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Research Article

Microbiological quality of commercial Thua Nao, a Thai fermented soybean product

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ABSTRACT

Thua Nao, a Thai fermented soybean product, is widely used as a condiment in many northern food. Although it is generally known that *Bacillus* species are predominant and thus play a key role during the soybean fermentation, information regarding other microbial population is limited. Besides, Thua Nao has currently been produced traditionally (i.e. by naturally occurring microbes) and this may raise a concern regarding food safety. This present study hence aimed to evaluate the microbiological quality of Thua Nao products sold in local markets of Chiang Rai and Phayao Provinces, Thailand. In total, twenty-four Thua Nao products (9 fresh and 15 dried samples) were collected from different local markets in Chiang Rai (20 samples) and Phayao (4 samples). Microbiological analyses were carried out for a total count of mesophilic aerobic bacteria (TMAB) and fungi; the presence of some pathogenic bacteria was also evaluated including *Bacillus cereus*, *Escherichia coli*, *Clostridium perfringens*, *Staphylococcus aureus*, and *Salmonella* species. Our data revealed that high levels of TMAB (>10^5 CFU/g) and moulds (>10^4 CFU/g) were found in most samples. *Escherichia coli*, *Salmonella* spp. and *Bacillus cereus* were detected in which *E. coli* was the most frequently isolated pathogenic bacteria accounting for 54.17% of the samples studied. However, our results showed that *C. perfringens* and *S. aureus* were not found in all samples. Based on this finding, commercial Thua Nao products prepared traditionally may not be hygienic. Practical guideline and monitoring of this microbial contamination should be implemented to ensure safer food for the consumers.

1. INTRODUCTION

Thua Nao, a Thai traditional fermented soybean, is popularly consumed in northern Thailand. It is used as a food condiment and due to its protein-rich nature, Thua Nao is often considered as a meat substitute. Thua Nao has been prepared by natural fermentation of the boiled soybeans. Traditionally, the fermentation is carried out in a bamboo basket, covered with banana leaves for 2 - 3 days at ambient temperature. Thua Nao is then cooked by steaming or roasting. The shelf-life of these fresh Thua Nao products is normally limited (ca. 2 days) by microbial activities which are strongly influenced by storage temperature[1]. Alternatively, fresh Thua Nao can be prepared into a flat disk and exposed to sunlight, resulting in the dried form of Thua Nao, known as Thua Nao Kab[2]. At present, the dried product is also available in

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the powder form. This post-fermentation process can prolong the shelf-life of Thua Nao product for several months. Similar fermented soybeans have been found in Asia such as Japanese Natto, Indian Kinema, Chinese Douche, and Korean Chungkukjang[3].

It has been well documented that bacteria in the genus *Bacillus* are responsible for the fermentation of these fermented soybean products. For example, several *Bacillus* species including *B. subtilis*, *B. pumilus*, *B. brevis*, *B. macerans*, *B. polymyxa*, and *B. licheniformis* can be isolated from Daddawa, Kinema, Thua Nao and Chungkukjang[2]. As a result, bacterial members of *Bacillus* species are a key target for screening of potential inoculum with an expectation to develop the fermentation process. However, it should be noted that, to date, only Natto has been produced commercially using the pure starter culture of *B. subtilis* strain *natto*. Besides, other microbial groups are also present and have been reported although the presence of fungi is often considered contamination as occurred in the case of Thua Nao[2]. Microbiological safety is thus considered as an important issue especially in developing countries like Thailand. To date, microbiological quality of some fermented soybeans (i.e., Kinema) and other legumes have been reported[4,5]; this information is, however, scarce for Thua Nao. The present study was hence performed to evaluate the microbiological quality of commercial Thua Nao in Chiang Rai and Phayao, Thailand. The data obtained were preliminary and further study is suggested in order to establish a safety guideline for these products.

2. MATERIALS AND METHODS

2.1 Thua Nao samples

Twenty-four commercial Thua Nao samples (9 fresh samples and 15 dried samples) were collected from local markets in Chiang Rai and Phayao (Table 1). In general, approximately 100 g of each sample was collected in sampling bags, transferred to the laboratory, and stored at 4°C until required.

2.2 Microbiological analysis

Twenty-five grams of each Thua Nao samples were aseptically weighed and mixed with 225 ml 0.1% peptone. The samples were then homogenized using a Stomacher (model 400; Seward Medical, UK) at 200 rpm for 2 min. Decimal dilutions were prepared with the same diluent. For microbial population analysis, spread plate technique was selected and used throughout the study. In general, 0.1ml of the samples from appropriate dilutions (between 10^{-5} and 10^{-8})

Table 1. Total count of mesophilic aerobic bacteria (TMAB) and moulds in retail Thua Nao samples (log CFU/g sample)

Samples	Sources	Types	TMAB	Moulds
TNF1	Chiang Rai (Phan)	Fresh	6.19 ± 0.26	6.00 ± 0.00
TNF2	Chiang Rai (Huai Sak)	Fresh	6.52 ± 0.57	5.59 ± 0.41
TNF3	Chiang Rai (Mae Chan)	Fresh	7.53 ± 0.55	6.22 ± 0.10
TNF4	Chiang Rai (Mae Lao)	Fresh	6.81 ± 1.32	4.30 ± 0.00
TNF5	Chiang Rai (Mae Sai)	Fresh	6.73 ± 0.18	7.29 ± 0.28
TNF6	Chiang Rai (Muang)	Fresh	7.41 ± 0.30	ND
TNF7	Chiang Rai (Muang)	Fresh	7.48 ± 0.04	ND
TNF8	Phayao (Thung Ton Si)	Fresh	6.87 ± 0.29	6.53 ± 0.59
TNF9	Phayao (Dok Khamtai)	Fresh	ND	4.00 ± 0.00
TND01	Chiang Rai (Phan)	Dried	7.08 ± 0.00	6.04 ± 0.06
TND02	Chiang Rai (Mae Sai)	Dried	7.97 ± 0.00	4.00 ± 0.00
TND03	Chiang Rai (Mae Chan)	Dried	7.45 ± 0.47	4.30 ± 0.00
TND04	Chiang Rai (Muang)	Dried	5.89 ± 0.26	6.13 ± 0.18
TND05	Chiang Rai (Mae Khao Tom)	Dried	5.53 ± 0.11	ND
TND06	Chiang Rai (Fah Thai)	Dried	ND	5.20 ± 0.85
TND07	Chiang Rai (Bandu)	Dried	6.45 ± 0.21	5.19 ± 0.16
TND08	Chiang Rai (Huai Sak)	Dried	7.66 ± 0.00	ND
TND09	Chiang Rai (Pa Daet)	Dried	7.22 ± 0.52	6.47 ± 0.45
TND10	Chiang Rai (Huai Sak)	Dried	6.99 ± 0.16	7.13 ± 0.03
TND11	Chiang Rai (Chiang Khong)	Dried	6.88 ± 0.57	7.04 ± 0.73
TND12	Chiang Rai (Fah Thai)	Dried	6.59 ± 0.17	ND
TND13	Chiang Rai (Bandu)	Dried	6.70 ± 0.01	ND
TND14	Phayao (Chun)	Dried	7.59 ± 0.01	6.31 ± 0.17
TND15	Phayao (Pong)	Dried	7.17 ± 0.13	7.18 ± 0.39

ND = Not determined.

were spread on nutrient agar (NA) and potato dextrose agar (PDA). The NA and PDA plates used for enumeration of total aerobic bacteria and fungi were incubated at 37 and 30ºC, respectively. Colony counting was then undertaken and expressed as colony forming units (CFU) per gram of sample.

To determine the different microbial groups, selective media were used, followed by incubation at a specific temperature for a certain period of time as described by Bacteriological Analytical Manual[6]. *Bacillus cereus* was enumerated by mannitol-egg yolk-polymyxin (MYP) agar plates and incubated at 35ºC for 24 h. Positive colonies (pink colonies surrounded by a zone of precipitation) were further biochemically tested including catalase (+), nitrate reduction (+), lysozyme (+), tyrosine (+), Voges-Proskauer (+), and utilization of glucose (+). Enumeration of *Staphylococcus aureus* was undertaken by using Baird-Parker medium and kept at 35ºC for 24 h. Typical black colonies with zones around were selected for catalase (+), coagulase (+), and utilization of glucose (+), and mannitol (+). For *Escherichia coli*, a diluting sample was spread on eosin methylene blue agar and incubated at 35ºC for 24 h. Presumptive colonies (metallic sheen appearance) were subsequently subjected to IMViC tests (Indole, +; Methyl red, +; Voges-Proskauer, -; Citrate, -). For enumeration of *Salmonella* species, the *Salmonella-Shigella* agar was used and the plates were incubated at 30ºC for 24 h. Representative colonies (clear red and black colonies) were selected and tested for the triple sugar iron test (+), motility (+), indole (-), and lysine test (+). For *Clostridium perfringens* enumeration, the diluted sample was spread on tryptose sulfite cycloserine agar. After incubation at 35°C for 24 h under anaerobic condition, black (or grey) colonies were subsequently confirmed using fermentation of lactose (+), gelatin liquefaction (+), motility (-) and nitrate reduction (-). This further characterization was performed as outlined by BAM[6] to confirm the presence of each pathogenic bacterial group.

3. RESULTS AND DISCUSSION

Results of microbiological analysis of 24 Thua Nao samples are shown in Table 1 and 2. It is evident that most Thua Nao samples (both fresh and dried forms) had high levels of TMAB (>10^5 CFU/g) and moulds (>10^4 CFU/g). The TMAB counts range from 6.19 – 7.53 log CFU/g and 5.53 – 7.97 log CFU/g for the fresh and dried Thua Nao products, respectively. Similar finding is also found for the moulds counts which range from 4.00 – 7.29 log CFU/g and 4.00 – 7.18 log CFU/g for the fresh and dried Thua Nao products,

Table 2. Presence of some pathogenic bacteria in commercial Thua Nao samples

Samples	*B. cereus*	*E. coli*	*C. perfringens*	*S. aureus*	*Salmonella* spp.
TNF1	-	+	-	-	-
TNF2	-	+	-	-	-
TNF3	-	+	-	-	-
TNF4	-	-	-	-	-
TNF5	-	+	-	-	+
TNF6	-	-	-	-	-
TNF7	-	-	-	-	-
TNF8	-	+	-	-	-
TNF9	-	+	-	-	+
TND01	-	-	-	-	-
TND02	-	+	-	-	-
TND03	-	-	-	-	+
TND04	-	-	-	-	-
TND05	-	-	-	-	-
TND06	-	+	-	-	-
TND07	-	+	-	-	-
TND08	-	-	-	-	-
TND09	-	-	-	-	-
TND10	-	+	-	-	-
TND11	-	+	-	-	-
TND12	+	-	-	-	-
TND13	+	-	-	-	-
TND14	-	+	-	-	-
TND15	-	+	-	-	-

Note: + and – indicate the presence and the absence of the pathogenic bacteria in Thua Nao samples.

respectively (Table 1). These results are expected considering that fresh Thua Nao samples were moistened and thus had higher contents of water than dried Thua Nao samples. Water content referred to water activity (a_w) is one of the key limiting factors important for microbial growth. In this point of view, a_w values of the fresh forms are generally higher than those of the dried forms. Mesophilic bacteria typically required a very high a_w value for their growth (a_w value > 0.91), were thus found at high levels in the fresh Thua Nao products although it should be noted that the highest TMAB counting occurred in the dried sample (7.97 log CFU/g of the TND02 sample). In contrast, fungi tend to be xerophilic and thus were found abundantly in both fresh and dried Thua Nao samples.

Further microbiological analysis was then carried out to determine the presence of some certain pathogenic bacteria including *B. cereus*, *E. coli*, *C. perfringens*, *S. aureus*, and *Salmonella* species. Our data revealed that *E. coli*, *Salmonella* spp. and *B. cereus* were detected in 13, 3, and 2 samples, respectively. Of these, *E. coli*, which could be detected from both fresh and dried samples, was the most frequently detected pathogenic bacteria accounting for 54.17% of the samples studied. *Salmonella* species were also found in both fresh (2 samples) and dried (1 sample) Thua Nao products accounting for 12.5% of the samples tested. There were only 2 dried Thua Nao samples (8.33%) that were contaminated with *B. cereus*. However, our results showed that *C. perfringens* and *S. aureus* were not found in all samples (Table 2).

In the Asian region, many kinds of fermented soybean products are present. To date, there is only Natto of Japan that has been produced using a pure starter culture and, thus its production process has been industrially controlled[7]. Other similar products including Thua Nao are still produced by artisanal techniques. The finding of high levels of TMAB and moulds in Thua Nao samples from this study is therefore not a surprise considering that its production procedure is not strictly controlled and its fermentation is proceeded by naturally occurring microbes. This finding is in agreement with the previous report describing a high concentration of the TMAB in Kinema[4], Korean fermented soybeans[8], Chinese Sufu[9], and Tempeh[10]. Fungi are also detected ranging from 2 – 6 log CFU/g in Sufu[11]. In Thailand, there is only a guideline of 'Community Product Standards' for Thua Nao product in which not much information is given for microbiological analyses[12]. However, Food and Drug Administration (FDA) of Thailand has provided a general guideline for food and related products to guarantee the public safety[13], which is in accordance with other general standard for contaminants and toxins in food and feed such as Codex[14] and International Commission on Microbiological Specifications for Foods (ICMSF)[15]. Thua Nao product considered as food and food ingredient, thus must be followed such guidelines. For example, based on the ICMSF specifications, the TAMB count of 4 log CFU/g of food sample is of acceptable quality and 4 – 6 log CFU/g is of marginal quality[12]. Our results, therefore, indicate that all Thua Nao samples were unacceptable (> 6 log CFU/g).

E. coli (and other Enterobacteriaceae members) is also a good indicator for hygienic quality of the food product. In this study, *E. coli* was detected in 13 out of 24 samples (ca. 54%). The similar finding is also found in Kinema, Sufu and Tempeh where the members of the Enterobacteriaceae widely occurred[4,9,10]. *B. cereus*, an endospore-forming bacilli causing diarrheal and emetic syndromes[16], were isolated from only two Thua Nao samples (ca. 8%). This appears to differ from the data of Nout et al.[4] and Han et al.[9] reporting that *B. cereus* could be isolated from all Kinema and Sufu samples. Frequent finding of *B. cereus* is also described from Korean soybean paste[17]. This discrepancy is of interest and further study should be undertaken. *Salmonella* species were detected in 3 Thua Nao samples (12.5%) and at present, no previous work has described an occurrence of *Salmonella* in the fermented soybean products. The present study failed to detect the presence of *C. perfringens* and *S. aureus*. Earlier studies also confirmed these results in Kinema and Sufu where *S. aureus* was absent although it should be noted that *C. perfringens* was found in 25% of Sufu samples tested[4,9].

4. CONCLUSIONS

This study reveals the microbiological quality of Thua Nao products commercially available in Chiang Rai and Phayao Provinces, Thailand. In general, high levels of TMAB and fungi were observed in all of the samples indicating that their manufacturing processes and shelf storage were not hygienic. Food safety is a major issue considering that foods contaminated by pathogenic microbes can cause adverse health

effects (or even life threatening) on consumers. Unfortunately, some variations regarding specifications of microbial parameters have been enacted by different countries. The data presented here are expected to be useful for the public health policymakers who can improve food standards and promote an awareness of food safety for the consumers.

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Conflict of interest

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