The Annals of the University Dunarea de Jos of Galati
Fascicle VI – Food Technology (2022), 46(2), 9-21

ORIGINAL RESEARCH PAPER

PHYLOGENIC IDENTIFICATION OF TOXIGENIC *BACILLUS CEREUS* IN CHILI AND WHITE PEPPER FROM BOGOR AREA, INDONESIA

HASIFA NANTEZA[1], RATIH DEWANTI-HARIYADI[1,2,*], SITI NURJANAH[1,2]

[1] *Food Science and Technology Department, IPB University, Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia*

[2] *Southeast Asian Food and Agricultural Science and Technology Center, IPB University, Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia*

[*]Corresponding author: ratihde@apps.ipb.ac.id

Received on 8 February 2022
Revised on 20 June 2022

Abstract

This study aimed to compare the occurrence and level of *Bacillus cereus sensu stricto* (*B. cereus*) in chili, and in previously isolated white pepper from traditional and supermarkets around Bogor, to determine the phylogenetic relationship between the obtained isolates based on their *16S rDNA* gene, and to determine their potential toxicity based on *ces* and *nheA* genes using Polymerase Chain Reaction. The highest presumptive *B. cereus* level in samples from traditional and supermarkets was 5.95×10^5 and 2.6×10^5 CFU/g respectively. The difference in *B. cereus* levels between the two market types was not significant. Ten presumptive isolates from chili and 10 from white pepper from our previous study were sequenced, subjected to BLAST analysis, and 13 were confirmed as *B. cereus sensu lato*. The sequences were phylogenetically analysed and tested for the possession of *nheA* and *ces* toxigenic genes. Based on the phylogenetic tree established, 12 of 13 isolates were related to *B. cereus*, sharing >98% similarity with reference strains. All 12 (100%) isolates owned the *nheA* gene; none of them possessed the *ces* gene. Absence of *ces* gene lessens the danger for emesis from these spices, nonetheless, the 100% presence of *nheA* gene presents a potential risk for *B. cereus* diarrheal syndrome.

Keywords: *Bacillus cereus*, chili, PCR, toxigenic genes, white pepper

Introduction

Chili (*Capsicum annum L.*) and white pepper (*Piper nigrum*) are widely produced and used as spices and or condiment in many Indonesian cuisines – more especially Ready-to-eat (RTE) foods. These spices are used in relatively small to substantial quantities and have the potential to contaminate many RTE foods with foodborne pathogens. Among foodborne pathogens associated with spices like chili and white

https://doi.org/10.35219/foodtechnology.2022.2.01

pepper is *B. cereus*, a facultatively anaerobic rod-shaped spore forming bacterium that has been reported to contaminate a variety of spices and herbs at levels of <3 to 1,600 MPN/g in USA (Hariram and Labbe, 2015), 1.6 x10^3 CFU/g in Germany (Frentzel *et al*. 2016), 3.1 x10^2 CFU/g in Latvia (Fogele *et al*., 2018) and >1.01 x10^2 CFU/g in Poland (Berthold-Pluta *et al*., 2019). In USA, the organism was reported to contaminate chili at levels of up to 23 MPN/g (Hariram and Labbe, 2015) and up to 11.3×10^5 CFU/g in Iraq (Jessim *et al*., 2017). Moreover, *B. cereus* spore concentrations of 240 MPN/g were reported to contaminate white pepper in USA (Hariram and Labbe, 2015) and up to 10^3 CFU/g in Turkey (Hampikyan *et al*., 2009).

Bacillus cereus is widely distributed in the environment especially in soil where such spices grow (Mathot *et al*., 2021). The bacterium can form endospores during storage or processing or upon exposure to extreme conditions; the bacterium is also capable of producing two types of toxins. The spores that germinate in the food may produce the emetic toxin (cereulide), an acid, heat and protease stable peptide that results in emesis characterized by nausea, vomiting and malaise. Cereulide toxin is encoded by the cereulide synthetase gene (*ces*) (Zhang *et al*., 2016). Additionally, when the vegetative cells in food are ingested, they may produce heat labile enterotoxins in the intestines that result in the diarrheal syndrome characterized by abdominal cramps and severe watery diarrhea. One such enterotoxins is the most widely distributed non hemolytic enterotoxin (*nhe*) encoded by the *nheA* gene.

Emetic and diarrheal syndromes constitute foodborne diseases caused by *B. cereus* and a number of them have been reported in various parts of the world. Hariram and Labbe, (2015) have described spice associated *Bacillus cereus* outbreaks in France, Belgium, United Kingdom, Hungary and Denmark. FAO/WHO (2014) stated that *B. cereus* is the second most outbreak causing microorganism in Low Moisture Foods (25.7%); the first being *Salmonella* (44.9%). In Indonesia, *B. cereus* is the second most food poisoning causing pathogen after *E.coli* and it was reported to have caused 34 food borne diarrheal outbreak events (19.4%) in the period between 2000 – 2015 (Arisanti *et al*., 2018). In 2019, 188 people were reported to have experienced diarrhea, nausea, and stomach cramps after consuming satay (a seasoned meat dish served with peanut sauce) that was contaminated with enterotoxigenic *B. cereus* in Yogyakarta (Son *et al.,* 2020).

Presently, there is no data on the potential toxicity of the pathogen in chili and white pepper from Indonesia. It is therefore important to determine the toxigenic potential of *Bacillus cereus* isolated from chili and white pepper which can potentially cause diarrheal and emetic foodborne illnesses and outbreaks. This study therefore aimed to: (1) compare the occurrence and level of *B. cereus* in chili and white pepper from traditional and supermarkets, (2) determine the phylogenetic relationship between the obtained isolate strains with the closest reference strains based on their *16S rDNA* gene and (3) determine the potential toxicity of these isolates based on *ces* and *nheA* genes.

Materials and methods

Sampling and sample preparation of chili

Twenty samples of powdered chili in packages of 10 to 50g were purchased from three traditional markets and three supermarkets around Bogor and transported to the laboratory in their original packages. Fifty-gram portions were weighed and diluted with 450 mL Butterfield's phosphate- buffered dilution water (Millipore corporation, USA) and homogenized using a BagMixer® (Inter-science, France) for 2 minutes. The samples were then serially diluted from 10^{-1} to 10^{-3}. In addition, 20 white pepper samples obtained from traditional and supermarkets around Bogor in our previous study were compared (Nanteza *et al*., 2022).

Isolation and quantification of presumptive B. cereus from chili samples

Isolation and quantification of *B. cereus* was conducted according to ISO 7932:2004. Mannitol egg yolk polymyxin (MYP) agar (Oxoid Ltd. UK) plates were inoculated by spreading 100 µL of each sample diluted to 10^{-3} on duplicate plates, followed by incubation for 18-24 hours at 30°C. Typical colonies (pink and bordered by a zone of precipitation) were quantified. Five typical colonies were taken from each plate, slanted on nutrient agar, then observed for mannitol fermentation, lecithinase production, catalase production, spore and Gram staining. Lecithinase production and mannitol fermentation were directly observed on MYP plates. For Gram staining, a small portion of a colony was transferred to a degreased microscope slide, pulverized in a small water globule, dried in air and heat fixed. It was then stained with a drop of crystal violet solution which was rinsed off with water after 1 minute. The smear was then stained with a drop of lugol's iodine and rinsed off with water after 1 minute. The slide was decolorized with 95% ethanol for 20 seconds, rinsed with water then counterstained with safranin for 1 minutes followed by rinsing. The slide was dried and observed under an electronic microscope (Olympus CX21, Hong Kong) at ×1000 magnification.

For spore staining, a small amount of colony was transferred to a degreased microscope slide, pulverized in a small water globule, dried in air and heat fixed. The smear was then stained with malachite green solution over boiling water for 10minutes. The excess dye was rinsed off with running water and the slide was dried. The slide was then stained with safranin solution for 20 seconds in order to stain the sporangia. It was then examined under an electronic microscope (Olympus CX21, Hong Kong) at ×1000 magnification with the aid of immersion oil.

Catalase reaction involved introducing a small portion of the colony onto a glass slide containing a drop of hydrogen peroxide (3% v/v). A rapid release of oxygen bubbles implied the production of catalase. The absence of catalase was evidenced by the lack of or production of weak bubbles.

DNA extraction from B. cereus isolates obtained from chili

Each pure colony was inoculated and incubated in sterile Brain Heart Infusion Broth (BHIB) (Oxoid Ltd-UK) for 24 hours at 30°C to obtain 1 x 10^9 CFU/ml bacterial cells. DNA was then extracted from these cells using Presto™ Mini gDNA bacteria kit (Gene-aid Biotech Ltd, Taiwan) according to manufacturer's instructions,

followed by measurement of DNA purity at ($A_{260/280}$) using Nanodrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA).

Bacillus cereus identification by 16S rDNA gene amplification

Isolates with the best DNA purity (ratio of the two absorbance values between 1.8 - 2.0) were tested in order to identify them as *Bacillus* sp. and subsequently test the homology of the isolates (phylogenetic analysis). Primer pairs in Table 1 were used for gene amplification. A final PCR reaction of 25 µL contained 2 × Taq Master Mix (12.5 µL) from Promega, USA, Nuclease Free Water (NFW) (9.5 µL), Promega, USA, forward and reverse primers (1 µL each) with a concentration of 0.4 µM and 1 µL of 100 ng template DNA (Sacchi *et al*., 2002) with modification. The PCR conditions included pre-denaturation at a temperature of 95°C for 3 minutes, 30 cycles with: denaturation for 30 seconds at a temperature of 94°C, annealing of primers for 45 seconds at a temperature of 51°C and extension for 1 minute at a temperature of 72°C. The final extension parameters were temperature of 72°C and time of 10 minutes. (Sacchi *et al.,* 2002) with modification. Two percent agarose gel electrophoresis was done at 90V for 45 minutes using an electrophoresis set from Bio Rad, USA. The resulting bands were visualized on a UV transilluminator (Bio-Rad, USA) and compared with standard bands on a 100 bp DNA ladder (Gene-aid Biotech Ltd, Taiwan).

Table 1. Primer sequences for *16S rDNA*, *ces* and *nheA* genes and their corresponding amplicon sizes.

Target gene	Primer sequence (5' → 3')	Amplicon size (bp)	Reference
16S rDNA	67-F: TGA AAA CTG AAC GAA ACA AAC	1,686	Sacchi *et al.*, 2002
	1671-R: CTC TCA AAA CTG AAC AAA ACG AAA 3´		
ces	*ces*-F: TTCCGCTCTCAATAAATGGG	634	Kim *et.al*., 2012
	ces-R: TCACAGCACATTCCAAATGC		
nheA	*nheA*-F: AGGTAAATGCGATGAGTAG	617	Zhang *et al.*, 2016
	nheA-R: TTGTTGAATGCGAAGAG		

16S rDNA gene Sequencing and phylogenetic analysis

Twenty DNA amplicons (10 obtained from chili and 10 from white pepper) and the forward and reverse primers used for PCR were sent to 1st Base Malaysia for sequencing using Sanger sequencing with ABI PRISM 7700 Sequence Detection System. Analysis of sequences was done using GeneStudio software, available at

https://sourceforge.net/projects/genestudio/. Basic Local Alignment Search Tool (BLAST) program from the NCBI website (https://www.ncbi.nlm.nih.gov/) was used to match the resulting forward sequences with thirteen *B. cereus* group*16S rDNA* sequences recovered from GenBank (www.ncbi.nlm.nih.gov). *Geobacillus* sp. 1Y (EF667358.1) was included as an out group. A phylogenetic tree was constructed do determine the degree of homology of isolates using the MEGA 11 (Molecular Evolutionary Genetics Analysis) 64-bit variant application available at (www.megasoftware.net.) and the maximum likelihood (ML) statistical method was employed with the Kimura two-parameter (K2P) model following alignment by MUSCLE, a multi sequences alignment method with reduced time and space complexity. Bootstraps statistical method, using 1000 replications, was used to estimate the degree of reliability of tree topologies.

Detection of nheA enterotoxin and ces emetic toxin genes using PCR

Presence of *ces* and *nheA* genes from isolates obtained from chili and white pepper was detected through PCR amplification using primer pairs in Table 1 and running conditions in Table 2. The PCR components used to amplify the *ces* gene were 12.5 µL of 2x master mix, 9.5 µL NFW, 1 µL of 0.7 µM forward and reverse primers and 1 µL of 100ng template DNA. (Kim *et al*., 2012). For *nhe*A amplification, a total quantity of 20 µL encompassing 10 µL of 2 × Taq master mix, 7.0 µL of NFW, 1 µL of 100ng template DNA and1 µL of each forward and reverse primers (Zhang *et al*., 2016) was used to perform the PCR.

Table 2. PCR Running conditions for *nheA* and *ces* gene amplification**.**

	***NheA*[1]**			***Ces*[2]**		
PCR Step	**Temperature (ºC)**	**Time (s)**	**No. of cycles**	**Temperature (ºC)**	**Time (s)**	**No. of cycles**
Pre denaturation	95	180		95	180	
Denaturation	94	30	30	94	30	35
Annealing	54	45	30	50	60	35
Extension	72	60	30	72	60	35
Final extension	72	300		72	300	

[1] Zhang *et al*., 2016, [2] Kim *et al*., 2012

Results and discussion

Fifteen of the 20 chili samples examined (75%) were found to contain presumptive *B. cereus* with a highest CFU/g of 8.5×10^3. Five samples (25%) did not contain any presumptive *B. cereus*. A total of 44 isolates from these samples were presumptively confirmed as *B. cereus.* They did not ferment mannitol but produced lecithinase and catalase, and their cells were rod shaped, Gram positive with centrally located spores when observed under a microscope at x1000 magnification (Olympus CX21, Hong Kong). Their colonies were pink in color, bordered by a precipitate zone as shown in Figure 1.

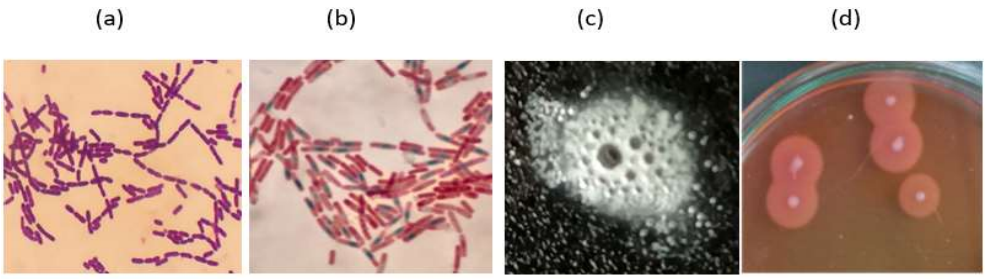

Figure 1. (a) Gram staining of *B. cereus* chili isolate, (b) Spore staining of *B. cereus* chili isolate showing central spores (c) Oxygen bubbles from catalase production and (d) Zone of precipitation of presumptive *B. cereus* on MYP agar.

Comparison of level and occurrence of presumptive B. cereus in chili and white pepper from traditional and supermarkets

The level of *B. cereus* in 20 white pepper samples from our previous study (Nanteza *et al*., 2022) and 20 chili samples from this study obtained from supermarkets (total n = 20) and traditional markets (total n = 20) were compared. Nineteen (95%) samples from traditional markets and fourteen (70%) from the supermarkets were found to contain presumptive *B. cereus*. The highest presumptive *B. cereus* level in samples from traditional markets was 5.95×10^5 CFU/g and the highest in samples from supermarkets was 2.6×10^5 CFU/g. One sample from traditional market and 6 samples from the supermarkets did not contain any *B. cereus* as shown in Figure 2.

The difference between means of samples obtained from traditional and supermarkets was statistically insignificant ($P > 0.05$) when tested using the Tukey multiple comparison test in Minitab at a 95% confidence level. However, samples from the traditional market had a higher mean CFU/g of 1.78×10^3 compared to 3.55×10^2 CFU/g from supermarkets.

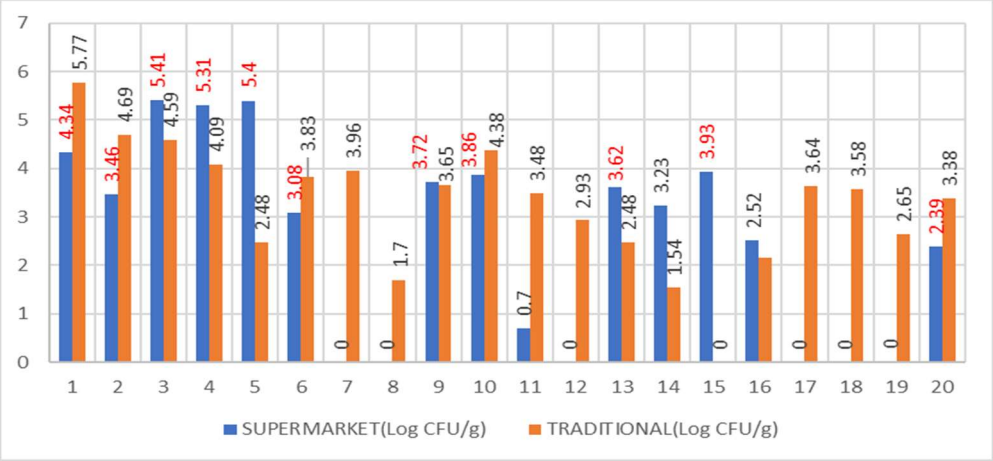

Figure 2. Occurrence and level of presumptive *B. cereus* in white pepper (1-10) and chili (11-20) from traditional markets and supermarkets.

Supermarkets would generally be expected to have lower microbial levels due to better handling, better hygiene and use of packaging that protects the spices from environmental contamination and limits oxygen entry to the product. The comparable level of *B. cereus* could be due to a longer holding time for supermarket products which gives adequate time for *B. cereus* to form spores thus comparing with samples from traditional markets where conditions are generally unhygienic with high temperatures and humidity that favor *B. cereus* growth, thus presenting a risk for contamination. Pathogens in spices and herbs have been reported to persist over time, with spore formers surviving up to 2.2 years (SPICED/European Commission, 2016), hence a possibility of prevalence in supermarket products that are usually held longer on the shelves. It is therefore important that precaution is taken when adding chili and white pepper to foods that undergo minimum processing or ready to eat foods regardless of the source of the spice in order to prevent multiplication of vegetative cells and germination of spores, which in turn result in production of diarrheal and emetic toxins and subsequent foodborne disease and outbreaks. According to Mathot *et al.* (2021), spores can be destroyed by heat treatment at 100°C applied for 16 minutes or by steam treatment using the vacuum-steam-vacuum (VSV) process for a period of 10 to 20 seconds at temperatures of 120 to 140°C. Microwaves, irradiation and fumigation are other less used methods due to causing loss of essential oils, less acceptance of the technology by consumers, and persistence of carcinogenic and mutagenic compounds from chemicals used in the fumigation process respectively.

Our findings relate to Fogele *et al*. (2018) who stated that spices like black pepper obtained from the local market in Latvia were more contaminated with *B. cereus* than those from supermarkets. On the other hand, there was no significant statistical difference in microbial levels of fresh produce from supermarkets and open air markets (Vital *et al.*, 2014).

Identification of isolates by PCR, sequencing and BLAST analysis

Ten isolates from chili and ten from white pepper from our previous study formed clear bands of about 1686bp after their DNA was amplified by PCR followed by separation and visualization of PCR products on agarose gel electrophoresis, Figure 3. The resulting PCR products were sequenced and 13 isolates were identified as *B. cereus sensu lato*. Analysis of the level of similarity of the 13 isolate sequences was done by matching them with reference strain sequences available in GenBank data center using BLAST program. Based on BLAST analysis, 12 isolates (M1, M3, M4, P4, P5, P8, P9, P10, M13, M14, P13, and P15) showed highest similarity (>98) to *16S rDNA* sequences of *Bacillus cereus sensu stricto* strains from NCBI. We therefore identify these isolates as *B. cereus* species. One isolate (M15) showed (99%) similarity to *Bacillus thuringiensis* strain ATCC 10792 and it was identified as *B. thuringiensis.*

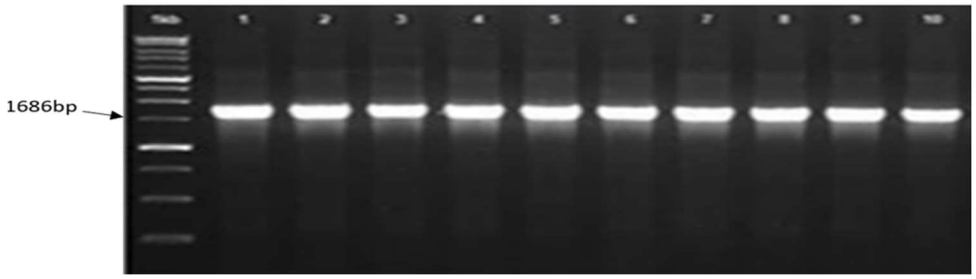

Figure 3. Visualization of *16S rDNA* gene bands on agarose gel electrophoresis. First bar is 100 bp ladder; lanes 1 to 10 are chili isolates in this study.

Phylogenetic identification of B. cereus isolated from white pepper and Chili

To prove the BLAST analysis results, a kinship analysis was carried out by constructing a phylogenetic tree. Results of the phylogenetic tree Figure 4, showed that isolates M1, M3, M4, M13 and M14 from chili and isolates P4, P5, P8, P9, P10, P13 and P15 from white pepper were on the same phylogenetic clade with several *B. cereus* strains, including ATCC 14580 accession number NR_074540.2, ATCC 14579 accession number NR_074540.1, JCM 2152 accession number NR_113266.1, NBRC 15305 accession number NR_112630.1 and CCM 2010 accession number NR_115714.1, thus, they are closely related to *B. cereus*. We therefore conclusively identify these isolates as *B. cereus* species. Although *B. pseudomycoides* was also located on the same clade, there was a larger phylogenetic distance from our strains therefore we could not identify them as *B. pseudomycoides*. *B. cereus* and *B. pseudomycoides* have been reported to be very close, only differing in their fatty acid composition and DNA and this could explain their location on the same phylogenetic clade. Isolate M15 (from chili) showed greater kinship to *B. thuringiensis* strain ATCC 10792 accession number NR_114581 thus proving the BLAST analysis results and it has closer kinship to *B. thuringiensis.*

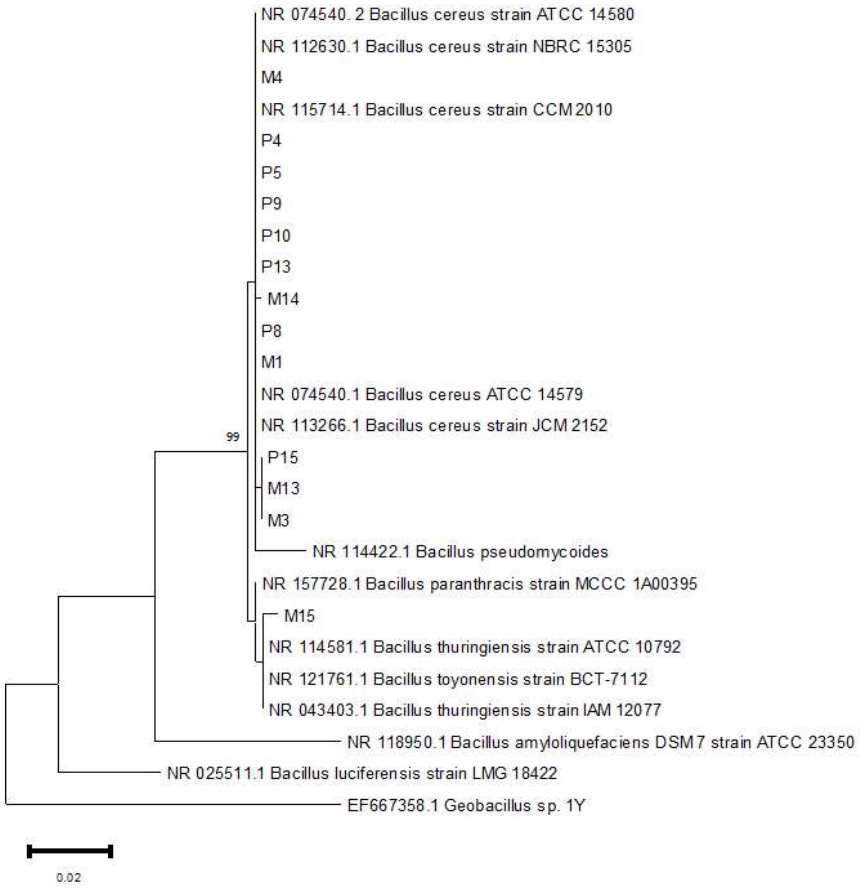

Figure 4. Maximum likelihood dendrogram showing the phylogenetic positions of the 13 isolates and other reference strains from *B. cereus* group based on *16S rDNA* gene. P codes are strains from white pepper and M codes are strains from chili. *Geobacillus* sp. 1Y (EF667358.1) was used as an outgroup.

A very close relationship has been reported between *Bacillus cereus sensu lato* (*Bacillus cereus* group members) including *Bacillus cereus sensu stricto* (*Bacillus cereus*) and different researchers have expressed the difficulty in differentiating between them based on morphological, biochemical, *16S rDNA* and phylogenetic testing. (Liu *et al*., 2015; Griffiths and Schraft 2017; Fayad *et al*., 2019). For example, conventionally, *B. cereus* and *B. thuringiensis* could be differentiated on the basis of production of paraspinal crystals. However, it has been reported that some plasmids encoding crystal formation may be lost during culturing yet authentic *B. cereus* cultures can acquire the ability to form crystals if grown in a culture containing *B. thuringiensis* (Bavykin *et al*., 2004), making their differentiation even more difficult. According to Fayad *et al.*, (2019), use of the maximum-likelihood phylogeny still could not differentiate between the two organisms. However, *(*Liu *et*

al., 2015) was able to differentiate them using a combination of methods among which were *16S rDNA* gene analysis and toxin-coding gene screening.

The phylogenetic tree also showed that the out group, reference strains and isolates obtained in this study evolved from a common ancestor with a divergence of 0.02, which indicates that the level of homology of *16S rDNA* nucleotide sequences of our isolates and nucleotide sequences of reference strains differ with a 2% constituent base sequence and this could indicate the level of mutation that has occurred between these organisms.

Potential toxigenicity of B. cereus isolated from white pepper and chili

Bacillus cereus produces four enterotoxins namely, Hemolysin BL (*Hbl*), nonhemolytic enterotoxin (*nhe*), enterotoxin FM (*entFM)* and cytotoxin K (*CytK*), which cause the diarrheal syndrome. Additionally, it also produces cereulide toxin which causes emesis. In this study, we only tested for the emetic toxin encoding gene, *ces* and the *nhe* toxin encoding gene *nheA* since it is the most widely distributed of the four enterotoxins.

All the twelve isolates analysed showed clear and thick bands in accordance to the amplicon's target size of 617 bp for *nheA* Figure 5, proving that these isolates possessed the *nheA* gene. Our findings closely collate with those reported in earlier studies. For example, 96.6% of the spice and herb samples contained *nheA* gene and produced the *nhe* toxin (Frentzel *et al*., 2016), 47 of 50 (94%) isolates from spices and herbs had the *nheA* gene (Fogele *et al*., 2018), 95.4% of 151 isolates from raw vegetables possessed *nheA* with 100% detection in bell pepper (Park *et al*., 2018), 99% of 97 isolates from cassava starch carried the *nheABC* gene (Sánchez-Chica *et al*., 2021) and 89% of the isolates from ready to eat food samples possessed the *nheA* gene (Yu *et al*., 2020).

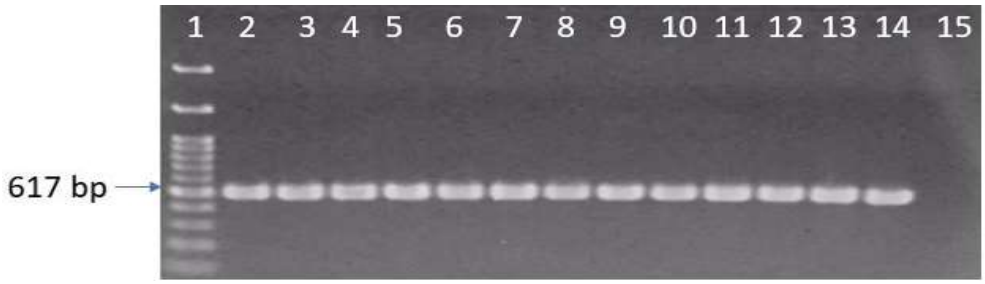

Figure 5. PCR amplicons on 2% agarose gel viewed under Bio-Rad trans-illuminator. Lane 1 is a 100 bp DNA ladder marker, lane 2 is positive control, lanes 3 to 9 are isolates P4, P5, P8, P9, P10, 13P, P15; lanes 10 to 14 are isolates M1, M3, M4, M13, M14 and lane 15 is negative control.

A 100% presence of the *nhe* toxin encoding gene in white pepper and chili presents a possible food safety concern and a risk for diarrheal food borne disease and outbreaks.

None of the 12 isolates showed any band at the target length of 634 bp for *ces* implying absence of the *ces* gene. The absence of the *ces* gene from our isolates may be because: (1) The emetic toxin has been detected especially in *B. cereus* strains isolated from starchy foods (especially rice and pasta) (Griffiths and Schraft, 2017) and this could explain why white pepper and chili in this study did not contain *ces*, the emetic toxin encoding gene. (2) Production of emetic toxins is limited to very few *B. cereus* strains (Fogele *et al.*, 2018), unlike the diarrheal toxin which is produced by almost all *B. cereus* evolutionary strains. Thus, our isolates possibly differed evolutionally from the emetic toxin producing strains. According to Wehrle et al. (2010), the incidence of ces gene in *B. cereus* species strains is low, generally less than 5%. In harmony with this, Chon *et al*. (2015), Fogele *et al*. (2018), Park *et al*. (2018) and Sánchez-Chica *et al*. (2021), did not find any *ces* genes in all the spice, ready to eat vegetables, vegetable and cassava starch samples they analyzed, respectively. Moreover, Ceuppens *et al*. (2011) and Frentzel *et al*. (2016) reported only 1.5% and 1.7% of their samples to contain the *ces* gene. However, Kim *et al*. (2013); Owusu-Kwarteng *et al*. (2017) and Yu *et al*. (2020) reported 13.2%, 9% and 7% of isolates from their red pepper, milk products and ready-to-eat food respectively to be positive for *ces*. The absence of the emetic toxin encoding gene (*ces*) reduces the potential risk for emesis from chili and white pepper from Bogor area.

Conclusions

The results of this study suggest a similar level of presumptive *B. cereus* in traditional and supermarkets. The absence of the emetic toxin encoding gene (*ces*) reduces the risk for emesis from chili and white pepper from Bogor area. However, the high contamination level coupled with 100% prevalence of the *nheA* gene in white pepper poses a potential food safety concern and a risk for *B. cereus* diarrheal food borne disease and outbreaks to people around Bogor.

It is recommended that ready to eat foods to which chili and or white pepper has been added should be consumed immediately or stored at temperatures below 4°C in order to prevent the multiplication of vegetative cells and germination of spores which could result in concentrations above 10^4 CFU/g, which is considered hazardous by the National Agency for Drug and Food Control (NADFC) and EFSA. Good sanitation and hygiene practices should also be ensured throughout the chili and white pepper supply chain.

Acknowledgments

The authors are grateful to the Directorate General for Higher Education, Ministry of Education and Culture, Indonesia for funding this research. We also appreciate the assistance rendered through the KNB scholarship.

References

Arisanti, R.R., Indriani, C., Wilopo, S.A. 2018. Kontribusi agen dan faktor penyebab kejadian luar biasa keracunan pangan di Indonesia: *kajian sistematis*. *Journal of Community Medicine and Public Health*. **34** (3), 99–106.

Bavykin, S.G., Lysov, Y.P., Zakhariev, V., Kelly, J.J., Jackman, J., Stahl, D.A., Cherni, A. 2004. Use *of 16S rRNA*, 23S rRNA, and gyrB gene sequence analysis to determine phylogenetic relationships of *Bacillus cereus* group microorganisms. *Journal of Clinical Microbiology*, **42**(8), 3711–3730.

Berthold-Pluta, A., Pluta, A., Garbowska, M., Stefańska I. 2019. Prevalence and toxicity characterization of *Bacillus cereus* in food products from Poland. *Foods*, **8**(7), 1–12.

Ceuppens, S., Rajkovic, A., Heyndrickx, M., Tsilia, V., Van De Wiele, T., Boon, N., Uyttendaele, M. 2011. Regulation of toxin production by *Bacillus cereus* and its food safety implications. *Critical Reviews in Microbiology*, **37**(3), 188–213.

Chon, J.W., Yim, J.H., Kim, H.S., Kim, D.H., Kim, H., Oh, D.H., Kim, S.K., Seo, K.H. 2015. Quantitative Prevalence and Toxin Gene Profile of *Bacillus cereus* from Ready-to-Eat Vegetables in South Korea. *Foodborne Pathogens and Disease*, **12**(9), 795–799.

FAO/WHO. 2014. Ranking of Low Moisture Foods in Support of Microbiological Risk Management: Preliminary report of FAO/WHO expert consultation on ranking of low moisture foods. *Part I – Main Report. Rome/ Geneva: FAO/ WHO*. https://ucfoodsafety.ucdavis.edu/sites/g/files/dgvnsk7366/files/inline-files/209893.pdf

Fayad, N., Awad, M.K., Mahillon, J. 2019. Diversity of *Bacillus cereus* sensu lato mobilome. *BMC Genomics*, **20**(1), 1–11.

Fogele, B., Granta, R., Valciņa, O., Bērziņš, A. 2018. Occurrence and diversity of *Bacillus cereus* and moulds in spices and herbs. *Food Control*, **83**(2018), 69–74.

Frentzel, H., Kraushaar, B., Krause, G., Bodi, D., Wichmann-Schauer, H., Appel, B., Mader, A. 2016. Phylogenetic and toxinogenic characteristics of *Bacillus cereus* group members isolated from spices and herbs. *Food Control*, **83**(2016), 90–98.

Griffiths, M.W., Schraft, H. 2017. *Bacillus cereus* Food Poisoning. In *Foodborne Diseases:* Dodd, C., Aldsworth, T., Stein R., 3rd ed., pp. 395-405. *Elsevier Inc.*

Hampikyan, H., Bingol, E.B., Colak, H., Aydin, A. 2009. The evaluation of microbiological profile of some spices used in Turkish meat industry. *Journal of Food, Agriculture and Environment*, **7**(3–4), 111–115.

Hariram, U., Labbe, R. 2015. Spore prevalence and toxigenicity of *Bacillus cereus* and *Bacillus thuringiensis* isolates from U.S. retail spices. *Journal of Food Protection*, **78**(3), 590–596.

ISO. 2004. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of presumptive *Bacillus cereus* - Colony-count technique at 30 °C. ISO 7932:2004/Amd.1:2020(E).

Jessim, A.I., Sabah, S., Fakhry, S.J., Alwash, S. 2017. Detection and determination of B*acillus cereus* in cooked rice and some types of spices with ribosomal *16SrRNA* gene selected from Iraqi public restaurants. *International Journal of Bio-resource and Stress Management,* **8**(3), 382-387.

Kim, H.J., Baek, S.Y., Lee, N., Oh, S.W. 2013. Organic acid resistance and toxin gene profiles of *B. cereus* isolated from red pepper powder. *Journal of Food Safety*, **33**(3), 319–326.

Kim, J., Forghani, F., Kim, J., Park, Y., Park, M., Wang, J., Park, J.H., Oh, D. 2012. Improved multiplex pcr assay for simultaneous detection of *Bacillus cereus* emetic and enterotoxic strains. *Food Science and Biotechnology* **21**(5), 1439–1444.

Liu, Y., Lai, Q., Göker, M., Meier-Kolthoff, J.P., Wang, M., Sun, Y., Wang, L., Shao, Z. 2015. Genomic insights into the taxonomic status of the *Bacillus cereus* group. *Scientific Reports*, **5**, 1–11.

Mathot, A.G., Postollec, F., Leguerinel, I. 2021. Bacterial spores in spices and dried herbs: The risks for processed food. *Comprehensive Reviews in Food Science and Food Safety*, **20**(1), 840–862.

Nanteza, H., Dewanti-Hariyadi, R., Nurjanah, S. 2021. Occurrence of *Bacillus cereus* in white pepper from Bogor area, Indonesia. Presented at International *Conference on Green Engineering, Food Agricultural Science and Technology* (accepted to be published in ICGEFAST proceedings.)

Owusu-Kwarteng, J., Wuni, A., Akabanda, F., Tano-Debrah, K., Jespersen, L. 2017. Prevalence, virulence factor genes and antibiotic resistance of *Bacillus cereus* sensu lato isolated from dairy farms and traditional dairy products. *BMC Microbiology*, **17**(1), 9–16.

Park, K.M., Jeong, M., Park, K.J., Koo, M. 2018. Prevalence, enterotoxin genes, and antibiotic resistance of *Bacillus cereus* isolated from raw vegetables in Korea. *Journal of Food Protection*, **81**(10), 1590–1597.

Sacchi, C.T., Whitney, A.M., Mayer, L.W., Morey, R., Steigerwalt, A., Boras, A., Weyant, R.S., Popovic, T. 2002. Sequencing of *16S rRNA* gene: A rapid tool for identification of *Bacillus anthracis*. *Emerging Infectious Diseases*, **8**(10), 1117–1123.

Sánchez-Chica, J., Correa, M.M., Aceves-Diez, A.E., and Castañeda-Sandoval, L.M. 2021. Enterotoxin gene distribution and genotypes of *Bacillus cereus* sensu lato isolated from cassava starch. *Toxins*, **13**(2), 131.

Son, K.L., Nugroho, A.S.D., Rahayujati, B., Gozali, L.K. 2020. Food poisoning outbreak caused by diarrhoeal *Bacillus cereus* in Tegalkenongo village, Bantul, Yogyakarta, Indonesia: A retrospective study. *Asia Pacific Family Medicine*, **18**(1), 1–5.

SPICED/European Commission. 2016. Securing the spices and herbs commodity chains in Europe against deliberate, accidental or natural biological and chemical contamination. 36(June).

Vital, P.G., Dimasuay, K.G.B., Widmer, K.W., Rivera, W.L. 2014. Microbiological quality of fresh produce from open air markets and supermarkets in the Philippines. *Scientific World Journal*, **2014**, 219534.

Wehrle, E., Didier, A., Moravek, M., Dietrich, R., Märtlbauer, E. 2010. Detection of *Bacillus cereus* with enteropathogenic potential by multiplex real-time PCR based on SYBR green I. *Molecular and Cellular Probes*, **24**(3), 124–130.

Yu, S., Yu, P., Wang, J., Li, C., Guo, H., Liu, C., Kong, L., Yu, L., Wu, S., Lei, T., Chen, M., Zeng, H., Pang, R., Zhang, Y., Wei, X., Zhang, J., Wu, Q., Ding, Y. 2020. A study on prevalence and characterization of *Bacillus cereus* in ready-to-eat foods in China. *Frontiers in Microbiology*, **10**(3043), 1–11.

Zhang, Z., Feng, L., Xu, H., Liu, C., Shah, N.P., Wei, H. 2016. Detection of viable enterotoxin-producing *Bacillus cereus* and analysis of toxigenicity from ready-to-eat foods and infant formula milk powder by multiplex PCR. *Journal of Dairy Science*, **99**(2), 1047–1055.

https://sourceforge.net/projects/genestudio/

https://www.ncbi.nlm.nih.gov/

www.ncbi.nlm.nih.gov

www.megasoftware.net.