The Nutritional Value and Antioxidant Activities of the Developed Thai Fermented Bhutan Oyster Mushroom (*Pleurotus eous*)

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Abstract

"Nham" Thai fermented sausage food product developed from Bhutan oyster mushroom (*Pleurotus eous*) and mixed with cooked Riceberry rice was compared to Bhutan oyster mushroom mixed with cooked white rice. The objectives of this experiment were to develop Nham Thai fermented food and observe the natural probiotic lactic acid bacteria, microbial pathogens contamination, antioxidant activity, and nutritional composition. The results indicated that fermented Bhutan oyster mushroom had pH value of 4.41 to 4.51, while the percentage of lactic acid was 0.46 to 0.55. Fermented Bhutan oyster mushroom had natural probiotic lactic acid bacteria at approximately 10^7-10^8 CFU/g. Microbial pathogens including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* sp., *Clostridium perfringens*, yeasts, and molds were not found in products. For total phenolic content and the percentage of free-radical inhibition, the fermented Bhutan oyster mushroom mixed with cooked Riceberry rice contained higher amounts than fermented cooked white rice. Moreover, the fermented Bhutan oyster mushroom mixed cooked Riceberry rice had high protein and dietary fiber while having low fat, low carbohydrate, and low energy. Hence, the development of "Nham" Thai fermented sausage could benefit from the use of Bhutan oyster mushroom mixed with cooked Riceberry rice in processing. It is suggested that this product could be used as an alternative vegetarian food.

Keywords: Fermented Bhutan oyster mushroom, Lactic acid bacteria, Antioxidant activities, Nutrient content, Dietary fiber

Introduction

Nham (Thai fermented sausage) is a popular Thai fermented food product. Traditionally, it is made from fresh pork, pork skins, herbs, and cooked rice. The mixture is wrapped with cleaned banana leaves or plastic bags and then fermented for 3 days at ambient temperature (Thailand). This method is one approach to food preservation that extends the shelf-life and good taste of food. However, this product is made from uncooked fresh pork. It is possible to contaminate pathogenic microbes such as *Salmonella* sp., *Staphylococcus aureus*, *Escherichia coli,* yeast, or mold (Petchsing & Woodburn, 1990; Osiriphun et al., 2004). They can attack the digestive tract of humans and negatively affect human health. The fermented pork is neither vegetarian food nor halal food. Nham was added nitrate nitrite or fresh garlic, which can reduce pathogen bacteria such as *Salmonella* sp. However, nitrate and nitrite are chemical food additives. For fresh garlic report revealed that at least 5% of fresh garlic decreased *Salmonella* sp. The advantage of adding garlic is that it stimulates the growth of lactic acid bacteria and decreases the pH effect to inhibit various food poisoning bacteria (Swetwiwathana, Leutz, Lotong, & Fischer, 1999).

Nowadays, Nham is modified to use mushrooms instead of fresh pork because it tastes like pork in fermented food and is also suitable for vegetarians. Edible mushrooms are one type of fungi. They are popular components in foods or flavor ingredients in Thai food. In addition, mushrooms have been an ingredient in the human diet due to their nutritional value, phenolic compounds, and medicinal properties. For phenolic compounds, the contents of the edible mushrooms have the ability to scavenge free-radicals in the metabolism of humans, thus playing a role in the control of cancer and other human diseases (Palacios et al., 2011). Furthermore, the properties of phenolic compound (phenolic acids) have been revealed to possess antibacterial and antifungal activities (Alves et al., 2012). *Pleurotus* mushroom is an edible mushroom that rapidly grows in Asia and is mostly cultured in Thailand (Ministry of Agriculture and Cooperatives, 2012). Some species such as the Bhutan oyster mushroom (*Pleurotus eous*) have benefits including low sodium, low cholesterol levels, and low calorific values. They are a potential source of prebiotics as well as highly nutritional, containing 1-4% protein, 0.1-0.4% fat, 0.6-2.4% fiber, 0.4-.09% ash and 89-92% moisture (Mattila et al., 2001; Yang, Lin, & Mau, 2001; Aida et al., 2009). As such, this mushroom is used in cooking for Thai food.

In Thailand and Southeast Asia, rice is the most important in terms of main food products. Recently, Thai purple rice, called Riceberry rice, has been developed to provide more nutrients than possible with white rice. Riceberry rice does not only have high nutrient values such as protein and crude fiber, but also has high antioxidant properties such as poly-phenol compounds, anthocyanin, vitamin E, and gamma-oryzanol (Pukdee, Kumar, Chaiwut, & Sripisut, 2016; Settapramote, Laokuldilok, Boonyawan, & Utama-ang, 2018).

No more information or literature is available concerning the antioxidant activity and nutrient composition of fermented Bhutan oyster mushroom sausage. Therefore, the objective of this experiment is to develop Nham Thai fermented food from Bhutan oyster mushroom cooked mixed with rice cooked or Riceberry rice cooked and then fermented for 3 days. Fresh garlic as Thai herb was added in the ingredients. Each treatment of the fermented Bhutan oyster mushrooms was observed for lactic acid bacteria (LAB) on MRS agar and the microbiological quality of food safety. Moreover, the pH, percentage of lactic acid, antioxidant activity, and nutrient composition in each treatment were measured. Ingredients were not supplemented with chemical food additive, seasoning or preservative. This research aimed to develop good quality Nham from Bhutan oyster mushroom as an alternative food product for consumers.

Methods and Materials

Nham preparation

Bhutan oyster mushroom (*Pleurotus eous*), rice, and Riceberry rice (*Oryza sativa*) used in this experiment were purchased from a local farmer in Phra Nakhon Si Ayutthaya, Thailand. Starting with a small size, the Bhutan oyster mushroom was cooked by steaming water (boiling water estimated at 95°C) then tap squeeze water. Rice and Riceberry rice were cooked separately using a rice cooker. The fermented oyster mushroom was modified based on a typical fermented mushroom sausage recipe (modified from Chockchaisawasdee et al., 2010). The mixture ratio of the ingredients is shown in Table 1. Start with 25 g of each mixture was put into cleaned plastic bags and sealed in a triangle shape, after which they were left to ferment at ambient temperature for 72 hours.

Table 1 The ratio of fermented Bhutan oyster mushroom sausage ingredients

Treatment No.	Bhutan oyster mushroom cooked (%)	Rice cooked (%)	Riceberry rice cooked (%)	Fresh chopped garlic (%)	Iodine salt (%)	Fresh peppermint (%)
1	79.0	10	-	10	1	-
2	78.5	10	-	10	1	0.5
3	79.0	-	10	10	1	-
4	78.5	-	10	10	1	0.5

Chemical analysis of fermented mushroom sausage

After 3 days of fermentation, 25 g of each treatment was taken and homogenized with 100 mL of sterile distilled water to test pH. The pH was determined in triplicate using a pH meter (AOAC, 2000). For lactic acid titration, the samples were taken as well as pH analysis, but phenolphthalein indicator and NaOH were used for titration following AOAC (2000), in triplicate.

Lactic acid bacteria examination and microbiological quality analysis of fermented mushroom sausage

25 g of each treatment was contained in a sterile bag and suspended in 225 mL of 0.1% sterile peptone saltwater then macerated for 60 seconds using a Stomacher. The homogenate was diluted 10-fold serial dilution in 0.1% sterile peptone saltwater (10^{-2} to 10^{-7}). The lactic acid bacteria were determined by using the pour plate technique and counted on MRS agar (dilution of 10^{-5} to 10^{-7}), incubated at 37°C for 48 hours. After incubation, the average number of 30-300 colonies was enumerated and the calculated results were shown as a colony-forming unit (CFU) per g. The yellow colonies showing a clearing zone around were selected and then 30 isolates of LAB were purified on MRS plate by the streak plate technique for morphology analysis and biochemical test (motility test, glucose fermentation, and catalase test) in triplicate (Holt et al., 1994; Khalil and Anwar, 2016).

For the microbiological quality test, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* sp., *Clostridium perfringens*, yeasts and molds count were evaluated as follows: BAM method [16]. Briefly, the *E. coli* was tested on eosin-methylene blue (EMB) agar plates and incubated at a temperature of 37°C for 24-48 hours. If the plate showed a colony, with or without a metallic sheen, the IMViC test was confirmed. The *S. aureus* was tested on Baird-Parker agar plates and incubated at a temperature of 37°C for 24-48 hours. If the plate showed a colony, the catalase test and coagulase test were confirmed. The *Salmonella* sp. was tested on *Salmonella Shigella* agar plates. The *C. perfringens* was tested on tryptose-sulfite-cycloserine (TSC) agar plates and incubated under anaerobic conditions. The yeasts and molds were tested on potato dextrose agar (PDA) plates and Dichloran rose bengal chloramphenicol (DRBC) agar plates and incubated at 30°C for 2-3 days (BAM, 1998).

Antioxidant activity and nutritional value analysis of fermented mushroom sausage

The samples of each treatment were homogenized and extracted using 10% of ethanol (ratio 1:20) then a shaker at a temperature of 60°C for 1 hour. After that, the extracted samples were taken for determination of total phenolic contents and antioxidant activity (DPPH assay), as modified from Jabłońska-Ryś et al. (2016). The total phenolic contents were observed by using 2 mL of extracted sample mixed with 8 mL of Folin-ciocalteu phenol reagent in the test tube for 3 min then 2 mL of 7.5% Na_2CO_3 was added in each sample tube of each treatment. Gallic acid was used for the preparation of a standard curve with a calibration range of 0-100 mg/L (coefficient of determination R^2 = 0.996). The results were revealed as mg of gallic acid equivalents (GAE) per 1 g of fermented mushroom sample. The DPPH radical scavenging activity was observed by 0.2 mL of ethanol extracted sample mixed with 2.8 mL of 0.1 mM DPPH ethanol solution, which was then mixed well and left to stand for 30 min in the dark. After that, the antioxidant activity was shown as a percentage of free-radical inhibition. Briefly, treatment No. 1 and treatment No. 3 were selected for determination of nutritional proximate composition of "Nham" fermented Bhutan oyster mushroom mixed with cooked rice or cooked Riceberry rice. The samples were analyzed for composition including moisture, fat, ash, protein, and dietary fiber, as described in a previous method (AOAC, 2016). Carbohydrates and calories (energy) were investigated following the method of analysis for nutrition labeling (Sullivan & Carpenter, 1993).

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA), SPSS23. The significant differences among the samples receiving the different treatments showed a significance level at $p<0.05$.

Results and Discussion

The chemical analysis of fermented mushroom sausage yielded the following results.

After fermentation (3 days), four fermented Bhutan oyster mushroom samples exhibited pH and lactic acid, as shown in Table 2. The results showed that the fermented mushroom samples had pH from 4.41±0.03 to 4.51±0.03 and level of lactic acid (%) from 0.46±0.00 to 0.55±0.00. It is possible that the pH and lactic acid of each treatment involved moisture content or mushroom content. A report has revealed that fermented mushroom sausage samples containing more mushrooms had higher moisture content, which might be suitable for the growth of lactic acid bacteria (Chockchaisawasdee et al., 2010). Also, *Pleurotus ostreatus* (oyster) and *Cantharellus cibarius* (chanterelle) are popular edible mushrooms fermented using *Lactobacillus plantarum* lactic acid bacteria (LAB) as starter cultures. After 3-4 days, the fastest increase of pH was seen in fermented mushrooms and remained unchanged throughout the storage period. The reduction of pH values in fermented mushrooms also reflected the elevation of the lactic acid content (Jabłońska-Ryś et al., 2016). Lactobacilli had a significant role in the production of lactic acid, which caused a decrease in the pH. Acidity resulted in protein coagulation and contributed to the firmness of the product. The lactic acid also gave the acidic taste and volatile compounds in Nham (Rotsatchakul, Visesanguan, Smitinont, & Chaiseri, 2009). Acidic pH is suitable for Nham production and helps prevent the growth of microbes such as *Staphylococcus aureus*. As well, sourdough is traditional fermented dough made from natural fermentation by LAB. This product showed a low pH of 4.5 involved in LAB communities such as *Weissella*, *Pediococcus* and *Lactobacillus* and their fermentation properties (Oshiro et al., 2020). The fermentation conditions (acidic pH and lactic acid content) were involved in the shelf-life as well as the storage period of Nham product, at which the finished product is microbiologically stable for six months under 4-6°C of refrigeration (Jabłońska-Ryś et al., 2019).

Table 2 The pH and the percentage of lactic acid of the fermented Bhutan oyster mushroom sausage, fermented at ambient temperature for 3 days, in triplicate

Treatment No.*	pH	Lactic acid (%)	Average of lactic acid bacteria (CFU/g)
1	$4.51^{a}\pm0.03$	$0.52^{b}\pm0.04$	1.84×10^{8}
2	$4.41^{c}\pm0.02$	$0.46^{d}\pm0.02$	1.39×10^{7}
3	$4.44^{bc}\pm0.03$	$0.55^{a}\pm0.02$	2.15×10^{8}
4	$4.49^{ab}\pm0.02$	$0.50^{c}\pm0.03$	1.32×10^{7}

*1) cooked Bhutan oyster mushroom + cooked rice, 2) cooked Bhutan oyster mushroom + cooked rice + fresh peppermint leaves, 3) cooked Bhutan oyster mushroom + cooked Riceberry rice, and 4) cooked Bhutan oyster mushroom + cooked Riceberry rice + fresh peppermint leaves

Values are mean ± SD and mean values followed by the same letters are not significantly different at ($p<0.05$)

^{abcd} are significantly different at ($p<0.05$) with in column

The lactic acid bacteria examination of fermented mushroom sausage

The four fermented Bhutan oyster mushroom sausage samples were examined for total lactic acid bacteria. Nham treatment No. 3 (cooked Bhutan oyster mushroom mixed with cooked Riceberry rice) enhanced the highest of total lactic acid bacteria by approximately 2.15×10^{8} CFU/g (Table 2). A reported showed that fermented mushroom (Sajor-caju mushroom) mixed with cooked riceberry rice had total lactic acid bacteria count of 2.83×10^{5} CFU/g (Wittanalai, Tanruean, & Mapoong, 2019). Some information reported that mushrooms are the raw material for lactic acid fermentation; this method is utilized for food preservation and lactic acid bacteria group as probiotics for health-promoting properties (Jabłońska-Ryś et al., 2019). For treatment No. 2 and treatment No. 4 showed a reduced decrease of the total lactic acid bacteria in the treatments that contained fresh peppermint mixed with ingredients (Table 2). The peppermint oil from leaves reported high antioxidant activity and high antibacterial activity against some gram-positive and gram-negative bacterial strains (Singh, Shushni, & Belkheir, 2015). It possible inhibit lactic acid bacteria growth. The lactic acid bacteria on the MRS plates in this work were observed for morphology and biochemical testing (data not shown). The colony morphology results indicated that all of the selected lactic acid bacteria for each treatment had cream colonies color, circular form, entire or undulated margins, smooth surface, flat elevation, and opaque optical characteristics. Cell morphology under a microscope (magnification of 1000x) showed that all of the selected lactic acid bacteria for each treatment were gram-positive (staining is purple) and the rod-shape cell belonged to a group of lactic acid bacteria (Holt et al., 1994; Khalil & Anwar, 2016). Besides, they also revealed non-motility, positive glucose fermentation, and negative catalase test (data not shown). As well the reported investigated that the morphological characteristics of lactic acid bacteria was isolated from Nham Hed (fermented mushroom) (Wittanalai et al., 2019). The lactic acid bacteria group of food products is widely studied because of their ability to promote probiotic properties and exert an antagonistic effect on gastroenteric pathogens, antitumoral activity, reduction of serum cholesterol, stimulation of the immune system, and stabilization of the gut microflora (Ljungh & Wadstrom, 2006; Naidu et al., 1999; Yan & Polk, 2011).

The microbiological quality analysis of fermented mushroom sausage

The quality control of microbial contamination in the fermented sausage called Nham is very important for consumers' health. Nham was originally made from uncooked pork that could contain contamination of pathogens such as *Staphylococcus aureus* and *Salmonella* spp., which are commonly found in poultry and pork meats (Osiriphun et al., 2004). In this experiment, we used cooked Bhutan oyster mushroom instead of uncooked pork for Nham fermented sausage production because mushroom has no reported microbial pathogen contamination. It is a part of the human diet due to its nutritional value, phenolic compounds, and medicinal properties. For the phenolic compounds content, edible mushrooms have the ability to scavenge free-radicals in the metabolism of humans, thus playing a role in the control of cancer and other human diseases (Palacios et al., 2011). They have also been revealed to possess antibacterial and antifungal activities. The results indicated that all the treatments that included Bhutan fermented mushroom did not contaminate microbial pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* sp., *Clostridium perfringens*, yeasts and molds (Table 3).

Table 3 The microbiological quality analysis of fermented Bhutan oyster mushroom sausage including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* sp., *Clostridium perfringens*, yeasts and molds, in triplicate

Treatment No.*	*E. coli* (CFU/g)	*S. aureus* (CFU/0.1g)	*Salmonella* sp. (CFU/25g)	*C. perfringens* (CFU/0.1g)	yeasts and molds (CFU/g)
1	<3	ND	ND	ND	<10
2	<3	ND	ND	ND	<10
3	<3	ND	ND	ND	<10
4	<3	ND	ND	ND	<10

ND means Non-detected

*1) cooked Bhutan oyster mushroom + cooked rice 2) cooked Bhutan oyster mushroom + cooked rice + fresh peppermint leaves 3) cooked Bhutan oyster mushroom + cooked Riceberry rice and 4) cooked Bhutan oyster mushroom + cooked Riceberry rice + fresh peppermint leaves

The compounds are fatty acid esters of *Pleurotus eous* extract, which have been reported to have biocompounds that are antibacterial against *S. aureus, Bacillus subtilis, B. cereus, Pseudomonas aeruginosa E. coli,* and *Klebsiella pneumonia* (Suseem & Mary, 2013). Moreover, mushrooms have been researched for properties of the bioactive compounds of mushroom extracts that have higher antimicrobial activity against both gram-positive and gram-negative bacteria (Alves et al., 2012). Therefore, we suggest that the process for fermented mushroom sausage in this research is suitable in terms of food safety and microbiological quality control, which are vital to maintaining the health of consumers (Vose, 1998).

The antioxidant activity and nutritional value analysis of fermented mushroom sausage

Research concerning edible mushrooms has reported that they possess high protein content and high fiber, but low-fat content. Moreover, a variety of studies concerning the antioxidant properties of cultivated and wild edible mushrooms are widely available. This research is the first instance in which the interested results revealed that the fermented mushroom sausage showed the total phenolic content as an antioxidant (Table 4), Interestingly, Nham from Bhutan oyster mushroom products was also investigated for their antioxidant properties (DPPH assay). Our results showed that the percentages of inhibition of free-radicals for treatment No. 3 (20.64±0.76) and treatment No. 4 (21.94±0.75) were not significant ($p<0.05$) (Table 4). As treatment No. 1 and treatment No. 2 decreased in antioxidant properties, they were statistically significant ($p<0.05$) compared with treatment No. 3 and treatment No. 4 (Table 4). Some research reported that the oyster mushroom or chanterelle mushroom mixed with *Lactobacillus plantarum* as lactic acid bacteria (LAB) for lactic acid fermentation had high concentrations of total phenolic content. Both of the fermented mushrooms had high scavenging activities against DPPH radicals with estimated values of 15-20 µmol TE/g DW (Jabłońska-Ryś et al., 2016; Jabłońska-Ryś et al., 2019). Moreover, Riceberry rice also has higher antioxidant properties than white rice (Pukdee et al., 2016; Settapramote et al., 2018).

Table 4 Total phenolic contents and free-radical inhibition of fermented Bhutan oyster mushroom sausage, fermented for 3 days, in triplicate

Treatment No.*	Total phenolic contents (mg GAE/g)	DPPH (% free-radical inhibition)
1	137.00^{d}±0.82	14.84^{c}±0.52
2	154.17^{b}±1.03	18.09^{b}±0.82
3	150.33^{c}±1.25	20.64^{a}±0.76
4	159.00^{a}±0.82	21.94^{a}±0.75

*1) cooked Bhutan oyster mushroom + cooked rice, 2) cooked Bhutan oyster mushroom + cooked rice + fresh peppermint leaves, 3) cooked Bhutan oyster mushroom + cooked Riceberry rice, and 4) cooked Bhutan oyster mushroom + cooked Riceberry rice + fresh peppermint leaves

Values are mean ± SD and mean values followed by the same letters are not significantly different at ($p<0.05$)

^{abcd} are significantly different at ($p<0.05$) with in column

After giving 20 people all four treatments of fermented Bhutan oyster mushroom products to taste using a blind test, most of the people preferred treatment No. 3. Some people did not like treatments No. 2 and treatment No. 4 as they had a peppermint odor (data not shown). Hence, we selected treatment No. 3 compared with treatment No. 1 to examine the nutrition proximate composition of fermented mushroom sausage (Table 5). The results indicated that the cooked fermented Bhutan oyster mushroom mixed with cooked Riceberry rice (treatment No. 3) had lower amounts of fat, carbohydrate, energy, and ash than the cooked fermented Bhutan oyster mushroom mixed with cooked rice (treatment No. 1) (Table 5). However, treatment No. 3 had high protein (Table 5). Interestingly, treatment No. 3 also had high dietary fiber (Table 5). In developing countries, mushrooms have been used as a significant protein source in place of meat. *Pleurotus* mushrooms have been reported to be a health promoter; the proximate composition of fresh mushrooms includes high protein, moisture content and dietary fiber, but low crude fat (Tolera & Abera, 2017). This is the first time, the results propose that the fermented Bhutan oyster mushroom mixed with cooked Riceberry rice is vegetarian food processing that enhance natural probiotic LAB, high nutritional value and antioxidant activities of edible mushroom.

Table 5 The nutrition proximate composition of the fermented Bhutan oyster mushroom sausage (treatment No. 1 and treatment No. 3) when fermented for 3 days, in duplicate

Parameters	Treatment No.*	
	1	3
protein (g/100g)	$2.34^{b}\pm0.06$	$2.87^{a}\pm0.07$
fat (g/100g)	$0.21^{a}\pm0.03$	$0.15^{b}\pm0.02$
carbohydrate (g/100g)	$15.73^{a}\pm0.12$	$12.76^{b}\pm0.09$
moisture (g/100g)	$74.27^{b}\pm0.22$	$78.42^{a}\pm0.16$
dietary fiber (g/100g)	$4.24^{b}\pm0.05$	$7.62^{a}\pm0.02$
energy (Kcal/100g)	$69.55^{a}\pm0.16$	$55.67^{b}\pm0.12$
ash (g/100g)	$4.21^{a}\pm0.05$	$2.38^{b}\pm0.04$

*1) cooked Bhutan oyster mushroom + cooked rice 3) cooked Bhutan oyster mushroom + cooked Riceberry rice

Values are mean ± SD and mean values followed by the same letters are not significantly different at ($p<0.05$)

^{ab} are significantly different at ($p<0.05$) with in row

Conclusion and Suggestions

The development of fermented sausage called Nham from cooked Bhutan oyster mushroom (*Pleurotus eous*) mixed with cooked Riceberry rice for comparison with cooked Bhutan oyster mushroom mixed with cooked white rice. The results indicated that Bhutan oyster mushroom fermented sausage mixed with cooked Riceberry had total lactic acid bacteria (LAB) as a probiotic of approximately 10^{7}-10^{8} CFU/g. In this experiment, the suitable processing of Bhutan oyster mushroom fermented sausage products found no microbial pathogens such as *E. coli*, *S. aureus*, *Salmonella* sp., *C. perfringens* and yeasts, and molds. Interestingly, high amounts of total phenolic contents as antioxidant activities were found in Bhutan oyster mushroom fermented sausages. However, the percentage of free-radical inhibition for fermented Bhutan oyster mushroom mixed with cooked Riceberry sausage is higher than that for sausage mixed with cooked rice. Also, this treatment had a good nutrition proximate composition. Therefore, we suggest that Bhutan oyster mushroom can be used as a beneficial source of protein and dietary fiber for Nham fermented sausage products. Second, cooked Riceberry rice has antioxidants suitable for use as an ingredient in fermented sausage. Finally, Bhutan oyster mushroom fermented sausage mixed with cooked Riceberry rice product can be an alternative food product that is beneficial for human health.

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Conflicts of interest

No conflicts of interest.

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