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Bacillus subtilis K-C3 as Potential Starter to Improve Nutritional Components and Quality of Shrimp Paste and Corresponding Changes during Storage at Two Alternative Temperatures

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Abstract: This study aimed to evaluate *Bacillus subtilis* K-C3 as a potential starter to improve shrimp paste quality, particularly in terms of nutritional profiles. The quality/characteristic changes of shrimp paste with and without inoculation during storage for 18 months when stored at low (4 °C) and room (28 °C) temperature were also investigated. The results found that this *B.* strain increased essential amino acids (EAAs) and polyunsaturated fatty acids (PUFAs), as well as antioxidant properties including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging activities, ferric reducing antioxidant power (FRAP) and metal chelating activity in the experimental shrimp paste compared to traditional shrimp paste ($p < 0.05$). The faster development of some characteristics of inoculated samples were also noted, as indicated by the higher total viable count (TVC), formal and amino nitrogen content, pH, and browning index, as well as biogenic amines, indicating different quality which may be further responsible for different product acceptability. The changes in quality/characteristics of shrimp paste were observed throughout the 18 months of storage. Shrimp paste stored at room temperature accelerated those changes faster than samples stored at low temperature ($p < 0.05$); however, the quality of them still meets the product's standard even storage for 18 months. Meanwhile, shrimp paste stored at a low temperature had an amount of yeast and mold over the limitation (>3.00 log CFU/g), indicating food spoilage. Thus, storage at room temperature can extend this product's shelf-life better than storage at low temperature. Overall, inoculation with *B. subtilis* K-C3, in conjunction with storage at room temperature, resulted in quality improvement and maintenance in shrimp paste, particularly in the aspects of nutritional profiles and safety concern, as the shrimp paste should have a shelf-life of at least 18 months.

Keywords: shrimp paste; starter culture; *Bacillus subtilis*; fermented food; inoculation

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1. Introduction

Shrimp paste is a traditional fermented food consumed as a condiment to enhance palatability in many Eastern and Southeast-Asian dishes. It is made by mixing small shrimp with salt, and the shrimp are then allowed to ferment in anaerobic conditions for 1 month or until the typical aroma is generated [1]. During processing/fermentation, proteolysis and lipolysis occur due to the activities of endogenous enzymes or microorganisms (bacteria, yeast, and mold), converting raw materials into products such as peptides, amino acids, aldehydes, organic acids, or amines, which are responsible for final product characteristics [2]. Shrimp paste is a good source of protein, polyunsaturated fatty acids

(PUFAs), peptides, and amino acids; the paste also possesses some biological activities such as antioxidant, antibacterial, ACE inhibitory activities, etc [3–5]. Interestingly, to date, little information regarding how those compositions and biological activities change during storage has been reported. This research gap, coupled with alteration by inoculation and temperature storage, is interesting to investigate.

In general, the traditional process of making shrimp paste is allowing the desirable, naturally occurring microorganisms to grow preferentially under controlled processes/conditions. Alternatively, a controlled process, in which isolated and characterized microorganisms are used as starter culture, is a potential approach to shorter fermentation time, as it improves certain product characteristics and guarantees the product safety as reported by many previous studies [6–8]. Recently, our previous research found that *Bacillus subtilis* K-C3, which is isolated from commercial Thai shrimp paste, has great potential for use as an inoculum for this product. This strain can survive and exhibit proteolytic, lipolytic, and chitinolytic activities throughout processing, which can accelerate the fermentation rate and govern some desirable characteristics, compared with the typical process [9,10]. However, the comparison of biological activities or compositions related to human health between inoculated and traditional products, as well as their changes during storage, has not been studied.

During storage, proteolysis and lipolysis—as well as other reactions, i.e., lipid oxidation or microbial metabolic activity—continuously occur, leading to the reduced quality of the product when the storage time is extended [11,12]. Our most recent prior research found that shrimp paste quality was continuously further governed during storage and exhibited the highest acceptability at 6 months (as indicated by the highest sensory scores) when stored at room temperature [12]. This indicated the continuous fermentation/degradation of the components during storage of this product. This leads to the hypothesis that there might be some differences in quality changes during storage between inoculated and traditional products. In general, there are many factors affecting the rate of alteration during storage, such as processing hygiene, microbial loads, type of packaging, and food storage method [13]. Among all, storage temperature is one of the most crucial parameters that leads to quality changes during storage. Nowadays, in most cases, shrimp paste is generally not labeled with a suggested shelf-life and storage condition. It is sometimes stored at room temperature or at refrigerated temperature until the paste is completely consumed. This study, therefore, aimed to determine how inoculation with *B. subtilis* K-C3 and storage temperature (4 and 28 °C) impact the composition changes of shrimp paste, particularly in the aspect of nutritional components, during storage for 18 months. The obtained knowledge will be beneficial for either the processor or consumer to produce and store shrimp paste of prime quality, particularly in the aspects of health benefits.

2. Materials and Methods

2.1. Preparation of Starter Culture

Bacillus subtilis K-C3 was isolated from commercial shrimp paste (Rayong, Thailand) from March (2018) and kept as lyophilized culture (−80 °C) until use. A loopful of this strain was cultured in 50 mL of sterile nutrient broth (Merck, Darmstadt, Germany), then it was incubated on a shaking incubator (180 rpm) at 37.5 °C for 48 h under aerobic conditions. The bacterial density for inoculation of shrimp paste was adjusted to 10^8 CFU/mL and used as inoculum.

2.2. Preparation of Shrimp Paste

Fresh shrimp (*Acetes vulgaris*) was mixed with salt at 5:1 (*w/w*) overnight. Salted shrimp was drained and ground using a blender (National, Tokyo, Japan) for homogeneity, then it was allowed to sun-dry until the moisture content decreased to 35–40% [14]. Subsequently, the inoculum was added to obtain the final cell concentrations of 10^4 CFU/g sample and was mixed thoroughly (Inoc). Dried shrimp without inoculum was prepared in parallel (Con). Thereafter, both with/without inoculated shrimp were compacted into earthen jars,

covered with cheesecloth, and allowed to ferment at room temperature (28–30 °C). After 30 days of fermentation, samples, which are ready for consumption, were collected for analyses and referred as day 0 (before storage).

To monitor the quality changes during storage, shrimp paste with and without inoculum was transferred into polypropylene container, which is the most traditional popular packaging used for this product, then covered with plastic lid. Samples were kept at 4 °C (low temperature) (Inoc-L, Con-L) and 28–30 °C (room temperature) (Inoc-R, Con-R) for 18 months. During storage, samples were periodically taken every 6 weeks to analyze the changes in microbial population, physicochemical, and antioxidant properties. The quantities of free amino acid contents, fatty acid profiles, and biogenic amines (BAs) of samples were comparatively measured only on day 0 and in the 12th month of storage.

2.3. Determination of Microbial Population

The amount of total viable count (TVC), lactic acid bacteria (LAB), and yeast and mold count were determined by using Plate count agar (PCA) (Difco); de Man, Rogosa, and Sharpe agar (MRS) (Sigma); and Potato dextrose agar (PDA) (Difco), respectively, as per the method of BAM [15]. An amount of 90 mL of shrimp paste (10 g) was mixed with 0.1% peptone water containing 10% NaCl using a Stomacher 400 lab blender (Seward Ltd., Worthing, UK) at high speed for 3 min. Then, samples were appropriately diluted in serial 10-fold steps and were cultivated in each specific media and incubated following the procedure of individuals. The microbial population was counted and reported as a colony forming unit/g (CFU/g) sample.

2.4. Physicochemical Measurements

Water activity (a_w) of samples was measured using a water activity analyzer (Thermoconstanter, Novasina, Switzerland). The pH of samples (which were prepared in a 1:10 ratio of samples:distilled water) was determined using a pH meter (Sartorius, Gottingen, Germany). Formal nitrogen, ammonia nitrogen, and amino nitrogen contents were investigated by the titration method followed the method of Thai Industrial Standard [16] and expressed as mg N/ g sample. For the measurement of the browning index (A_{420}), samples (1 g) were homogenized with 25 mL of distilled water. The mixture was centrifuged (Beckman Coulter, Avanti J-E Centrifuge, Palo Alto, CA) at 10,000×g for 15 min, then the absorbance of supernatant was measured at 420 nm using the UV-1601 spectrometer (Shimadzu, Kyoto, Japan) [10]. The thiobarbituric acid reactive substances (TBARS) value was measured by using 0–2 ppm of malonaldehyde bis (dimethyl acetal) for standard curve and was reported as a mg malonaldehyde (MDA)/kg sample as per the method of Pongsetkul et al. [1].

2.5. Determination of Antioxidant Activities

Water extracts of shrimp paste were prepared via the same method of the measurement of the browning index (as described above). The extracts were approximately diluted with distilled water prior to assay. Then, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging activities, as well as ferric reducing antioxidant power (FRAP), were evaluated using 50–600 µM Trolox as standard and were expressed as µmol Trolox equivalents (TE)/g samples, whereas metal chelating activity was evaluated using 0–30 µM EDTA as standard and expressed as a µmol EDTA equivalent (EE)/g sample, as per the method of Faithong and Benjakul [17].

2.6. Determination of Free Amino Acid Composition

In brief, samples (25 g) were extracted using 6% $HClO_4$ (*v/v*), neutralized using 0.1 NaOH, and then filtrated. The filtrates were used to determine their free amino acid composition using an amino acid analysis system (Prominence, Shimadzu, Kyoto, Japan) equipped with a column (Shim-pack Amino-Li, 100 mm 9 6.0 mm i.d.; column temperature, 39 °C, Shimadzu) and pre-column (Shim-pack ISC-30/S0504 Li, 150 mm 9 4.0 mm i.d.,

Shimadzu). Free amino acids were identified using a fluorescence detector (RF-10AXL; Shimadzu) and reported in terms of a mg/g sample [18].

2.7. Determination of Fatty Acid Profiles

Lipids of samples were extracted by the Bligh and Dyer method [19], then converted into fatty acid metyl esters (FAMEs) as per the method of Saito et al. [20]. Subsequently, FAMEs were analyzed using a gas chromatography system (Hewlett-Packard 7890A; Agilent Technologies, Santa Clara, CA, USA) fitted with a capillary column (SP 2560, Supelco Inc., Bellefonte, PA, USA, 100 m × 0.25 mm i.d., 0.20-μm film thickness) and a flame ionization detector according to the conditions described by Metcalfe et al. [21]. The FA profile of samples was expressed as g/100 g total FA.

2.8. Determination of Biogenic Amines (BAs)

BAs including tryptamine (Try), β-phenethylamine (Phe), putrescine (Put), cadaverine (Cad), histamine (His), tyramine (Tyr), spermine (Spm), and spermidine (Spd) were measured as per the method of Sang et al. [22] with slight modification. Briefly, samples were extracted using 10% trichloroacetic acid (TCA) and adjusted to the final volume of 50 mL. Then, the extract (1 mL) was mixed with 200 μL of 2 M NaOH, 300 μL of saturated $NaHCO_3$, and 2 mL of 10 mg/mL dansyl chloride, followed by incubation at 45 °C, 40 min. After that, the mixture was centrifuged, filtered, and applied to HPLC analysis using an Alliance 2690 HPLC unit (Agilent, Palo Alto, CA, USA), which consisted of a Sulfire-C18 column (4.6 × 200 mm, 5 μm) coupled to a quaternary pump and a diode array detector at the wavelength of 254 nm. BAs were reported as mg/kg sample.

2.9. Statistical Analysis

All experiments were run in triplicate and expressed as mean ± standard deviation (SD). Data were analyzed by using two-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) at the significance level of 95% ($p < 0.05$). All statistical analyses were performed using SPSS version 25 (IBM Corp. USA).

3. Results and Discussion

3.1. Microbial Population

Figure 1 shows the amount of TVC, LAB, and yeast and mold evaluated in shrimp pastes during storage for 18 months. At day 0 (before storage), TVC of the inoculated sample (5.93 log CFU/g) was significantly higher than the sample without inoculation (4.70 log CFU/g) ($p < 0.05$), whereas there was no significant difference in LAB and yeast and mold contents between samples with/without inoculation, accounting for 1.73–1.86 and 0.40–0.62 log CFU/g, respectively ($p > 0.05$). As shown in Figure 1a, the TVC of all samples increased when stored for 6 months ($p < 0.05$), then remained constant when the storage time was extended up to 18 months ($p > 0.05$). At 18 months of storage, all samples contained a comparable amount of TVC, which was in the range of 7.69–8.02 CFU/g, suggesting that storage temperature as well as inoculation with *B. subtilis* K-C3 strain did not affect to the total bacteria of samples, particularly when the storage time was extended. This could be explained by the report of Phithakpol [23], who stated that total microorganisms of fermented food mostly reached the equilibrium of 5.00–8.00 log CFU/g. In contrast, LAB of samples stored at room temperature (28 °C) (Inoc-R and Con-R) was significantly higher than samples stored at a low temperature (4 °C) (Inoc-L and Con-L) for all of the 6, 12, and 18 months of storage ($p < 0.05$) (Figure 1b). This was due to the fact that the optimal temperature for LAB is 27–33 °C [24]. The difference in the LAB of samples may further differentiate quality changes of shrimp paste when stored in different storage conditions. The amounts of TVC and LAB found in our samples were in the same ranges reported by previous authors [12,25,26].

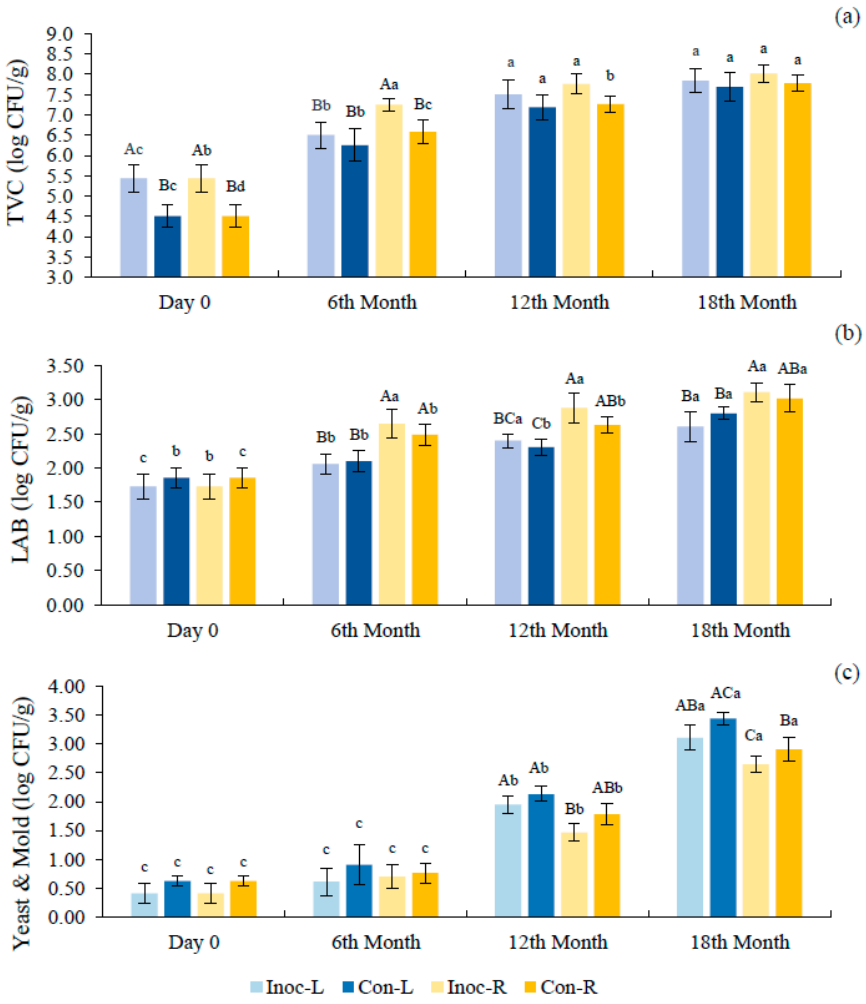

Figure 1. Total viable count (TVC) (**a**), lactic acid bacteria (LAB) (**b**), and yeast and mold count (**c**) of shrimp paste with/without *B. subtilis* K-C3 inoculation during 18 months of low (4 °C) and room (28 °C) temperature storage. Different lowercase letters indicate significant differences ($p < 0.05$), as affected by storage time. Different uppercase letters indicate significant differences ($p < 0.05$) between samples at the same storage time.

All samples had no change in yeast and mold content during storage for 6 months ($p > 0.05$) (Figure 1c). However, the significant increase was obtained after 6 months and exhibited the highest amount at the end of the 18th month, accounting for 2.65–3.44 log CFU/g ($p < 0.05$). According to the Thai Community Product Standard for shrimp paste product [27], yeast and mold count should be lower than or equal to 3.00 log CFU/g. It was found that Inoc-L and Con-L had an amount of yeast and mold over the standard limitation when stored for 18 months, indicating the spoilage of these samples in the aspect of food safety. In general, yeast is considered to be "osmophilic" (high osmotic pressure-loving), whereas mold is considered to be "xerophilic" (dry-loving), which means it is able to grow at low water activity (a_w), in the wide range of 0.62–0.80 [28]. However, favorable conditions for growth, particularly for mold, is an a_w of 0.80–0.85 with a high relative humidity more than 80% [29]. This possibly supports our results that low temperature may accelerate the yeast and mold growth more easily and led to product spoilage faster than storage at room temperature. This was also coincidentally correlated with the higher a_w of shrimp paste, which increased to the appropriate range for yeast and mold growth (0.80–0.85) (Table 1). Interestingly, this finding may indicate that a low or refrigerated temperature (4 °C) may not be appropriate for fermented shrimp paste storage.

Table 1. Water activity (a_w), pH, browning index (A_{420}) and TBARS value of shrimp paste with/without *B. subtilis* K-C3 inoculation during 18 months of low (4 °C) and room (28 °C) temperature storage.

Parameters	Storage Time	Inoc-L	Con-L	Inoc-R	Con-R
a_w	0	0.70 ± 0.02^{d}	0.68 ± 0.02^{d}	0.70 ± 0.02^{b}	0.68 ± 0.02^{c}
	6	0.74 ± 0.02^{ABc}	0.72 ± 0.01^{Bc}	0.76 ± 0.03^{Aa}	0.74 ± 0.01^{ABb}
	12	0.77 ± 0.01^{b}	0.76 ± 0.02^{b}	0.78 ± 0.01^{a}	0.77 ± 0.01^{a}
	18	0.83 ± 0.01^{Aa}	0.82 ± 0.01^{ABa}	0.80 ± 0.01^{Ba}	0.79 ± 0.02^{Ba}
pH	0	7.11 ± 0.02^{Ac}	7.04 ± 0.04^{Bc}	7.11 ± 0.02^{Ac}	7.04 ± 0.04^{Bc}
	6	7.33 ± 0.04^{ABb}	7.20 ± 0.04^{Cb}	7.39 ± 0.05^{Ab}	7.29 ± 0.03^{Bb}
	12	7.37 ± 0.02^{Bb}	7.28 ± 0.03^{Cb}	7.46 ± 0.02^{Aa}	7.40 ± 0.06^{Ba}
	18	7.48 ± 0.04^{a}	7.44 ± 0.02^{a}	7.42 ± 0.06^{a}	7.40 ± 0.04^{a}
Browning index	0	0.45 ± 0.03^{Ac}	0.38 ± 0.02^{Bd}	0.45 ± 0.03^{Ad}	0.38 ± 0.02^{Bd}
	6	0.48 ± 0.02^{ABc}	0.45 ± 0.02^{Bc}	0.52 ± 0.03^{Ac}	0.50 ± 0.02^{Ac}
	12	0.59 ± 0.04^{ABb}	0.53 ± 0.03^{Bb}	0.64 ± 0.04^{Ab}	0.58 ± 0.05^{ABb}
	18	0.74 ± 0.03^{ABa}	0.68 ± 0.05^{Ba}	0.81 ± 0.05^{Aa}	0.70 ± 0.03^{Ba}
TBARS	0	0.78 ± 0.03^{d}	0.76 ± 0.03^{d}	0.78 ± 0.03^{d}	0.76 ± 0.03^{d}
	6	0.84 ± 0.04^{Bc}	0.84 ± 0.02^{Bc}	0.91 ± 0.02^{Ac}	0.90 ± 0.03^{Ac}
	12	1.12 ± 0.04^{Cb}	1.01 ± 0.05^{Db}	1.29 ± 0.02^{Ab}	1.23 ± 0.02^{Bb}
	18	1.82 ± 0.06^{Ca}	1.79 ± 0.04^{Ca}	2.15 ± 0.04^{Aa}	2.01 ± 0.05^{Ba}

Mean ± SD from triplicate determinations. Different uppercase letters in the same row indicate the significant difference ($p < 0.05$) between samples at the same storage time. Different lowercase letters in the same column indicate the significant difference ($p < 0.05$) as affected by storage time.

3.2. Physicochemical Properties

3.2.1. a_w and pH

Water activity (a_w) of fresh shrimp paste with/without inoculation was 0.68–0.70 (Table 1), which was in the range of intermediate moisture foods (IMFs) [30]. The a_w of all samples gradually increased as storage time increased ($p < 0.05$) and reached the maximum at the end of the 18th month (0.79–0.83). Among all samples, the highest a_w was obtained in Inoc-L and Con-L, indicating that low temperature storage resulted in a greater rate of a_w increasing. This could further affect other physical/microbiological changes, i.e., the growth of yeast and mold, which can alter the product's quality/safety. However, a_w of all samples was < 0.85 throughout 18 months of storage, indicating samples still reached the quality requirements of fermented shrimp paste [29]. From the results, there was no evident difference in a_w and its change between shrimp paste with/without inoculation. In contrast, inoculated shrimp paste had a significantly higher pH than shrimp paste without inoculum, as the pH values were 7.11 and 7.04, respectively ($p < 0.05$). The pH of all samples continuously increased as storage time increased ($p < 0.05$). The increase in pH of shrimp paste during extended fermentation periods or storage time has been reported by others [12,26,31]. The slight shift to basic pH of shrimp paste is a result of the accumulation of degradation products or volatile base compounds, i.e., trimethylamine and ammonia caused by endogenous or microbial enzymes [12]. The higher pH of inoculated samples, particularly during the early storage period, might be due to the inoculum with the *Bacillus* strain, which possesses higher proteolytic activity and accelerates the fermentation rate faster than traditional samples, thus resulting in the higher accumulation of degraded basic products. This was well associated with the increase in ammonia nitrogen content (Figure 2b) and biogenic amines (Table 4), the basic nitrogen components that occur during storage. The increase in pH of shrimp paste during an extended fermentation period or storage time has been also reported by Sripokar et al. [26]. The different rate of pH and microbial population change among all samples in this study revealed the significant importance of both inoculation and storage temperature on the quality alteration of shrimp paste during storage.

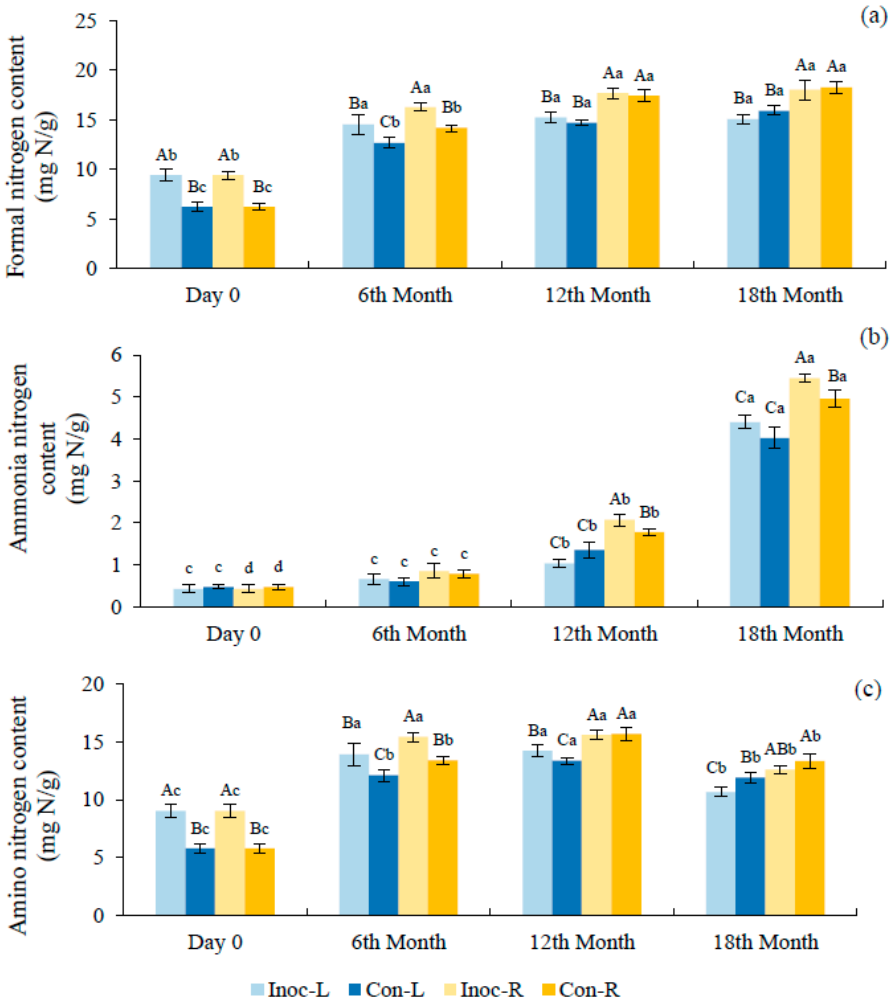

Figure 2. Formal nitrogen (**a**), ammonia nitrogen (**b**), and amino nitrogen (**c**) contents of shrimp paste with/without B. subtilis K-C3 inoculation during storage for 18 months at low (4 °C) and room (28 °C) temperature. Different lowercase letters indicate significant differences ($p < 0.05$) as affected by storage time. Different uppercase letters indicate significant differences ($p < 0.05$) between samples with the same storage time.

3.2.2. Formal, Ammonia, and Amino Nitrogen Contents

Contents of formal, ammonia, and amino nitrogen of shrimp pastes during storage are determined (Figure 2a–c). At day 0, formal nitrogen content of samples with inoculum (9.44 mg N/g) was higher than samples without inoculum (6.23 mg N/g) ($p < 0.05$). Formal nitrogen content is an index used to determine the level of the cleavage of peptides, which can monitor the level of protein hydrolysis [32]. Our results, therefore, indicated that, compared to the traditional process, the *Bacillus* strain can accelerate the rate of fermentation in terms of protein hydrolysis. Formal nitrogen content of all samples clearly increased when the samples were stored for 6 months ($p < 0.05$), then remained constant throughout storage for up to 18 months ($p < 0.05$). The higher formal nitrogen content was observed in samples stored at room temperature (Inoc-R and Con-R), compared to those stored at a low temperature ($p < 0.05$). This indicated the continuous hydrolysis of protein caused by both endogenous and microbial enzymes during storage, which caused hydrolysis at a faster rate when stored at room temperature. Ammonia nitrogen content indicates the breakdown of protein and peptides into free amino acid or volatile nitrogen, which is responsible for the odor/flavor of fermented products [33]. In this study, samples at day 0 and 6th months of storage contained low amounts of ammonia nitrogen content (< 1 mg N/g). This value increased intensively as storage time increased to more than

6 months of storage and reached the highest value of >4 mg N/g at the end of 18 months. It was also observed that Inoc-R and Con-R had higher ammonia nitrogen content than Inoc-L and Con-L at all period tests ($p < 0.05$). This helps to confirm that room temperature storage had a greater impact on hydrolysis of shrimp paste protein during storage. Amino nitrogen content represents the amount of primary amino groups in samples, which is related to the degradation of polypeptides [32]. At day 0, in contrast to traditional samples, a higher amino nitrogen content was found in inoculated samples ($p < 0.05$). This result reconfirmed that inoculation by the *Bacillus* strain can accelerate the peptide cleavages, leading to a faster fermentation rate. During storage, amino nitrogen content increased when stored for 6 months, then conversely decreased when the storage time was prolonged for up to 18 months ($p < 0.05$). This might be due to the degradation of short-chain peptides or amino acids into other nitrogenous compounds such as biogenic amines, etc. With this explanation, the results were coincidental with the increase in biogenic amines when the storage time was extended (Table 4).

3.2.3. Browning Index (A_{420})

A greater development of the browning index (A_{420}) was found in inoculated shrimp pastes, as shown in Table 1. As storage time increased, the browning index of all samples gradually increased and reached the highest value at 18th months of storage (0.68–0.81) ($p < 0.05$). The development of browning, which occurred at a higher level in inoculated samples and when stored at room temperature (28 °C), was in accordance with the breakdown of proteins, determined by formal nitrogen content (Figure 2a). Small peptides or free amino acids, which are generated during fermentation/storage, contribute to brown color development via the Maillard reaction [26]. Our results suggested that inoculation with *B. subtilis* K-C3 can accelerate Maillard reaction during fermentation of shrimp paste. Moreover, this reaction continuously occurred during storage, in which the storage at room temperature facilitated this reaction more than when the shrimp paste was stored at low temperature. The different rate of browning or Maillard reaction directly resulted in different quality/characteristics of shrimp paste. It has been noted that Maillard reaction products (MRPs) play a profound role in brown color development, which is a desirable characteristic [33]. Furthermore, MRPs of shrimp pastes possess some biological properties, particularly antioxidants [34].

3.2.4. TBARS Value

The TBARS value, which is used as the index of lipid oxidation, of shrimp paste during storage was evaluated (Table 1). At the beginning, shrimp paste with and without inoculum had a comparable TBARS value of 0.76–0.78 mg MDA/kg. A gradual increase of TBARS as storage time increased was obtained in all samples ($p < 0.05$), suggesting lipid oxidation continuously occurred during storage. A higher accumulation of TBARS was found in Inoc-R and Con-R at all period tests ($p < 0.05$), indicating a greater lipid oxidation when stored at room temperature. Shrimp *A. vulgaris*, a raw material, is a good source of unsaturated fatty acids, accounting for 30.41% [1], thus, shrimp is prone to oxidation. This reaction can diminish the quality of shrimp paste, especially odor/flavor, because lipid oxidation products are normally associated with an off-flavor in many fermented foods [12,33,35]. Normally, the acceptable limit of TBARS is less than 2 mg MDA/kg, and higher than the limit is considered to be rancid [36]. In this study, the TBARS value of Inoc-R and Con-R exceeded this limitation when stored for 18 months, accounting for 2.15 and 2.01 mg MDA/kg, respectively. This may suggest reduced quality in terms of odor/flavor when the shrimp paste is stored at room temperature for 18 months. In contrast, samples stored at a low temperature had TBARS values lower than the limitation throughout the 18 months of storage.

3.3. Antioxidative Activities

Shrimp paste has been noted as a good source of antioxidants [10,17,26,37]. In this study, the antioxidative activities of water extract of the samples and its changes during storage were monitored, as shown in Figure 3. Overall, inoculation with *B. subtilis* K-C3 and extending storage time improved the antioxidant activities of shrimp pastes. At day 0, shrimp paste had DPPH radical scavenging activity in the range of 6.29–10.55 μmol TE/g, which was found at the higher end of the range in inoculated samples ($p < 0.05$) (Figure 3a). This activity increased as storage time increased up to 18 months, but the inoculated samples still had higher values in all storage tests ($p < 0.05$). However, temperature storage did not significantly govern this activity, as evidenced by the comparable value that was obtained when the shrimp paste was stored at either low or room temperature ($p > 0.05$). The results indicated that inoculated samples possess the ability to donate the hydrogen atom to free radicals, such that the propagation process could be retarded [17] better than in traditional shrimp paste. For ABTS radical scavenging activity (Figure 3b) and FRAP (Figure 3c), there was no significant difference in activities between samples with and without inoculation at day 0 ($p < 0.05$). However, inoculated samples possessed significantly higher ABTS radical scavenging activity during storage for up to 18 months ($p < 0.05$). A gradual increase in ABTS radical scavenging activity and FRAP was obtained in all samples when storage time increased ($p < 0.05$). ABTS assay indicates both hydrophilic and lipophilic antioxidants, which determine the capability of hydrogen donating antioxidants (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavengers of lipid peroxyl radicals) [38]. FRAP determines the reducing ability or the capability of a substance to provide the electron to a free radical [17]. With these various antioxidant approaches, it can be claimed that shrimp paste, particularly when it was inoculated with *B. subtilis* K-C3, had antioxidant capacity, such that it could retard the propagation chain of oxidation by direct confrontation with the free radicals.

The metal chelating activity, which indicates the ability of a substance to chelate redox-active metals known as pro-oxidants, of shrimp paste during storage was also investigated (Figure 3d). In general, excessive accumulation of metal ions, i.e., iron, copper, chromium, cobalt, and others, lead to oxidative stress by increasing the formation of reactive oxygen species (ROS), which are responsible for lipid peroxidation, protein modification, and other effects [39]. From the results, a higher metal chelating activity was also found in inoculated samples throughout the 18 months of storage (except in the 12th month of storage) ($p < 0.05$). Similar to other assays, there was no difference in metal chelating activity between samples stored in low and room temperature ($p > 0.05$). Overall, the result reconfirmed that shrimp paste is a good source of natural antioxidants with different modes of action in the prevention of lipid oxidation, which mostly showed greater activity when the storage time was prolonged, whether it was stored at low or room temperature. These properties can be effectively improved by inoculation with *B. subtilis* K-C3 strain. A higher fermentation rate, particularly that caused by protein hydrolysis, when added with the inoculum may hydrolyze or liberate more short-chain peptides or amino acids, which are able to catch up with radical, metal pro-oxidants, etc., thereby resulting in retardation of oxidation. The improvement of antioxidant capacities by inoculation with the *Bacillus* strain during processing/fermentation has been established in many fermented foods such as fermented soybean [40–42], fermented shrimp head [38,43], or fermented shrimp paste [10], etc.

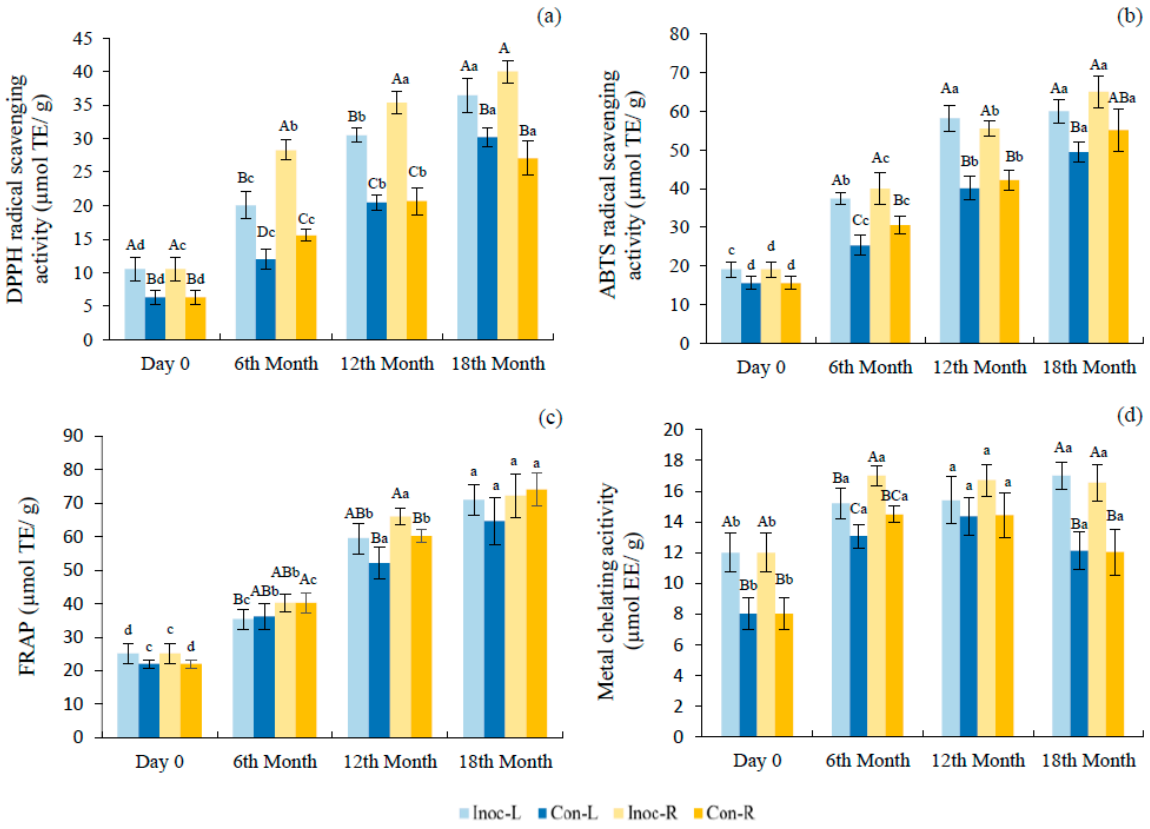

Figure 3. DPPH radical scavenging activity (**a**), ABTS radical scavenging activity (**b**), ferric reducing antioxidant power (FRAP) (**c**), and metal chelating activity (**d**) of water extracts from shrimp paste with/without *B. subtilis* K-C3 inoculation during storage for 18 months at low (4 °C) and room (28 °C) temperature. Different lowercase letters indicate significant differences ($p < 0.05$) as affected by storage time. Different uppercase letters indicate significant differences ($p < 0.05$) between samples at the same storage time.

3.4. Free Amino Acid Composition

Table 2 presents free amino acid content of shrimp pastes with/without inoculation of *B. subtilis* K-C3 at 0 and 12 months of storage. The initial total free amino acid content of 70.81 mg/g was obtained in inoculated samples, which appeared to be higher than samples without inoculation (64.83 mg/g) ($p < 0.05$). In this product, Glu/Gln were found as the predominant amino acids, accounting for 14.14–18.06 mg/g, followed by Asp/Asn, Lys, Ala, and Gly, respectively. The amino acid profiles of our samples were similar to *terasi* (Indonesian shrimp paste) [7], *belacan* (Malaysian shrimp paste [44], or *kapi* (Thai shrimp paste) [25], etc. In general, amino acid profiles are associated with aroma and nutritional values in fermented products [37]. A significant correlation between amino acid profiles and sensory acceptability was obtained in shrimp paste products, as reported by Cai et al. [45]. In this study, umami/sweetness amino acids, i.e., Glu/Gln, Asp/Asn, Ala, and Gly, were found at a higher concentration in inoculated samples (43.02 mg/g) compared to traditional shrimp paste (39.18 mg/g), indicating the possibility of improving the sensory acceptability, particularly the flavor/taste, of the product when it is challenged with the *B. subtilis* K-C3. In the aspect of nutritional values, higher amounts of essential amino acids, i.e., Lys and Ile, were obtained in inoculated samples (22.37 mg/g), compared with traditional product (18.64 mg/g) ($p < 0.05$). Additionally, a lower Leu/Ile ratio and P-PER were also found in inoculated samples ($p < 0.05$), indicating the good balancing profile of amino acids that are added with the *Bacillus* strain. Cai et al. [45] described that some diseases of humans are due to amino acid imbalance. For example, high leucine impairs the metabolism of tryptophan and niacin, which might be a factor in the development

of pellagra. Adeyeye [46] also reported that the Leu/Ile balance is more important than dietary excess of Leu alone in regulating the metabolism of tryptophan and niacin and, hence, the disease process of pellagra. Moreover, amino acids may be associated with other functional properties, i.e., antioxidants. Carrasco-Castilla et al. [47] reported that the presence of Asp/Asn, Glu/Gln, His, and Cys contents were responsible for chelation of iron. Peng et al. [48] stated that Asp and Glu plays a function on the iron chelation of protein hydrolysate. From our results, therefore, the higher Glu/Gln content of inoculated samples was coincidentally correlated with higher metal chelating activity (Figure 3d).

Table 2. Free amino acid contents (mg/g) of shrimp paste with/without *B. subtilis* K-C3 inoculation during 18 months of low (4 °C) and room (28 °C) temperature storage.

Amino Acids	Day 0				Month 12th			
	Inoc-L	Con-L	Inoc-R	Con-R	Inoc-L	Con-L	Inoc-R	Con-R
EAAs *								
His	0.80 ± 0.19 ^{a}	0.62 ± 0.22	0.80 ± 0.19 ^{a}	0.62 ± 0.22	0.42 ± 0.09 ^{Ab}	0.50 ± 0.11 ^{A}	0.29 ± 0.06 ^{Bb}	0.36 ± 0.12 ^{AB}
Ile	3.44 ± 0.55 ^{A}	2.01 ± 0.35 ^{Bb}	3.44 ± 0.55 ^{A}	2.01 ± 0.35 ^{Bb}	3.02 ± 0.48	2.87 ± 0.20 ^{a}	3.15 ± 0.12	3.11 ± 0.22 ^{a}
Leu	3.43 ± 0.40	3.95 ± 0.47	3.43 ± 0.40	3.95 ± 0.47	3.03 ± 0.52 ^{B}	3.91 ± 0.32 ^{A}	3.22 ± 0.25 ^{B}	3.68 ± 0.34 ^{AB}
Lys	6.61 ± 0.99 ^{Aa}	4.61 ± 0.25 ^{Ba}	6.61 ± 0.99 ^{Aa}	4.61 ± 0.25 ^{Ba}	3.03 ± 0.32 ^{Ab}	2.16 ± 0.15 ^{Bb}	2.15 ± 0.10 ^{Bb}	2.03 ± 0.08 ^{Bb}
Met	1.26 ± 0.32	1.08 ± 0.21	1.26 ± 0.32	1.08 ± 0.21	1.44 ± 0.28	1.26 ± 0.09	1.38 ± 0.10	1.16 ± 0.19
Phe	1.70 ± 0.22	1.77 ± 0.41	1.70 ± 0.22 ^{b}	1.77 ± 0.41	2.02 ± 0.26 ^{B}	1.92 ± 0.14 ^{B}	2.55 ± 0.19 ^{Aa}	2.01 ± 0.10 ^{B}
Thr	0.05 ± 0.00 ^{b}	0.00	0.05 ± 0.00 ^{b}	0.00	0.12 ± 0.02 ^{a}	0.00	0.35 ± 0.04 ^{a}	0.00
Trp	1.64 ± 0.23	1.59 ± 0.33	1.64 ± 0.23	1.59 ± 0.33 ^{a}	1.99 ± 0.14 ^{A}	1.78 ± 0.21 ^{A}	2.15 ± 0.30 ^{A}	1.00 ± 0.03 ^{Bb}
Val	3.44 ± 0.98	3.01 ± 0.25 ^{a}	3.44 ± 0.98	3.01 ± 0.25 ^{a}	3.03 ± 0.20 ^{A}	2.21 ± 0.27 ^{Bb}	2.88 ± 0.22 ^{A}	1.93 ± 0.14 ^{Bb}
Total EAAs	22.37 ± 1.48 ^{Aa}	18.64 ± 1.02 ^{Ba}	22.37 ± 1.48 ^{Aa}	18.64 ± 1.02 ^{Ba}	18.10 ± 0.93 ^{ABb}	16.61 ± 0.79 ^{BCb}	18.12 ± 0.70 ^{Ab}	15.28 ± 0.78 ^{Cb}
NEAAs **								
Ala	5.42 ± 0.36 ^{B}	6.42 ± 0.38 ^{A}	5.42 ± 0.36 ^{B}	6.42 ± 0.38 ^{A}	6.00 ± 0.43 ^{A}	6.40 ± 0.32 ^{A}	5.30 ± 0.20 ^{B}	6.12 ± 0.35 ^{A}
Arg	3.02 ± 0.55 ^{B}	4.44 ± 0.39 ^{A}	3.02 ± 0.55 ^{B}	4.44 ± 0.39 ^{A}	3.11 ± 0.41 ^{B}	4.72 ± 0.21 ^{A}	3.29 ± 0.22 ^{B}	4.46 ± 0.31 ^{A}
Asp/Asn	10.12 ± 0.93	9.05 ± 0.42 ^{a}	10.12 ± 0.93 ^{a}	9.05 ± 0.42 ^{a}	9.15 ± 0.66 ^{A}	7.56 ± 0.54 ^{Bb}	8.88 ± 0.45 ^{Ab}	7.12 ± 0.32 ^{Bb}
Cys	0.26 ± 0.09 ^{a}	0.40 ± 0.18 ^{a}	0.26 ± 0.09 ^{a}	0.40 ± 0.18 ^{a}	0.10 ± 0.02 ^{Bb}	0.21 ± 0.04 ^{Ab}	0.03 ± 0.00 ^{Cb}	0.09 ± 0.01 ^{Bb}
Gly	5.95 ± 0.76	5.02 ± 0.44 ^{b}	5.95 ± 0.76	5.02 ± 0.44	6.55 ± 0.49	5.93 ± 0.33 ^{a}	6.15 ± 0.61	5.89 ± 0.44
Glu/Gln	18.06 ± 1.01 ^{A}	14.14 ± 1.02 ^{Bb}	18.06 ± 1.01 ^{Ab}	14.14 ± 1.02 ^{Bb}	19.23 ± 0.92 ^{A}	16.11 ± 0.95 ^{Ba}	20.15 ± 1.02 ^{Aa}	17.03 ± 0.78 ^{Ba}
Pro	3.02 ± 0.56 ^{b}	3.99 ± 0.27 ^{b}	3.02 ± 0.56	3.99 ± 0.27	4.44 ± 0.26 ^{Aa}	4.42 ± 0.16 ^{Aa}	3.56 ± 0.29 ^{B}	3.97 ± 0.30 ^{B}
Ser	0.40 ± 0.05 ^{Bb}	0.56 ± 0.09 ^{Ab}	0.40 ± 0.05 ^{Bb}	0.56 ± 0.09 ^{Ab}	1.01 ± 0.22 ^{ABa}	1.12 ± 0.10 ^{Aa}	0.89 ± 0.12 ^{Ba}	1.15 ± 0.06 ^{Aa}
Tau	0.16 ± 0.02 ^{A}	0.12 ± 0.01 ^{B}	0.16 ± 0.02 ^{A}	0.12 ± 0.01 ^{B}	0.22 ± 0.06 ^{A}	0.12 ± 0.02 ^{B}	0.16 ± 0.05 ^{A}	0.10 ± 0.01 ^{B}
Tyr	2.03 ± 0.09 ^{a}	2.05 ± 0.21	2.03 ± 0.09	2.05 ± 0.21 ^{a}	1.88 ± 0.15 ^{b}	1.79 ± 0.10	1.93 ± 0.09	1.79 ± 0.19 ^{b}
Total NEAAs	48.44 ± 1.23 ^{b}	46.19 ± 0.95 ^{b}	48.44 ± 1.23	46.19 ± 0.95	51.69 ± 1.01 ^{a}	48.38 ± 0.77 ^{a}	50.34 ± 1.22	47.72 ± 0.64
Total AAs	70.81 ± 2.95 ^{A}	64.83 ± 2.35 ^{B}	70.81 ± 2.95 ^{A}	64.83 ± 2.35 ^{B}	69.79 ± 2.03 ^{A}	64.99 ± 2.99 ^{AB}	68.46 ± 2.90 ^{A}	63.00 ± 1.79 ^{B}
Total umami/ sweetness AAs ***	43.02 ± 1.50 ^{A}	39.18 ± 2.02 ^{B}	43.02 ± 1.50 ^{A}	39.18 ± 2.02 ^{B}	46.50 ± 1.74 ^{A}	41.54 ± 2.06 ^{B}	45.28 ± 2.00 ^{A}	41.28 ± 1.56 ^{B}
Leu/Ile ratio	1.00 ± 0.22 ^{B}	1.97 ± 0.39 ^{A}	1.00 ± 0.22 ^{B}	1.97 ± 0.39 ^{Aa}	1.00 ± 0.32	1.36 ± 0.44	1.02 ± 0.28	1.18 ± 0.25 ^{b}
P-PER ****	0.88 ± 0.10 ^{B}	1.11 ± 0.13 ^{A}	0.88 ± 0.10 ^{B}	1.11 ± 0.13 ^{A}	0.71 ± 0.17 ^{B}	1.12 ± 0.22 ^{A}	0.79 ± 0.12 ^{B}	1.01 ± 0.10 ^{A}

* EAAs: essential amino acids (Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Threonine (Thr), Tryptophan (Trp), and Valine (Val)). ** NEAAs: non-essential amino acids (Alanine (Ala), Arginine (Arg), Aspartic acid/Asparagine (Asp/Asn), Cysteine (Cys), Glycine (Gly), Glutamic acid/Glutamine (Glu/Gln), Proline (Pro), Serine (Ser), Taurine (Tau), and Tyrosine (Tyr)). *** Total umami/sweetness amino acids: Asp/Asn, Glu/Gln, Ala, Gly, Pro, Ser, and Thr. **** P-PER: –0.468 + 0.454 (Leu) – 0.105 (Tyr). Mean ± SD from triplicate determinations. Different uppercase letters indicate a significant difference between samples at the same storage time ($p < 0.05$). Different lowercase letters indicate a significant difference of the same sample between day 0 and 12th month of storage ($p < 0.05$).

In the 12th month of storage, total amino acid contents of all samples remained constant, accounting for 63.00–69.79 mg/g ($p > 0.05$). During storage, some amino acids increased, i.e., Glu/Gln, Pro, Ser; some amino acids decreased, i.e., Lys and Cys; whereas some amino acids remained constant. This was due to protein hydrolysis, which occurred continuously and liberated some amino acids out from the peptide chains. In another way, some nitrogenous substances such as amines or volatile acids might be further degraded by microbial action or by enzymatic decomposition, thus resulting in the changes in the free amino acid content, in either an increase or a decrease [37]. However, it was observed that inoculated samples, both those stored at low temperature and those stored at room temperature (Inoc-L and Inoc-R, respectively), still contained higher essential amino acids and total umami/sweetness amino acids, in addition to having a lower P-PER, compared

to shrimp pastes without inoculation (Con-L and Con-R). This tendency indicates the improvement of the amino acid profiles in terms of nutritional and flavor/taste aspects throughout storage by inoculation with *B. subtilis* K-C3.

3.5. Fatty Acid Profile

The fatty acid profiles in crude lipids extracted from shrimp pastes at day 0 and in the 12th month of storage are shown in Table 3. At the beginning of storage, shrimp pastes with/without *B. subtilis* K-C3 inoculation contained no difference in total FAs, SFA, MUFA, and PUFA ($p > 0.05$). PUFA was found to be dominant, accounting for 39.38–39.42% of total FAs, followed by SFA and MUFA, which accounted for 37.36–37.72 and 21.35–21.47% of total FAs, respectively. Among all FAs, shrimp paste contained four dominant FAs (>10% of total FAs) including palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1(n-9)), and eicosapentaenoic acid (EPA) 20:5(n-3). Moreover, arachidonic acid (AA) (20:4(n-6)) and docosahexaenoic acid (DHA) (22:6 (n-3)) were also obtained to a high extent, accounting for 9.02–9.10 and 7.19–7.28%, respectively. Thus, it can be claimed that shrimp paste is a good source of PUFA, particularly n-3 FAs, which are beneficial for human health [49]. PUFAs, i.e., EPA and DHA, are good fats responsible for a variety of health benefits. PUFAs are protective against cardiovascular diseases (CVDs) and are essential for the formation of neuron synapses in the fetal brain; they also promise antihypertensive, anticancer, antioxidant, anti-inflammatory, antidepression, antiaging, and antiarthritic effects [50]. In general, the human body needs PUFAs to function but cannot make them, so an individual must obtain PUFAs from their diet [49]. A high amount of PUFAs has been also found in *Julu* (Philippine shrimp paste) [49,51], anchovy (*Stolephorus* sp) fish paste [31], *kapi* (Thai shrimp paste) [1], etc. To date, no information on FA profiles of inoculated shrimp paste has been reported. From the results, there is no marked difference in FAs among shrimp paste with and without inoculum; however, some differences in FA compositions were observed. It was found that eicosatetraenoic acid (ETA) (20:4(n-3)) and docosapentaenoic acid (DPA) (22:5(n-3)) were obtained only in inoculated samples. Galvez et al. [52] stated that a precursor of long-chain n-3 PUFA can be converted to EPA, DPA, or DHA through the biochemical processes of elongation and desaturation involved with certain microorganisms. Thus, inoculation with the *B. subtilis* strain may lead to different microbial profiles, which further lead to different biochemical processes and results in different FA profiles.

There was no change in total FAs when the shrimp paste was stored for 12 months, such that PUFA was still found to be dominant ($p > 0.05$). Interestingly, some FAs, i.e., capric acid (10:0) or caprylic acid (8:0), slightly disappeared, whereas some FAs, i.e., cis-13-docosenoic acid (22:1(n-9)) and nervonic acid (24:1(n-9)) were firstly formed in the 12th month of storage, indicating FA profiles of shrimp paste were continuously governed during storage. It was found that PUFA content was gradually increased ($p < 0.05$), particularly when the shrimp paste was stored at room temperature (Inoc-R and Con-R), whereas SFA and MUFA contents were constant ($p > 0.05$). The changes of FAs during storage have not yet been studied in any previous studies; however, some reports exhibited its changes during prolonged fermentation periods. Montano et al. [49] reported that the amount of PUFA was slightly increased and reached 34.7 g/100 g lipids after fermentation for 6 months. The increase in PUFA and decrease in MUFA were also obtained for prolonged fermentation time of fish paste for 32 days [31]. These corresponded well with our results. However, Cai et al. [45] stated that the SFA of shrimp paste increased, whereas MUFA and PUFA decreased for an increased fermentation period of low-salt shrimp paste. Peralta et al. [37] reported that there were no changes in any FAs of shrimp paste during fermentation for 360 days, except a slight increase in DHA was obtained. Thus, a fluctuation of the changes in FAs during fermentation or even storage was noted. It was postulated that the decrease in some FAs were plausibly consumed by microorganisms during prolonged fermentation/storage. On the contrary, the increase in some FAs was possibly caused by

some salt-tolerant microorganisms as well. Overall, the contents of FAs, particularly PUFAs, after storage for 12 months confirmed the nutritional values of shrimp paste to some extent.

Table 3. Fatty acid (FA) profiles (%) of shrimp paste with/without *B. subtilis* K-C3 inoculation during 18 months of low (4 °C) and room (28 °C) temperature storage.

Fatty Acids	Day 0				Month 12th			
	Inoc-L	Con-L	Inoc-R	Con-R	Inoc-L	Con-L	Inoc-R	Con-R
SFA *								
8:0	0.15 ± 0.02 ^{Ba}	0.33 ± 0.12 ^{A}	0.15 ± 0.02 ^{B}	0.33 ± 0.12 ^{Aa}	0.02 ± 0.00 ^{Bb}	0.25 ± 0.09 ^{A}	0.00	0.12 ± 0.04 ^{Ab}
10:0	0.06 ± 0.05	0.17 ± 0.09	0.06 ± 0.05	0.17 ± 0.09	0.00	0.00	0.00	0.00
12:0	1.47 ± 0.12 ^{B}	2.05 ± 0.24 ^{A}	1.47 ± 0.12 ^{B}	2.05 ± 0.24 ^{A}	1.33 ± 0.21	1.65 ± 0.22	1.25 ± 0.32	1.61 ± 0.22
14:0	2.00 ± 0.20	1.96 ± 0.20	2.00 ± 0.20	1.96 ± 0.20	2.05 ± 0.11 ^{A}	2.03 ± 0.12 ^{A}	1.78 ± 0.07 ^{B}	1.74 ± 0.18 ^{B}
16:0	21.15 ± 1.43	20.62 ± 0.56	21.15 ± 1.43	20.62 ± 0.56	21.19 ± 0.50	21.01 ± 0.32	20.92 ± 0.44	20.60 ± 0.43
18:0	12.22 ± 1.02	12.16 ± 0.70	12.22 ± 1.02	12.16 ± 0.70	11.93 ± 0.45	12.05 ± 0.50	11.54 ± 0.30	11.62 ± 0.34
20:0	0.23 ± 0.09	0.31 ± 0.12	0.23 ± 0.09	0.31 ± 0.12	0.17 ± 0.03	0.20 ± 0.05	0.11 ± 0.04	0.16 ± 0.07
22:0	0.08 ± 0.01	0.12 ± 0.03	0.08 ± 0.01	0.12 ± 0.03	0.12 ± 0.04	0.13 ± 0.03	0.09 ± 0.02	0.11 ± 0.04
Total SFA	37.36 ± 1.30	37.72 ± 0.96	37.36 ± 1.30	37.72 ± 0.96	36.81 ± 0.78	37.32 ± 0.63	35.69 ± 0.88	35.96 ± 0.70
MUFA								
16:1 (n-7)	7.65 ± 0.67	8.03 ± 0.40	7.65 ± 0.67	8.03 ± 0.40	7.42 ± 0.40	7.67 ± 0.36	7.30 ± 0.34	7.54 ± 0.20
18:1 (n-7)	2.51 ± 0.34	2.38 ± 0.27	2.51 ± 0.34	2.38 ± 0.27	2.50 ± 0.21	2.41 ± 0.50	2.32 ± 0.33	2.35 ± 0.22
18:1 (n-9)	10.39 ± 0.41	9.98 ± 0.50	10.39 ± 0.41	9.98 ± 0.50	10.02 ± 0.47	10.01 ± 0.50	10.03 ± 0.22	9.94 ± 0.35
20:1 (n-9)	0.92 ± 0.21	0.96 ± 0.15	0.92 ± 0.21 ^{a}	0.96 ± 0.15	0.67 ± 0.09 ^{A}	0.78 ± 0.17 ^{A}	0.51 ± 0.05 ^{Bb}	0.83 ± 0.12 ^{A}
22:1 (n-9)	0.00	0.00	0.00	0.00	0.19 ± 0.03 ^{B}	0.09 ± 0.01 ^{C}	0.31 ± 0.09 ^{A}	0.26 ± 0.03 ^{A}
24:1 (n-9)	0.00	0.00	0.00	0.00	0.03 ± 0.00 ^{B}	0.00	0.11 ± 0.03 ^{A}	0.05 ± 0.02 ^{B}
Total MUFA	21.47 ± 0.88	21.35 ± 0.56	21.47 ± 0.88	21.35 ± 0.56	20.83 ± 0.50	20.96 ± 0.72	20.58 ± 0.49	20.97 ± 0.61
PUFA (n-6 series)								
18:2 (n-6)	3.98 ± 0.28	3.55 ± 0.30	3.98 ± 0.28	3.55 ± 0.30	4.05 ± 0.22	4.01 ± 0.20	4.12 ± 0.22	3.98 ± 0.23
18:3 (n-6)	2.67 ± 0.19 ^{A}	2.13 ± 0.14 ^{B}	2.67 ± 0.19 ^{A}	2.13 ± 0.14 ^{B}	2.33 ± 0.20	2.08 ± 0.21	2.42 ± 0.25	2.05 ± 0.26
20:2 (n-6)	1.01 ± 0.09 ^{B}	2.33 ± 0.11 ^{A}	1.01 ± 0.09 ^{Bb}	2.33 ± 0.11 ^{A}	1.22 ± 0.09 ^{B}	2.30 ± 0.16 ^{A}	1.54 ± 0.14 ^{Ba}	2.40 ± 0.10 ^{A}
20:4 (n-6) (AA) **	9.02 ± 0.32	9.10 ± 0.40	9.02 ± 0.32 ^{b}	9.10 ± 0.40	9.22 ± 0.32 ^{AB}	9.13 ± 0.22 ^{B}	9.79 ± 0.30 ^{Aa}	9.46 ± 0.20 ^{AB}
22:4 (n-6)	0.43 ± 0.04	0.49 ± 0.08	0.43 ± 0.14	0.49 ± 0.08	0.32 ± 0.12	0.45 ± 0.11	0.30 ± 0.06	0.39 ± 0.09
PUFA (n-3 series)								
18:3 (n-3)	2.22 ± 0.44	2.30 ± 0.23	2.22 ± 0.44	2.30 ± 0.23	2.16 ± 0.29	2.25 ± 0.15	2.12 ± 0.07	2.32 ± 0.09
20:4 (n-3)	0.08 ± 0.01	0.00	0.08 ± 0.01 ^{b}	0.00	0.05 ± 0.01 ^{B}	0.00	0.22 ± 0.02 ^{Aa}	0.00
20:5 (n-3) (EPA)	12.61 ± 0.71	12.20 ± 0.55	12.61 ± 0.71	12.20 ± 0.55	12.99 ± 0.30 ^{AB}	12.50 ± 0.41 ^{B}	13.38 ± 0.32 ^{A}	13.14 ± 0.30 ^{A}
22:5 (n-3)	0.21 ± 0.02 ^{b}	0.00	0.21 ± 0.02 ^{b}	0.00	0.43 ± 0.09 ^{a}	0.00	0.57 ± 0.13 ^{a}	0.00
22:6 (n-3) (DHA)	7.19 ± 0.40	7.28 ± 0.32	7.19 ± 0.40	7.28 ± 0.32	7.12 ± 0.44	7.27 ± 0.23	7.26 ± 0.18	7.35 ± 0.24
Total PUFA	39.42 ± 1.03	39.38 ± 0.67	39.42 ± 1.03 ^{b}	39.38 ± 0.67 ^{b}	39.89 ± 0.60 ^{B}	39.99 ± 0.66 ^{B}	41.72 ± 0.53 ^{Aa}	41.09 ± 0.61 ^{Aa}
Total FAs	98.25 ± 1.33	98.45 ± 1.05	98.25 ± 1.33	98.45 ± 1.05	97.53 ± 0.99	98.27 ± 1.35	97.99 ± 1.20	98.02 ± 1.06

* SFA, MUFA, and PUFA mean saturated fatty acid, monounsaturated fatty acid, and polyunsaturated fatty acid, respectively. ** AA, EPA, and DHA mean arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, respectively. Mean ± SD from triplicate determinations. Different uppercase letters indicate the significant difference between samples at the same storage time ($p < 0.05$). Different lowercase letters indicate the significant difference of the same sample between day 0 and 12th month of storage ($p < 0.05$).

3.6. BAs

Table 4 presents BA contents in shrimp paste at day 0 and in the 12th month of storage. The initial inoculated shrimp pastes had BA contents of 48.45 mg/kg, which was higher than shrimp paste without inoculation (38.67 mg/kg) ($p < 0.05$). At day 0, only three Bas, namely tryptamine (Try), cadaverine (Cad), and histamine (Him), were observed in shrimp paste without inoculation (Con-L and Con-R). However, another two BAs, namely tyramine (Tyr) and spermine (Spm), were found only in inoculated samples (Inoc-L and Inoc-R). BAs are produced by substrate-specific enzymes from microorganisms via decarboxylation of amino acids or by amination and transamination of aldehydes and ketones, which are found in many fermented foods [53]. For fermented foods, BAs are mainly generated in the course of microbial metabolisms and are intensively produced during prolonged fermentation periods or storage time. Thus, the higher BAs in inoculated samples were possibly associated with the higher microbial population (Figure 1a), which was governed by the addition of the *Bacillus* strain.

Table 4. Biogenic amines (BAs) of shrimp paste with/without *B. subtilis* K-C3 inoculation during storage for 18 months at low (4 °C) and room (28 °C) temperature.

BAs	Day 0				Month 12th			
	Inoc-L	Con-L	Inoc-R	Con-R	Inoc-L	Con-L	Inoc-R	Con-R
Try *	22.35 ± 2.02 ^{b}	20.02 ± 1.95 ^{b}	22.35 ± 2.02 ^{b}	20.02 ± 1.95 ^{b}	88.09 ± 1.26 ^{Ba}	90.11 ± 3.03 ^{Ba}	99.98 ± 5.05 ^{Aa}	101.02 ± 4.04 ^{Aa}
Phe	0.00	0.00	0.00	0.00	20.11 ± 1.12 ^{A}	23.22 ± 2.40 ^{A}	15.41 ± 2.11 ^{B}	15.65 ± 1.55 ^{B}
Put	0.00	0.00	0.00	0.00	60.35 ± 2.87 ^{D}	70.52 ± 2.15 ^{C}	80.38 ± 1.05 ^{B}	90.12 ± 2.56 ^{A}
Cad	2.41 ± 0.29 ^{b}	2.03 ± 0.16 ^{b}	2.41 ± 0.29 ^{b}	2.03 ± 0.16 ^{b}	50.02 ± 3.01 ^{Ba}	54.41 ± 2.66 ^{Ba}	62.22 ± 3.14 ^{Aa}	60.01 ± 4.18 ^{Aa}
Him	18.33 ± 0.69 ^{Ab}	16.62 ± 0.80 ^{Bb}	18.33 ± 0.69 ^{Ab}	16.62 ± 0.80 ^{Bb}	70.03 ± 3.22 ^{a}	70.49 ± 3.14 ^{a}	72.02 ± 3.25 ^{a}	75.55 ± 3.92 ^{a}
Tyr	1.41 ± 0.30 ^{b}	0.00	1.41 ± 0.30 ^{b}	0.00	20.55 ± 2.19 ^{Aa}	10.47 ± 1.01 ^{B}	17.03 ± 1.40 ^{Aa}	10.15 ± 1.05 ^{B}
Spm	3.95 ± 0.67	0.00	3.95 ± 0.67	0.00	0.00	0.00	0.00	0.00
Spd	0.00	0.00	0.00	0.00	3.09 ± 0.77 ^{A}	1.05 ± 0.30 ^{B}	4.41 ± 0.68 ^{A}	0.00
Total BAs	48.45 ± 3.08 ^{Ab}	38.67 ± 2.40 ^{Bb}	48.45 ± 3.08 ^{Aba}	38.67 ± 2.40 ^{Bb}	312.24 ± 5.79 ^{Ba}	320.27 ± 6.02 ^{B}	351.45 ± 6.14 ^{Aa}	352.50 ± 6.38 ^{Aa}

* Tryptamine (Try), β-phenethylamine (Phe), Putrescine (Put), Cadaverine (Cad), Histamine (Him), Tyramine (Tyr), Spermine (Spm), and Spermidine (Spd). Mean ± SD from triplicate determinations. Different uppercase letters indicate a significant difference between samples at the same storage time ($p < 0.05$). Different lowercase letters indicate a significant difference of the same sample between day 0 and 12th month of storage ($p < 0.05$).

In the 12th month of storage, the contents of BA of all samples sharply increased to 312.24–352.50 mg/kg ($p < 0.05$). All BAs found at day 0 significantly increased, except spermine (Spm), which disappeared. Moreover, another three BAs were firstly found in stored products, including β-phenethylamine (Phe), putrescine (Put), and spermidine (Spd). Ma et al. [54] reported that spermine was detected only in the early period of fish sauce fermentation, whereas spermidine, which was not detected in the early period (3–6 months), was formed during the medium (6–12 months) and late period (12–18 months) of fermentation. The increase in BAs as fermentation/storage time increased was pronounced in many previous studies [12,22,53]. In general, a high level of BAs induces toxicological risks and health troubles. BA are potential precursors for the formation of carcinogenic N-nitroso compounds [55]. Histamine has been implicated as the causative agent in several outbreaks of food poisoning, whereas tyramine and β-phenylethylamine have been noted as the initiators of hypertensive crisis [55]. Cadaverine and putrescine can enhance the toxicity of histamine and react with nitrite to form carcinogenic nitrosamines [13]. Among all BAs, histamine was found to be predominant in our samples, accounting for 16.62–18.33 mg/kg at day 0 and increasing to 70.03–75.55 mg/kg when stored for 12 months ($p < 0.05$). In general, the maximum level of histamine in fish products was set at 50 mg/kg by the FDA [56] and 200 mg/kg by EC [57]. A somewhat high level of histamine has been detected in other fermented fish products, i.e., fish sauce [54], anchovy fermented paste [58], shrimp paste [12], etc.

The use of selected starter culture can inhibit the accumulation of BAs effectively in certain products, including fish sauce inoculated with *Staphylococcus carnosus* FS19 and *Bacillus amyloliquefaciens* FS05 [6], dried sausage inoculated with *Staphylococcus xylosus* [59], etc. However, from our results, there was no difference between BAs of shrimp paste with and without inoculation ($p > 0.05$). This might infer that our *B.* strain may not help to reduce the accumulation of BAs in shrimp paste. On the contrary, it was observed that storage temperature markedly effected the changes in BAs of this product. The results showed that shrimp paste stored at room temperature (Inoc-R and Con-R) had significantly higher BAs compared to samples stored at a low temperature ($p < 0.05$). In fact, storage temperature is an important factor for preventing or inhibiting the formation of BA in foods [13]. The formation of putrescine, tyramine, and cadaverine in Edam cheese was reduced when ripened at 5 °C, compared to traditional ripening at 10 °C [60]. Based on hazard analysis and critical control points (HACCP) regulated by the FDA [56], a low temperature of <4.4 °C is recommended for the storage of fish-based products to prevent histamine formation. This supported the results of the lower BAs found in samples stored at a low temperature. However, the FDA set the limitation of BAs in fermented fishery products at <500 mg/kg [56]; thus, our shrimp pastes stored for 12 months at both low and

room temperature, which had BAs of 312.24–351.45 mg/kg, were still within the standard limitation and were, thus, claimed to be safe to consume.

4. Conclusions

Inoculation with *B. subtilis* K-C3 significantly accelerated the fermentation rate and developed some characteristics, i.e., pH, formal and amino nitrogen content, browning index, and microbial population, in addition to improving some nutritional components, i.e., essential amino acids, PUFAs, and antioxidant properties of shrimp paste compared to traditional shrimp paste. These characteristics continuously changed throughout 18 months of storage, resulting in reduced shrimp paste quality. Interestingly, the results suggested that room temperature storage (28 °C) exhibited a higher rate of quality change compared to low temperature storage (4 °C). However, the quality of the shrimp paste still meets the standard regulations of this product even if it is stored for 18 months. In contrast, shrimp paste stored at low temperature storage (4 °C) for 18 months was defined as spoiled because an excessive yeast and mold count was observed, suggesting it was an inappropriate condition for shrimp paste storage. Further research should focus on the safety properties of the *B. subtilis* K-C3 strain in addition to seeking greater insights into the correlation between sensory profiles, particularly taste or flavors, before use as a starter culture in the industry.

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