evaluate the efficiency of the best experimental dose of probiotic *B. Subtilis* in the diets to increase shrimp (*L. vannamei*) flesh at the lowest cost, increase productivity and contribute to solving the food problem as one of the goals of sustainable development "Egypt Vision 2030".

MATERIALS AND METHODS

1. Ethical approval

The present experimental protocol and design were approved by the Research Ethics Committee (REC) in the Suez Canal University (REC183/2022), Egypt.

2. Probiotic *Bacillus subtilis* preparation

The probiotic *B. subtilis* preparation is described in Fig. (1); it was secluded from the intestine of farmed shrimp (*L. vannamei*), then cultivated in the nutrient broth for 2 days at 37°C, diluted by the formula $[N \cdot V \text{ (for standard solution)} = N_1 \cdot V_1 \text{ (for diluted solution)}]$; where N is the concentration and V is the volume. After the serial dilution, we cultured the last solution dilution and stored the plate in the incubator for 2 days. Then, we selected the *B. subtilis* colony and cultured it again using the plate streaking method. After another incubation, we examined it under a microscope to confirm our selection. Afterwards, we cultured it for the last time in nutrient broth. Finally, we prepared our experimental doses using the spectrophotometer (Lambda, EZ201, Germany) according to the standard of 0.5 McFarland at 600 nm. We added our five doses to the diet and dried them with different doses of bacteria very well by using an air blower (TOTAL, TB2066, China). The different dried experimental diets were used for feeding the different experimental groups (Shen *et al.*, 2010).

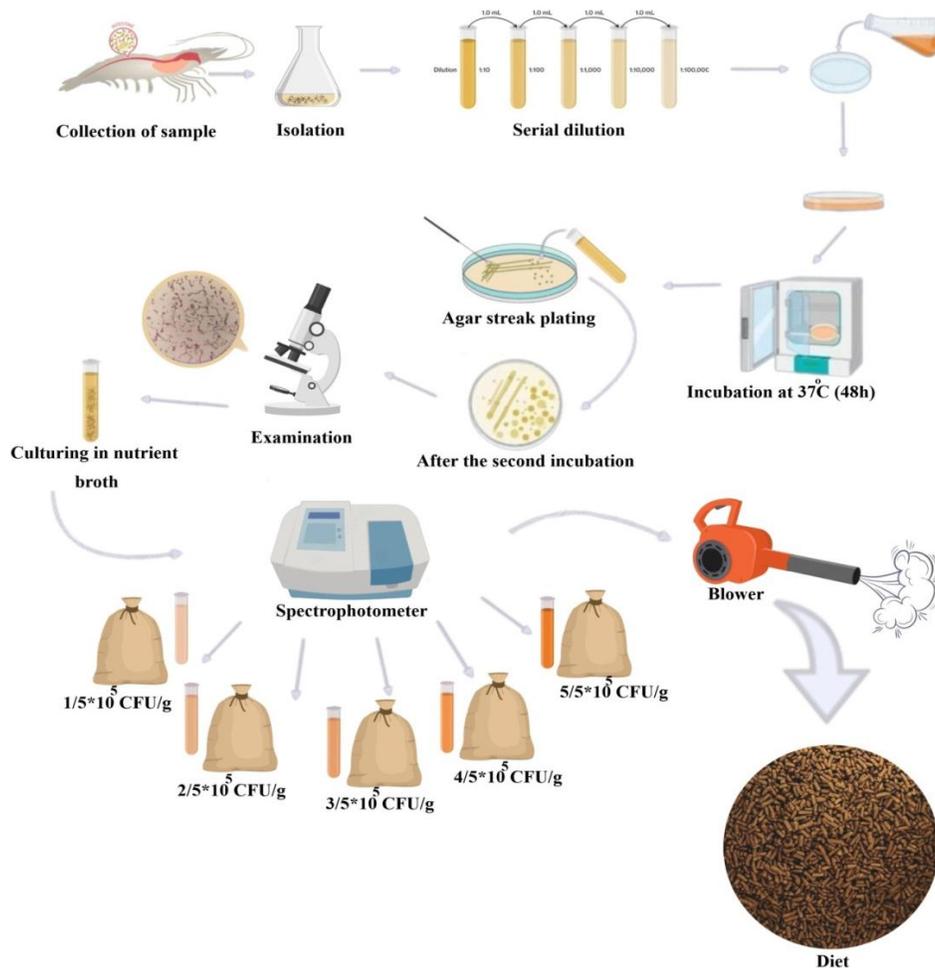


Fig. 1. Design mechanism for the probiotic *B. subtilis* preparation for adding the five doses to the diets for shrimp

3. Sample adaptation

Sixty *Litopenaeus vannamei* were grown in a private El-Ratmah farm for aquaculture in Damietta, Egypt and then transported to the cages in plastic bags filled with water and oxygen. Six hapa nests (1*1*1 m³) (Fig. 2) were divided into randomly six replicate groups (T0_a, T1, T2, T3, T4, and T5). Sixty white leg shrimp (*L. vannamei*) were used for the current experiment, with initial weights between 9.88 & 9.98g, while their lengths varied between 4.50 & 6.00cm. They were stocked in each hapa nest. The preparation of a control diet for shrimp is presented in Table (1).

Table 1. Shrimp diet components with no treatment additives

Component	g/kg diet	Component	g/kg diet
Crude protein	41.00	Shrimp meal	240.00
Crude fat	7.50	Fish meal	240.00
Crude fibre	3.40	Fish oil	50.00
Crude ash	8.10	Soybean meal	110.00
Wheat flour	80.00	Mix vitamins	40.00
Rice bran	80.00	Dry matter	100.00
Total = 1000.00 g/kg diet			
<i>L. vannamei</i> were fed their diet twice a day at 2% of the body weight			

4. Experimental design

Seventy *Litopenaeus vannamei* were randomly divided into seven groups with ten shrimp in each group, as seen in Fig. (2):

Group 1 (T0_a): (Control-farmed group) 10 adult shrimps (*L. vannamei*) feeding a diet without any additions for two months.

Group 2 (T1): 10 shrimps (*L. vannamei*) feeding a diet with the addition of the probiotic *B. subtilis* (1/5*10⁵ CFU/g) for two months of treatment.

Group 3 (T2): 10 shrimps (*L. vannamei*) feeding a diet with the addition of the probiotic *B. subtilis* (2/5*10⁵ CFU/g) for two months of treatment.

Group 4 (T3): 10 shrimps (*L. vannamei*) feeding a diet with the addition of the probiotic *B. subtilis* (3/5*10⁵ CFU/g) for two months of treatment.

Group 5 (T4): 10 shrimps (*L. vannamei*) feeding a diet with the addition of the probiotic *B. subtilis* (4/5*10⁵ CFU/g) for two months of treatment.

Group 6 (T5): 10 shrimps (*L. vannamei*) feeding a diet with the addition of the probiotic *B. subtilis* ($5/5 \times 10^5$ CFU/g) for two months of treatment.

Group 7 (T0_b): (Control-natural group) 10 adult shrimps (*L. vannamei*) obtained from the Mediterranean Sea at the end of two months (the experimental period).

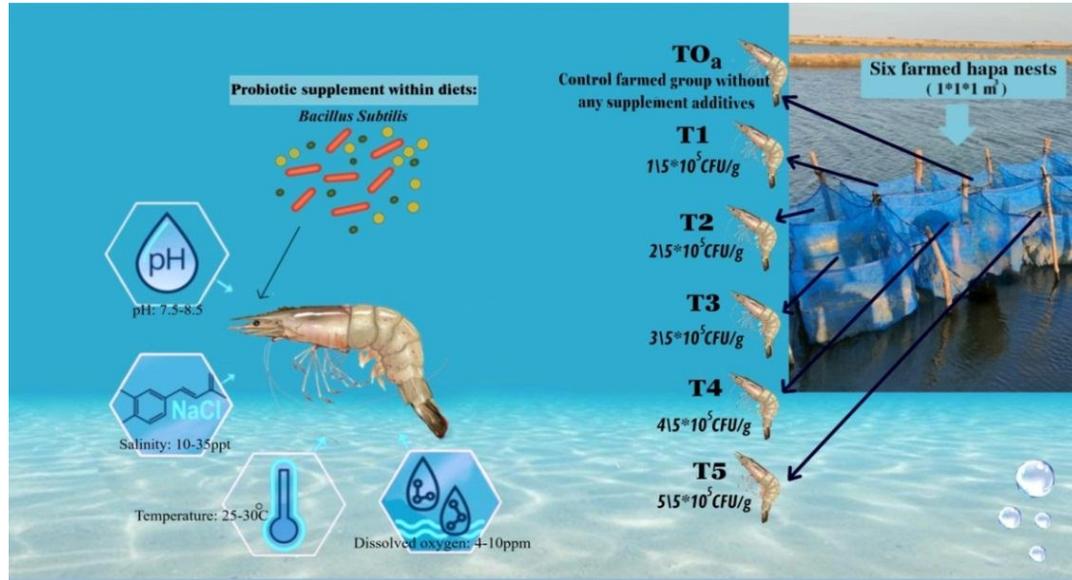


Fig. 2. Design mechanism for sample adaptation of the different experimental groups

During the current experiment (two months), the measurements of certain conditions in all experimental hapa nests were measured. The PH (7.5-8.5) was measured by using the PH device, JENCO 6230, USA; salinity (10-35 ppt) was measured by using the salinometer, JENCO 3250, USA; temperature (25-30°C) was measured by using the temperature device, JENCO 3250, USA, and the dissolved oxygen (4-10 ppm) was measured by the dissolved oxygen device, Pocketester, USA (Fig. 2).

At the end of the two-month experiment, five samples of *L. vannamei* from each hapa nest were randomly collected (Fig. 3). All experimental shrimps were weighed to evaluate the growth parameters. Additionally, shrimp samples of muscles, hepatopancreas, intestine and antennae were gathered for histological examinations; whereas, muscle samples were collected for SCoT analysis.

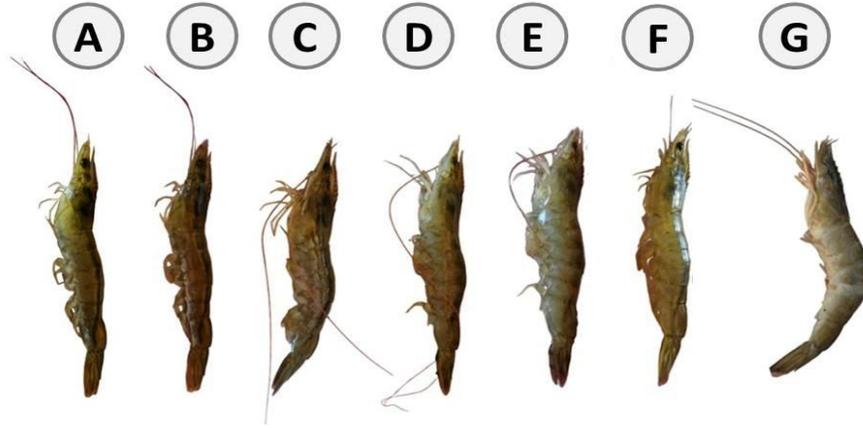


Fig. 3. Photograph of shrimp samples of *L. vannamei* for the different seven experimental groups; A: T0_a, B: T1, C: T2, D: T3, E: T4, F: T5, G: T0_b.

4.1. Growth parameters

The growth performances for shrimps were calculated using the following equations:-

Survival rate (SR) (%) = $100[\text{no. of surviving shrimp} / \text{no. of stock experimental shrimp in each group}]$

Wet weight gain (WWG) (g) = $[\text{final weight(g)} - \text{initial weight(g)}]$

Wet weight gain (WWG) (%) = $100[\text{final weight(g)} - \text{initial weight(g)}] / \text{initial weight(g)}$

Daily weight gain (DWG) (g/fish/day) = $[\text{final weight(g)} - \text{initial weight(g)}] / \text{experimental period (day)}$

Specific growth ratio (SGR) (% per day) = $100[\text{final weight(g)} - \text{initial weight(g)}] / \text{experimental period (day)}$

Food conversion ratio (FCR) (g) = $[\text{total feed given(g)} / \text{wet weight gain(g)}]$

In addition, the following lengths were measured for shrimp: the initial length without a tail (cm), the final length without a tail (cm), the length with a tail (cm), the carapace's initial length (cm), the carapace's final length (cm), the initial length of an antenna (cm) and the final length of an antenna (cm).

4.2. Histological preparations

The shrimp histological samples were put in a tank of neutral formalin (10%) for fixation. After forty eight hours, we washed these samples with distilled water to remove the excess fixative. Then, we dehydrated these samples by using alcohol, cleared them with xylene and embedded the formed tissues in Paraplast wax. The sections (4-5 μ) were cut by the rotatory microtome (Leica, Germany). We stained the prepared slides with

hematoxylin and eosin stains, and then we examined the stained slides under a light microscope (Leica, Germany). Finally, we examined five slides from each group to confirm the observations and used a camera attached to the microscope for analyzing the data of the intestine for villi length and muscular thickness through the camera soft (EUROMEX, CMEX-10PRO, Holland). The three readings were randomly taken for each group.

4.3. Molecular preparations

4.3.1. DNA extraction

According to the protocol of **Porebski *et al.* (1997)**, DNA extraction was carried out with some modifications: A modified CTAB (hexadecyl trimethyl ammonium bromide) procedure was adopted.

4.3.2. DNA concentration

The estimation of genomic DNA concentration was done by comparing the intensity and the size of each current sample band with a DNA ladder. The 2µl of DNA samples were run on 1% agarose gel. To estimate a DNA concentration, there is a need for setting a comparison between the different bands in the DNA size marker and the degree of fluorescence of the DNA.

Table 2. The sequences for six different SCoT primers

Primer name	Sequence
SCoT-1	5'-ACGAC <u>ATGG</u> CGACCACGC-3'
SCoT-2	5'-ACCAT <u>GGCT</u> ACCACCGGC-3'
SCoT-3	5'-ACGAC <u>ATGG</u> CGACCCACA-3'
SCoT-4	5'-ACCAT <u>GGCT</u> ACCACCGCA-3'
SCoT-5	5'-CAAT <u>GGCT</u> ACCACTAGCG-3'
SCoT-6	5'-CAAT <u>GGCT</u> ACCACTACAG-3'

The variability of genes is detected by six SCoT primers in Table (2). According to **Elian *et al.* (2021)**, the amplification reaction was done in 25µl reaction volume, which contains 12.5 µl Master Mix (sigma), 2.5 µl primer (10 pmol), 2.5 µl template DNA (10 ng), and 7.5 µl dH₂O. A Perkin-Elmer/GeneAmp® PCR System 9700 (*PE Applied Biosystems*) that was programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94°C was used in PCR amplification. Each cycle consisted of a denaturation step at 94°C for 45sec., an annealing step at 50°C for 50sec., and an elongation step at 72°C for 1min. The primer extension segment was extended to 7 min at 72°C in the final cycle. The amplification products were resolved by electrophoresis in 1.5% of an agarose gel that contain ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. PCR products

were visualized on UV light and photographed using a System of Gel Documentation (BIO-RAD 2000). For SCoT analysis, only clear and unambiguous bands were visually scored as either present (1) or absent (0) for all samples, and the final data sets included both polymorphic and monomorphic bands. Then, a binary statistic matrix was constructed. Dice's similarity matrix coefficients were then calculated between genotypes using the unweighted pair group method with averages of arithmetic (UPGMA). This matrix was used to build a tree of phylogenetic (dendrogram) performed according to the Euclidean similarity index using the PAST software version 1.91 (Hammer *et al.*, 2001).

5. Statistical analysis

According to Ludbrook (1991), we calculated the bio-results data by the use of the 16.0 version SPSS as the mean \pm SE for the experimental groups, the different letters indicate the different significance for P -value < 0.05 (Duncan multiple comparison analysis).

RESULTS

1. Growth status and shrimp survival

As demonstrated in Table (3), the survival ratio of all treatment groups showed better improvements than the T0_a. The highly significant final shrimp weight was detected in T3 and T4 groups (P -value < 0.01). The significant differences in the wet weight gain (WWG) affected T2, T3, T4 and T5. The daily weight gain (DWG) and specific growth ratio (SGR) were recorded as highly significant in the T3 and T4 experimental treatment groups (P -value < 0.01), while the food conservation rate (FCR) showed its best results in T4 group. The shrimp's final length with tail (FL with tail), and the carapace's final length were highly significant (P -value < 0.01) in the T4 group, followed by the T3 group, compared to the control-farmed group (T0_a).

The survival ratio (SR) (%), initial weight (InW) (g), final weight (FW) (g), wet weight gain (WWG) (g), wet weight gain WWG) (%), daily weight gain (DWG) (g/fish/day), specific growth ratio (SGR) (% per day), food conversion ratio (FCR) (g), initial length without a tail (InL without a tail) (cm), final length without a tail (FL without a tail) (cm), initial length with a tail (InL) (cm), final length with a tail (FL) (cm), the initial length of a carapace (InL carapace) (cm), the final length of a carapace (FL carapace) (cm), the initial length of an antenna (InL antenna) (cm), and the final length of an antenna (FL antenna) (cm) showed significant differences (P < 0.05) among the experimental groups by different superscript letters (Duncan multiple test).

Table 3. Twelve growth parameters of seven experimental groups showing the effect of five doses of *B. subtilis* on the shrimp's healthy

Parameters	Experimental treatment groups					
	T0 _a	T1	T2	T3	T4	T5
SR (%)	60%	70%	80%	80%	90%	80%
InW (g)	9.92±0.03	9.98±0.08	9.97±0.09	9.98±0.09	9.94±0.08	9.88±0.07
FW (g)	19.92±0.26 ^{ab}	18.86±0.45 ^b	19.88±0.23 ^{ab}	20.04±0.46 ^a	20.32±0.30 ^a	19.76±0.36 ^{ab}
WWG (g)	10.00±0.22 ^a	8.90±0.39 ^b	9.91±0.17 ^a	10.16±0.34 ^a	10.48±0.31 ^a	9.88±0.30 ^a
WWG (%)	100.74±1.94 ^a	89.16±3.42 ^b	99.43±1.38 ^a	101.65±2.72 ^a	105.46±2.46 ^a	99.95±2.42 ^a
DWG (g/fish/day)	0.33±0.01 ^a	0.30±0.01 ^b	0.33±0.01 ^a	0.34±0.01 ^a	0.35±0.01 ^a	0.33±0.01 ^a
SGR (% per day)	1.10±0.02 ^a	0.97±0.04 ^b	1.10±0.02 ^a	1.11±0.03 ^a	1.14±0.02 ^a	1.08±0.02 ^a
FCR (g)	2.00±0.05 ^b	2.27±0.11 ^a	2.02±0.04 ^b	1.98±0.07 ^b	1.91±0.06 ^p	2.03±0.06 ^b
InL without tail (cm)	4.50±0.00	4.50±0.00	4.50±0.00	4.50±0.00	4.50±0.00	4.50±0.00
InL with tail (cm)	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00
FL without tail (cm)	12.24±0.20 ^a	12.26±0.32 ^a	12.42±0.21 ^a	12.66±0.21 ^a	12.66±0.24 ^a	12.30±0.21 ^a
FL with tail (cm)	14.40±0.19 ^b	14.70±0.34 ^{ab}	14.80±0.25 ^{ab}	15.10±0.19 ^{ab}	15.30±0.25 ^a	14.60±0.24 ^{ab}
InL carapace (cm)	2.50±0.00	2.50±0.00	2.50±0.00	2.50±0.00	2.50±0.00	2.50±0.00
FL carapace (cm)	4.90±0.19 ^b	5.30±0.30 ^{ab}	5.50±0.35 ^{ab}	6.00±0.22 ^a	6.00±0.22 ^a	5.60±0.24 ^{ab}
InL antenna (cm)	7.00±0.00	7.00±0.00	7.00±0.00	7.00±0.00	7.00±0.00	7.00±0.00
FL antenna (cm)	19.00±0.71 ^a	17.56±0.95 ^a	19.10±0.68 ^a	19.40±0.51 ^a	19.70±0.54 ^a	19.30±0.54 ^a

2. Histological findings

The hepatopancreas structures are shown in Fig. (4). The natural group is ideal in the structure of hepatopancreatic tubules which had tubule lumen, vacuoles, E, B, R, E, and M cells that appeared obviously (Fig. 4G). Hepatopancreatic tubules of the control-farmed group and T1, T2, T3, T4, and T5 groups treated with five doses of *B. subtilis* are explained in Figs. 4B, 4C, 4D and 4F. In contrast, the T4 group showed the best results as as the control-natural group (T0_b) (Fig. 4E).

The intestine layers are displayed in Fig. (5). The control-natural group had the ideal intestine structure composed of 4 main layers: serosa, muscular, submucosa and mucosa (Fig. 5G). Intestinal layers of the control-farmed group and T1, T2, and T3 groups treated with three doses of *B. subtilis* are exhibited in Figs. (5B, C, D), while the group treated with T5 showed the worst results (Fig. 5F). In contrast, the group treated with T4 (Fig. 5E) recorded the best results (it was like the control-natural group T0_b) (Fig. 5G). The better result with a highly significant P -value ≤ 0.001 was recorded in T4 group in terms of the intestinal villi length and intestinal muscular thickness, compared to the other experimental groups such as the control-natural group (T0_b) (Figs. 5H, I).

The skeletal shrimp muscles are shown in Fig. (6). The control-natural group (T0_b) had the ideal structure since it shows collagen bundles with a peripheral elongated nucleus and an appearing sarcoplasm (Fig. 6G). While, the control-farmed group (T0_a) showed abnormal torn bundles with stretched nuclei and most exited in the sarcoplasm (Fig. 6A). After treating the shrimp with *Bacillus subtilis* (T1), we found torn bundles with nuclei in the sarcoplasm but less than the control-farmed group (Fig. 6B). Upon

increasing the treating dose to T2, a slight improvement was detected in the tissue (Fig. 6C). While, by increasing the dose to T3, we found some torn bundles compared to the other treated groups (Fig. 6D). The T4 group (Fig. 6E) demonstrated the best result as the control-natural group (T0_b) (Fig. 6G). Finally, the last treated group with T5 showed tearing in bundles and numerous nuclei in the sarcoplasm (Fig. 6F).

The shrimp antenna transitional tissues are represented in Fig. (7). The ideal natural group (T0_b) had the intact structure of the antenna, as it showed the best nerve cells, epithelial cells, and muscles, and the collagen bundles were attached to the cuticle (Fig. 7G). While, the control-farmed group (T0_a) had separators between the cuticle, muscles and collagen bundles (Fig. 7A). The groups treated with T1, T2, and T3 (Figs. 7B, C, D) were slightly improved, respectively. The group treated with the T4 group illustrated the best results as the muscles and collagen bundles (Fig. 7E) were like the control-nature group (T0_b). Finally, the last treated group with the T5 group showed the worst result as muscles and collagen bundles were highly separated from the cuticle (Fig. 7F).

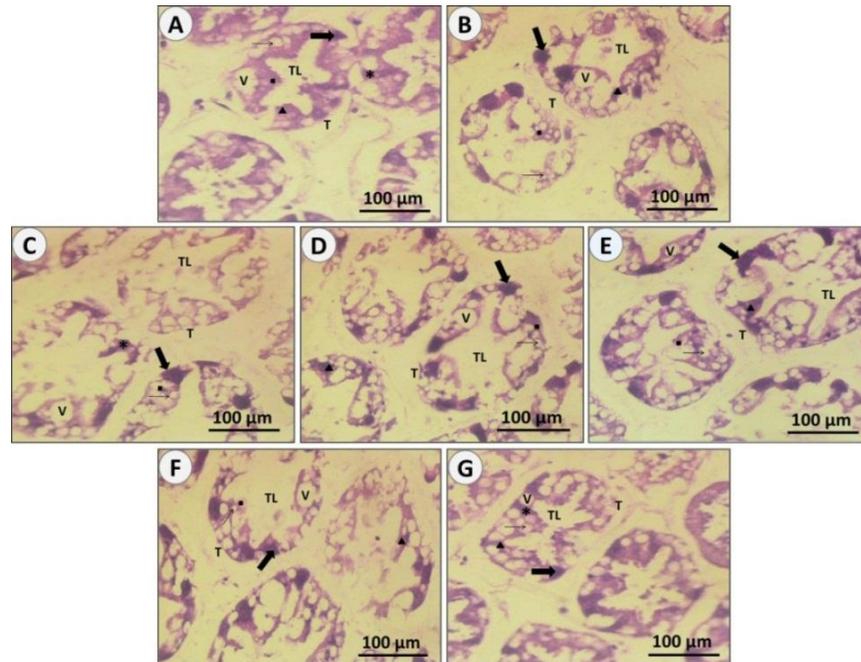


Fig. 4. Hepatopancreas photomicrograph sections of *L. vannamei*. (A): T0_a, (B): T1, (C): T2, (D): T3, (E): T4, (F): T5, and (G): T0_b. V: vacuole, TL: tubule lumen, T: tubule, Asterisk: F-cell, Thin arrow: B-cell, Black square: R-cell, Thick arrow: E-cell, and head arrows: M- cell. [H&E, Scale bar=100μm]

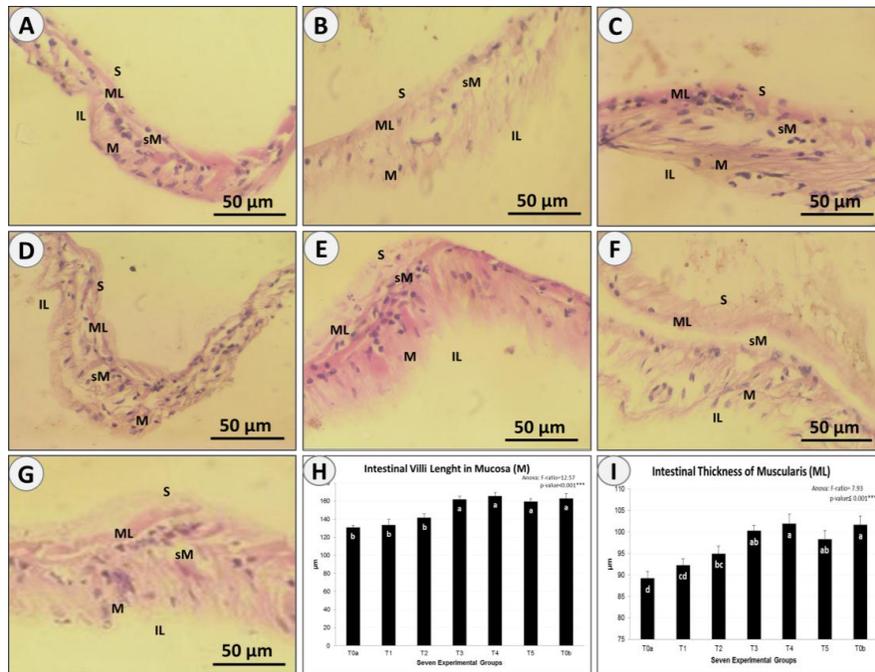


Fig. 5. Intestine photomicrograph of *L. vannamei*. (A): T0_a, (B): T1, (C): T2, (D): T3, (E): T4, (F): T5, and (G): T0_b. S: serosa, ML: muscularis, sM: submucosa, M: mucosa, and IL: intestinal lumen. [H&E, Scale bar=50 μ m]. (H, and I): Bars meaning Mean \pm SE for intestinal villi length and thickness of the muscular layer, the different letters for P -value \leq 0.001 were highly significant in T4, compared to seven experimental groups (Duncan multiple' test).

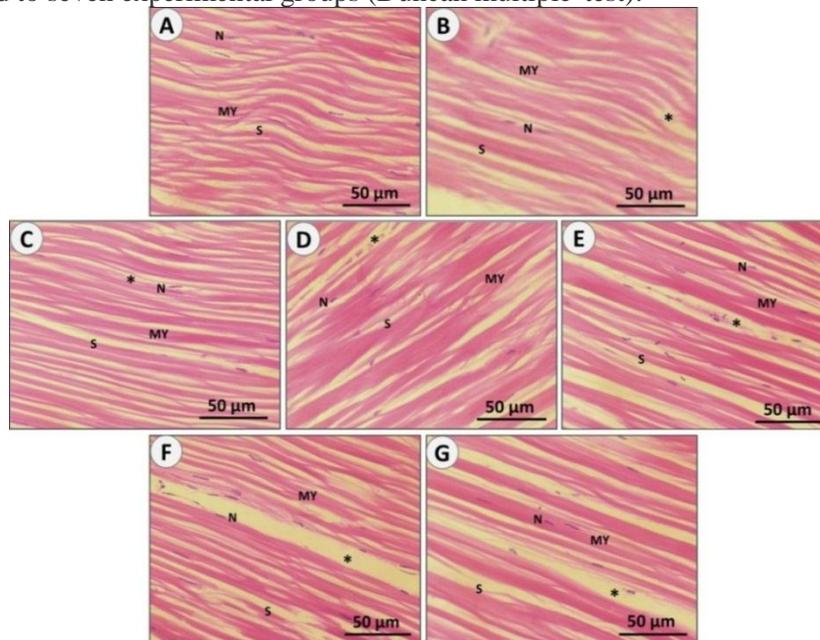


Fig. 6. Muscle photomicrograph longitudinal section of *L. vannamei*. (A): T0_a, (B): T1, (C): T2, (D): T3, (E): T4, (F): T5, and (G): T0_b. N: nucleus, S: sarcoplasm, and MY: myofibril of striated muscle. [H&E, Scale bar=50 μ m]

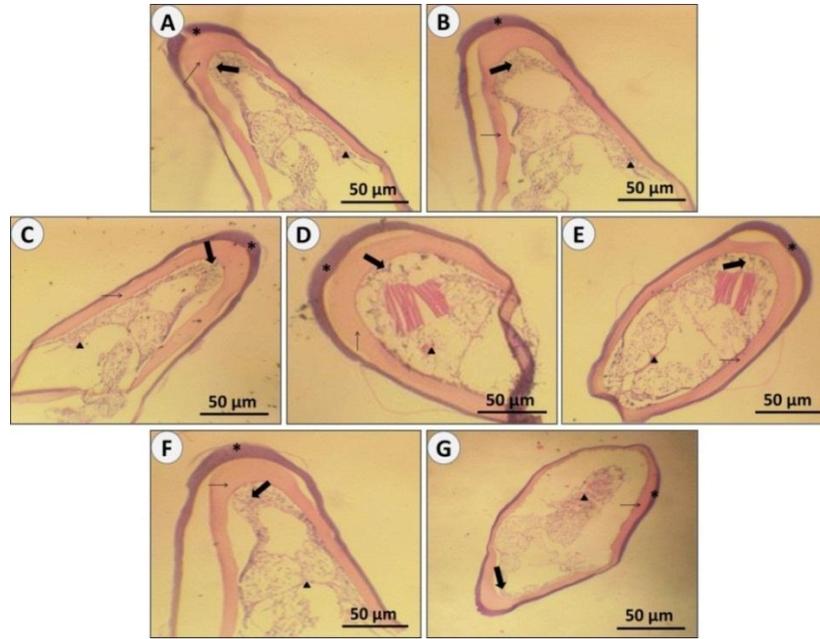


Fig. 7. Antenna photomicrograph transitional sections of *L. vannamei*. (A): T0_a, (B): T1, (C): T2, (D): T3, (E): T4, (F): T5, and (G): T0_b. Asterisk: Cuticle, Thin arrow: Muscle and collagen bundles, Thick arrow: Epithelial cells, and Black triangle: Nerve cells. [H&E, Scale bar=50µm]

3. SCoT findings

We genetically studied *L. vannamei* by using the SCoT-PCR technique as the six SCoT primers to estimate the genetic applications in shrimps under study. The appearance of DNA bands by the six SCoT primers from the genomic DNA of seven experimental shrimp groups was separated using an agarose gel electrophoresis, and it is displayed in Figs. (8, 9) and Tables (4, 5). Finally, the examination of DNA bands was performed by a gene profile computer program.

The significant amplification of all six SCoT primers detected different strong bands and their polymorphism % among the seven experimental shrimp (Fig. 8). The total numbers of amplified bands are 74 bands observed by six SCoT primers (Table 4). The size ranging for the bands amplified from 118 bp in the SCoT-2 to 2001 bp in the SCoT-4. The polymorphism among seven experimental shrimp groups was 65%, as illustrated in Table (4). The similarity for genetics among T0_a and T0_b groups was 76%, among T1 and T0_b was 0.76%, among T2, and T0_b was 81%, among T3, T4 and T0_b was 83%, and among T5 and T0_b was 82%, as demonstrated in Table (5), showing the difference among the seven shrimp groups..

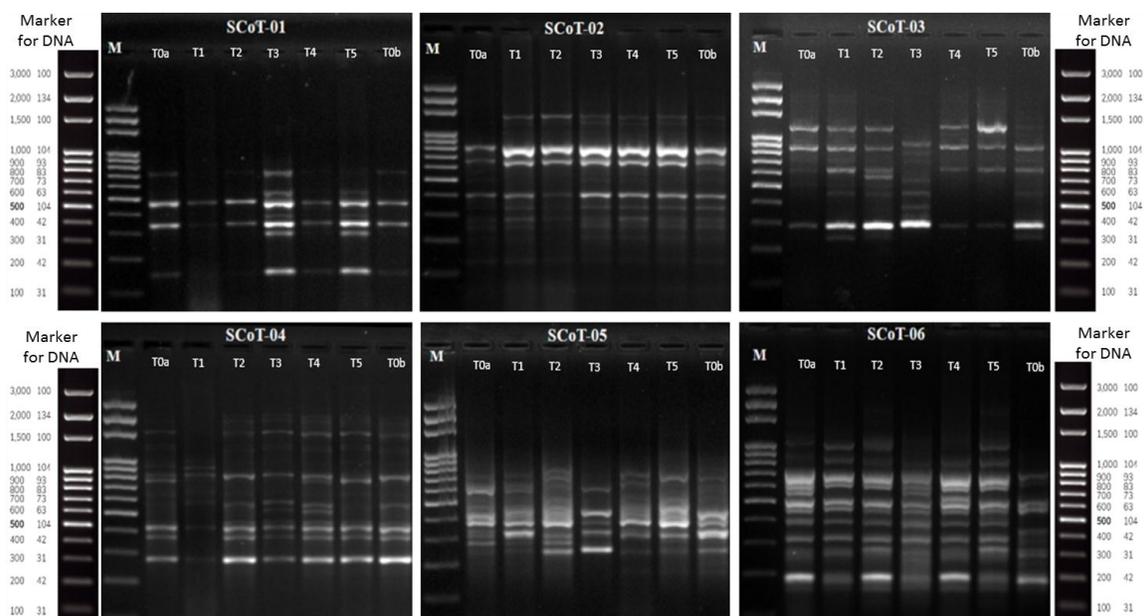


Fig. 8. SCoTs analysis for the seven experimental groups using six different primers for SCoTs: (SCoT-01, SCoT-02, SCoT-03, SCoT-04, SCoT-05, and SCoT-06), and the M means the marker for DNA.

Table 4. Six codes for SCoT primers for seven experimental groups, the total bands' number for amplification, ranging the molecular weights of total molecular bands size by base pair (bp), total numbers of monomorphic bands, total numbers for polymorphic bands without unique bands, total numbers for bands of unique, total numbers for polymorphic bands with unique bands, and the total of polymorphism % for showing the difference among the experimental groups.

Primers code	Amplified bands' numbers							Amplified bands total	Amplified bands size (bp)	Monomorphic Bands' numbers	Polymorphic Bands (without Unique Band), numbers	Unique Bands' numbers	Bands of Polymorphic and Unique together	Polymorphism %
	T0 _a	T1	T2	T3	T4	T5	T0 _b							
SCoT-1	4	3	3	7	6	7	4	7	144 bp - 752 bp	3	4	0	4	57%
SCoT-2	10	10	10	12	12	12	13	13	118 bp - 1430 bp	9	3	1	3	31%
SCoT-3	6	7	8	6	4	4	8	12	211 bp - 1206 bp	1	7	4	7	92%
SCoT-4	8	5	9	11	12	9	9	13	251 bp - 2001 bp	2	11	0	11	85%
SCoT-5	11	9	11	6	10	8	8	13	157 bp - 756 bp	4	9	0	9	69%
SCoT-6	11	11	11	12	10	11	10	16	156 bp - 1045 bp	7	7	2	7	56%
Total	50	45	52	54	54	51	52	74	118 bp - 2001 bp	26	41	7	41	65%

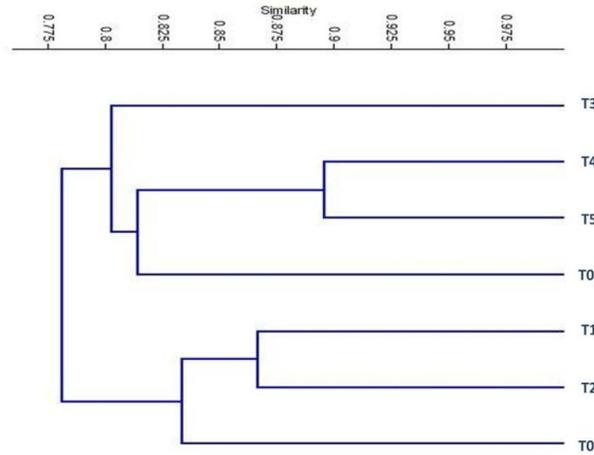


Fig. 9. The dendrogram graph explaining an analysis of the phylogenetic tree for seven experimental groups which was measured by using the molecular six SCoT markers.

Table 5. The average similarities (%) for genetics analyzed by using six SCoT primers for the seven shrimp experimental groups.

	T0_a	T1	T2	T3	T4	T5	T0_b
T0_a	1.00						
T1	0.84	1.00					
T2	0.82	0.87	1.00				
T3	0.75	0.71	0.74	1.00			
T4	0.81	0.77	0.83	0.81	1.00		
T5	0.79	0.75	0.85	0.78	0.90	1.00	
T0_b	0.76	0.76	0.81	0.83	0.83	0.82	1.00

The similarities of genetics for the seven experimental shrimp groups; T0_a: control-farmed group, T1, T2, T3, T4, and T5: different treated groups exposed to five doses of *B. subtilis* in diets, and T0_b: control-natural group.

DISCUSSION

Aquatic probiotics means microorganisms that are provided to aquatic organisms that help them survive underwater and have beneficial effects, including the improvement of growth rates and immune responses against various diseases (**Javanmardi *et al.*, 2020**). The probiotic *B. Subtilis* plays an animated role in the eco-friendly aquaculture factor for the elaboration of the growth rates as it increases the survival rate and improves the mechanism of digestive enzyme, absorption and immune responses in fish and shrimp (**Zhou *et al.*, 2009**; **Nimrat *et al.*, 2012**; **Sun *et al.*, 2018**; **Jjx *et al.*, 2019**). The current experiment coincides with that of **Mirbakhsh *et al.* (2021)**, who reported the

impact of a certain strain of *B. subtilis* on the growth changes in all treatment groups of *L. vannamei*, but they deduced that 10^8 CFU/ml is the positively affected group in the growth performance. Additionally, **Truong et al. (2021)** detected the impact of *B. subtilis* on *L. vannamei* and demonstrated the increase in the growth rate caused by the addition of *B. subtilis*, and the greatest effect was shown in 10^4 CFU/ml. Moreover, **Cao et al. (2022)** illuminated the impact of *B. subtilis* on the growth rates of *Penaeus vannamei* and showed that, the additional mixture of 0.5% *B. subtilis* and yeast had the best effect, followed by the group exposed to 0.5% *B. subtilis*, as it improved the digestion reaction which, in turn, promoted the excretion of enzymes for digestion, increased the growth rates by enhancing wet weight gain and specific growth ratio results. In this respect, **Azhdari et al. (2022)** explained the impacts of a mixture (3×10^6 CFU of *B. subtilis*, and 10^5 CFU of *Lactobacillus plantarum*) of probiotics per gram in the exchanged water in which the *L. vannamei* lived, and the authors recorded the highest weight. Therefore, the addition of *B. subtilis* in the current study promoted the increase in the growth rates of *L. vannamei*, especially the T4 group which was fed with a probiotic *B. subtilis* as a supplement with a shrimp aquaculture diet ($4/5 \times 10^5$ CFU/g), aligned with a better observed significant amelioration ($P < 0.05$).

The invertebrates are dependent on their immunity mechanism and resistance against the pathogenic organisms on innate or non-adaptive immunity, and by interacting with probiotics, they increase their immune responses efficiency by producing the molecule signals transduction to the pathogens (**Rengpipat et al., 2000**). The hepatopancreas of crustaceans plays an animated role in their digestion mechanism, such as improving nutrient absorption, metabolism, energy production for organ growth and further improvement of immune responses (**Ruiz et al., 2020; Zhu et al., 2021**). The invertebrate intestine plays an important role in nutrition mechanism and immunity (**Duan et al., 2019**). While, the skeletal muscle of shrimp is necessary for support, and movement and provides the human with a good source of protein. **Panini et al. (2017)** postulated that, the muscle mass of shrimp contains high protein myofibrils, and it is interspersed with connective tissues. On the other hand, the antenna helps in sensation and is considered the best indicator to examine shrimp's health condition in its aqua-environment. It was reported that, the histological structure improved as a result of adding *B. subtilis* to *L. vannamei* (**Jian et al., 2012; Jjx et al., 2019**). The best results of probiotics are in the digestive system in the larva stage of *Penaeus vannamei* (**Wang, 2007**). The intestinal flora of fish can be regulated by various types of gastric Bacillus, as well as helping them to grow for other beneficial bacteria, while, at the same time, working to inhibit some other types of dangerous bacteria (**Sun et al., 2011**). Many studies have confirmed that probiotics added to the diets of aquatic species help in altering and preventing diseases by improving the immune mechanisms and histological structures (**Chiu et al., 2007; Wang, 2007; Zhang et al., 2009; Ai et al., 2011; Liu et al., 2014**). **Cao et al. (2022)** showed the better effects of 5% of *B. subtilis* added to the diet

on hepatopancreas and dried protein content in tissues of *Penaeus vannamei* muscle. **Won *et al.* (2020)** demonstrated the effects of adding a group of 10^8 CFU of *B. subtilis* /g to a shrimp diet on the *L. vannamei* and detected an improvement in the growth rates, antibiotic agent activity of enzymes for digestion and in the defense against diseases as well as an enhancement in the health of gut architecture tissues ($P < 0.05$), evidenced with the highly significant villi height of mucosal intestine, and the intestinal muscular layer thickness. **Zheng *et al.* (2018)** explained that, the probiotic addition improved the intestinal length of the villi and the thickness of an intestinal muscular layer, thus leading to an enhancement in the absorption capacity of the shrimp intestine. Therefore, previous studies are consistent with the impact of the present study in improving histological structures in the intestine, muscles and antenna due to the addition of a probiotic *B. subtilis* to the diet of shrimps, especially in the T4 group which was exposed to $4/5 \times 10^5$ CFU of a probiotic *B. subtilis* as a supplement with a shrimp aquaculture diets.

Various studies have detected the beneficial impacts of probiotics in the diets of aquatic species on gene expression (**Chiu *et al.*, 2007; Mirbakhsh *et al.*, 2021**). In this context, **Won *et al.* (2020)** illustrated the impact of *B. subtilis* on the immune genes, showing a high increase in shrimp-fed on 10^8 of *B. subtilis*, *Pediococcus pentosaceus*, and *Lactococcus lactis*, respectively. Numerous studies agree that using SCoT is the best genetic analysis technique since it shows good results and it is easy to get valuable data. The SCoT meaning a Start Codon Targeting is repeatable in the exiting of the translated starting code which is ATG (**Collard and Mackill, 2009**). The SCoT analysis technique is important and successful in making comparisons among various species and is widely used in the field of plants (**Cabo *et al.*, 2014; Que *et al.*, 2014; Vivodík *et al.*, 2016**). However, during our research for data, we did not find any study related to polymorphism using the SCoT analysis technique on the shrimp tissues for comparison study, at least in Egypt. Whereas, the SCoT analysis technique is studied more in plant tissues, and recently some studies inserted using this technique for fish tissues. In the current experiment, it was important to use the SCoT analysis technique to estimate its effectiveness among shrimp tissues in the present experimental groups. In the studies of **Etminan *et al.* (2016)** and **Etminan *et al.* (2018)**, the authors assessed that the power of discrimination of the SCoT analysis technique is higher when distinguishing between species that are close to each other. The study of the molecular composition of aquatic species is important and useful in estimating the stocks, determining and improving breeding programs according to available stocks, managing sustainable crops, and preserving genetic biodiversity (**Dinesh *et al.*, 1993; Tassanakajon *et al.*, 1998**).

In the current study, we detected different strong bands and their polymorphism % is 65% among the seven experimental shrimps due to the addition of a probiotic *B. subtilis* to shrimp diets, especially in the T3 and T4 groups (83%), compared to T0_b.

CONCLUSION

Isolation of bacteria was successfully done from the intestine of *L. vannamei* which enabled us to get five doses from *B. subtilis*, and the dose of T4 ($4/5 \times 10^5$ CFU/g) showed the best impact on the growth parameters for experimental shrimp. This was confirmed in hepatopancreas, intestine, muscles, and antenna tissues since they are similar to those of the control-natural group (T0_b). In addition, this outcome was proved by using the SCoT-PCR primers analyses.

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