RESEARCH ARTICLE

Improving Nham Hed Quality and Safety through Co-Starter Fermentation with *Lactiplantibacillus plantarum* NH48/12 and *Saccharomyces cerevisiae* TISTR 5110

Pramuan Saithong[1], Jirawut Permpool[2],
Wanida Tewaruth Chitisankul[3] and Supachai Nitipan[2,*]

[1]Department of Applied Microbiology, Institute of Food Research and Product Development, Kasetsart University, Bangkok 10900, Thailand

[2]Department of Biology, Faculty of Science, Thaksin University, Phattalung Campus, Phattalung 93210, Thailand

[3]Department of Nutrition and Health, Institute of Food Research and Product Development, Kasetsart University, Bangkok 10900, Thailand

(*Corresponding author's e-mail: nisupachai@tsu.ac.th)

Abstract

This study evaluated the effects of *Lactiplantibacillus plantarum* NH48/12 and *Saccharomyces cerevisiae* TISTR 5110 as co-starter cultures in Nham Hed fermentation, focusing on fermentation dynamics, microbial stability, biochemical composition, and sensory characteristics. The addition of L. plantarum NH48/12 at 4% (w/w) significantly enhanced acidification, rapidly reducing pH within 24 hours and promoting lactic acid bacteria (LAB) proliferation. Co-starter fermentation with *S. cerevisiae* TISTR 5110 accelerated yeast establishment, reaching 8 log CFU/g within 2 days but did not significantly affect pH, acidity, or LAB populations. Free amino acid (FAA) analysis revealed increased levels of key flavor-enhancing amino acids, while volatile organic compound (VOC) profiling identified 2 unique compounds— butanoic acid, ethyl ester, and benzeneacetaldehyde— exclusively in co-starter fermented samples, contributing to improved aroma and taste. Microbiological safety assessment confirmed the absence of *Escherichia coli*, *Staphylococcus aureus*, *Clostridium perfringens*, *Bacillus cereus*, and S*almonella* spp., indicating the effectiveness of fermentation conditions in controlling pathogens. Sensory evaluation showed that co-starter fermentation significantly improved odor, taste, and overall consumer acceptance. These findings support the application of co-starter cultures to enhance fermentation efficiency, product quality, and safety in Nham Hed production. Future studies should investigate the biochemical pathways involved in flavor development to further optimize fermentation conditions.

Keywords: *Lactiplantibacillus plantarum* NH48/12, *Saccharomyces cerevisiae* TISTR 5110, Nham Hed, Co-starter fermentation, Microbial safety, Volatile compounds, Sensory quality

Introduction

Pleurotus pulmonarius, also known as the Indian oyster or phoenix mushroom, belongs to the Pleurotus family and is the third most cultivated edible mushroom globally [1]. Oyster mushrooms are nutritious and contain various beneficial compounds such as polysaccharides, dietary fiber, ergosterol, vitamin B, and minerals [2-4]. However, fresh mushrooms spoil quickly due to their high-water content, fast respiration rate, and naturally occurring microflora [5], highlighting

the need for preservation methods to extend their shelf life.

Nham Hed is a traditional Thai fermented oyster mushroom produced by fermenting steamed mushrooms with garlic, pepper, salt, and cooked rice as a carbon source. This fermentation process not only preserves the mushrooms but also enhances their nutritional value through the digestive activities of microorganisms. Traditional fermentation is spontaneous, relying on the microflora naturally present in the raw materials [6], and

typically requires 7 - 10 days. The quality of the final product depends largely on the microbial population in the raw material. Starter culture fermentation has been widely applied to improve quality consistency across major food groups, including dairy products [7], fermented fruits and vegetables [8-9], fermented fish [6,10], and fermented mushrooms [11].

Lactiplantibacillus plantarum (formerly *Lactobacillus plantarum*) is widely recognized for its beneficial role in fermented food production [12], contributing to product stability by lowering pH and producing bacteriocins [13], which inhibit the growth of harmful and spoilage microorganisms. *L. plantarum* NH48/12, isolated from traditionally fermented Nham Hed, has also shown potential as a probiotic [14]. However, the rapid production of lactic acid by LAB starter cultures can causes a significant drop in pH, potentially inhibiting aroma-producing microorganisms [15].

Several studies have explored the use of yeast to enhance the aroma of fermented foods [16,17]. *S. cerevisiae* is well-known for its ability to produce aroma compounds and has been used to improve the odor and flavor of fermented products [18,19]. This study prepared a starter culture of *L. plantarum* NH48/12 and *S. cerevisiae* TISTR 5110 and evaluated their optimal concentrations on the chemical and biological properties of Nham Hed.

Materials and methods

Raw materials

Fresh *P. pulmonarius* mushrooms were purchased from a local farm in Phatthalung Province, Thailand and identified by comparing their morphological features to the standard classification [20].

Microorganisms

L. plantarum NH48/12, known for its probiotic properties, was isolated from fermented oyster mushroom [14]. LAB were cultured in MRS medium under semi-anaerobic conditions at 30°C for 24 h. *S. cerevisiae* TISTR 5110 was obtained from the Thailand Institute of Scientific and Technological Research (TISTR) and cultured in yeast extract peptone dextrose (YPD) medium at 30°C with shaking at 150 rpm.

Preparation of mushroom mixture

The fresh mushrooms were sorted, cleaned, and washed under cold running water before steaming for 15 min and squeezing the excess water. For fermentation, 1 kg of steamed mushrooms was mixed with 150 g of steamed rice, 100 g of crushed garlic, and 25 g of NaCl.

Preparation of starter cultures

Two hundred fifty-milliliter cultures of *L. plantarum* NH48/12 (8 log CFU/mL) and *S. cerevisiae* TISTR 5110 (9 log CFU/mL) were centrifuged at 8000× g for 15 min at 4°C. Pellets were washed twice with sterile 0.85% NaCl and resuspended in 100 mL of 10 % skimmed milk (Difco, BD, USA). The suspensions were freeze-dried using a Low Freeze-drier (Leybold, Belgium), with the temperature gradually raised from −45 to 25°C over 30 h at 0.9 mbar, followed by an additional 15 h at 0.15 mbar to ensure complete dehydration.

Lactic acid bacteria fermentation optimization

The optimal concentration of LAB starter culture was tested using 250 g of mushroom mixture divided into five treatments: T1 (spontaneous fermentation), T2 (1% (w/w) *L. plantarum* NH48/12), T3 (2% (w/w) *L. plantarum* NH48/12), T4 (3% (w/w) *L. plantarum* NH48/12), and T5 (4% (ww) *L. plantarum* NH48/12). Each treatment was mixed thoroughly, portioned into 10 g samples, sealed in plastic bags, and incubated at 30 ± 2°C for 10 days. Samples were collected daily for chemical and microbiological analyses. All treatments were conducted in triplicate, with three independent fermentation batches analyzed at each time point.

Co-starter cultures fermentation optimizations

The optimal concentration of 4% (w/w) *L. plantarum* NH48/12 was used to assess the optimal concentration of *S. cerevisiae* TISTR 5110. A 250 g aliquot of the mushroom mixture was prepared with 4 % (w/w) *L. plantarum* NH48/12 and divided into four treatments: T1 (*L. plantarum* NH48/12), T2 (*L. plantarum* NH48/12 + 0.1 % (w/w) *S. cerevisiae* TISTR 5110), T3 (*L. plantarum* NH48/12 + 0.2% (w/w) *S. cerevisiae* TISTR 5110), and T4 (*L. plantarum* NH48/12 + 0.3% (w/w) *S. cerevisiae* TISTR 5110). Samples were sealed and incubated as above, with analyses conducted in triplicate.

Comparison of final Nham Hed products

The final comparison was based on the best-performing treatments identified from prior optimization experiments: 1) Spontaneous fermentation (SF) for 7 days, (2) 4% *L. plantarum* NH48/12 (LpF) for 4 days, and (3) co-culture of 4% *L. plantarum* NH48/12 with 0.1% *S. cerevisiae* TISTR 5110 (CF) for 4 days. These treatments were selected based on superior microbial stability, fermentation efficiency, and enhanced biochemical profiles. Treatments were performed in triplicate, with three independent batches analyzed at each time point.

Chemical analysis

pH and titratable acid

Nham Hed samples (5 g) were homogenized (Ultra-Turrax T18D, IKA, Staufen, Germany) with 15 mL of deionized water at 10,000 rpm for 1 min. The pH was measured with a digital pH meter (Suntex SP-2300, Taipei, Taiwan). Titratable acidity was determined according to AOAC (2005) by titrating with 0.1 M NaOH using phenolphthalein as the indicator and expressed as the percentage equivalent of lactic acid (w/w).

Free amino acid (FAA) analysis

Nham Hed samples (5 g) were homogenized in 25 mL of 5% (w/v) trichloroacetic acid and stored at 4°C for 2 h. The homogenate was filtered through Whatman No. 1 filter paper and centrifuged at 10,000 × g for 5 min at 4°C. The supernatant was subjected to pre-column derivatization with o-phthalaldehyde and 9-fluorenylmethyl chloroformate for free amino acid (FAA) analysis using reversed-phase high-performance liquid chromatography (RP-HPLC) (Agilent 1100, Agilent Technologies, USA) with a C18 Waters column. The mobile phase consisted of solvent A (0.5% tetrahydrofuran, 0.8% sodium acetate, 0.0225% triethylamine, pH 7.2) and solvent B (400 mL 2% sodium acetate-acetic acid solution, 800 mL acetonitrile, 800 mL methanol, pH 7.2). A gradient elution was performed by decreasing solvent A from 92 to 40% over 0 - 27.5 min then to 0% over 27.5 - 31.5 min. After a 2.5-minute hold, solvent A was returned to 92% from 34 to 35.5 min. The FAAs were identified and quantified by comparing retention times and peak areas with standards (Sigma Chemical Co., St. Louis, MO, USA).

Volatile organic compound analysis

Five g of Nham Hed samples were homogenized in 4 mL of saturated sodium chloride solution in a headspace vial and stirred. A Solid-Phase Microextraction (SPME) fiber (65 μm, PDMS/DVB; Supelco, Bellefonte, PA, USA) was then exposed to the vial's headspace for 30 min at 60°C, followed by desorption at 250°C for 3 min. The volatile compounds were analyzed using a Headspace-Gas Chromatography-Mass Spectrometer (HS-GC-MS) (Agilent 7890B/5977), equipped with a DB-WAX column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, Palo Alto, CA, USA). Mass spectrometry was performed using electron ionization (EI) mode at 70 eV with scanning from 30 to 500 m/z. Helium was used as the carrier gas. The volatile compounds were identified using the NIST 2005 and Wiley 7 standard libraries and quantified based on peak areas.

Microbiological analysis

Nham Hed samples (5 g) were mixed aseptically with 45 mL 0.85% (w/w) sterile saline solution in sterile plastic pouches and then homogenized for 2 min using a stomacher (BM-400P, Shanghai Truelab Lab-Sci Co., Ltd., Shanghai, China). Ten-fold serial dilutions of the samples were made using a sterile saline solution 0.85% (w/w). A 1 mL aliquot of each dilution was plated in triplicate on the appropriate growth media. Total plate counts (TPC) were incubated at 37°C for 48 h on Plate Count Agar. Lactic acid bacteria (LAB) counts were incubated anaerobically at 30°C for 48 h on MRS agar. Yeast counts were incubated at 28°C for 72 h on YPD agar.

Enterobacteriaceae counts were incubated at 37°C for 24 h on violet red bile dextrose agar. *Staphylococcus* counts were incubated at 30°C for 72 h on mannitol salt agar (MSA). *Salmonella* spp. counts were incubated at 37°C for 48 h on hektoen enteric (HE) agar. *Bacillus* were incubated at 37°C for 24 h on tryptone soy broth (TSB).

Microbiological counts were reported as colony-forming units per gram (Log10 CFU/g).

Sensory evaluation

The sensory evaluation of Nham Hed samples was performed by 30 adult panelists from the Institute of Food Research and Product Development (IFRPD),

Kasetsart University, Thailand. All panelists had prior experience in sensory evaluation of food products. Participants were fully informed about the study's objectives and procedures and provided written informed consent prior to participation. The samples used had been verified for microbiological safety and nutritional quality. No personally identifiable information was collected, and the study was classified as minimal risk with no inclusion of vulnerable populations. All procedures followed the ethical principles outlined in the Declaration of Helsinki and national research ethics guidelines. The samples were served in randomized order, and panelists evaluated appearance, odor, taste, texture, and overall liking using a 9-point hedonic scale (1 = dislike extremely, 9 = like extremely). Data are presented as mean ± standard deviation (SD), and significant differences were identified.

Statistical analysis

All data represent means ± standard deviation of 3 biological replicates (n = 3). Data normality and homogeneity of variances were verified using the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) was performed to determine significant differences among treatment means, followed by Duncan's multiple range test at a significance level of $p < 0.05$. All statistical analyses were carried out using SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Starter culture preparations

The lyophilized starter cultures of *L. plantarum* NH48/12 and *S. cerevisiae* TISTR 5110 were successfully prepared in 10% skimmed milk, with viable cell presence confirmed by SEM. **Figure 1** displays the distinct rod-shaped morphology of *L. plantarum* NH48/12 and the oval morphology of *S. cerevisiae* TISTR 5110. Viable cell counts on MRS and YPD agar indicated 7 - 8 log CFU/g for LAB and 8 - 9 log CFU/g for yeast, respectively.
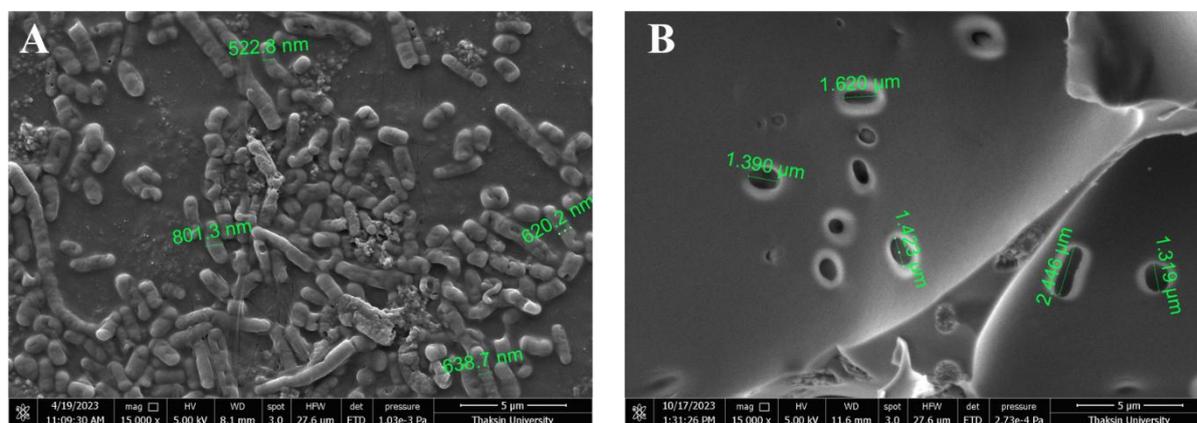

Figure 1 Scanning electron microscope (SEM) images of (A) *L. plantarum* NH48/12 and (B) *S. cerevisiae* TISTR 5110.

Optimization of L. plantarum NH48/12 concentration starter culture

The effects of various concentrations of *L. plantarum* NH48/12 starter culture (0, 1, 2, 3, and 4% w/w) on pH, titratable acidity, and microbiological quality during Nham Hed fermentation were evaluated. Starter culture fermentations showed a rapid pH decline from an initial 6.4 - 6.5 to a final range of 3.2 - 3.7 within 24 h, followed by a gradual decrease throughout the fermentation period. By contrast, the pH in spontaneous fermentation decreased only slightly over 120 h (**Figure 2(A)**). Microbiological analysis revealed a notable advantage of the 4 % w/w *L. plantarum* NH48/12 starter culture, which demonstrated the most robust and rapid LAB population growth. LAB counts in this group surged to 8 - 10 log CFU/g within the first 3 days, peaking by day 5 and stabilizing at 6 - 7 log CFU/g by day 7, outperforming all other concentrations. By comparison, spontaneous fermentation showed a slower LAB increase, reaching a peak of 8 log CFU/g on day 6, followed by a slight decline on day 7 (**Figure 2(B)**). Yeast and total plate count populations (**Figures 2(C)**

and **2(D)**) followed a similar trend in the starter culture fermentations, with an initial increase during the first 3 days, followed by a decline as fermentation progressed. In spontaneous fermentation, yeast populations increased modestly over the first 5 days before gradually decreasing, highlighting slower and less controlled microbial activity compared to the starter culture

groups. These findings indicated that the 4% w/w *L. plantarum* NH48/12 starter culture was the most effective concentration for improving fermentation performance and maintaining stable microbial populations under controlled concentration fermentation.
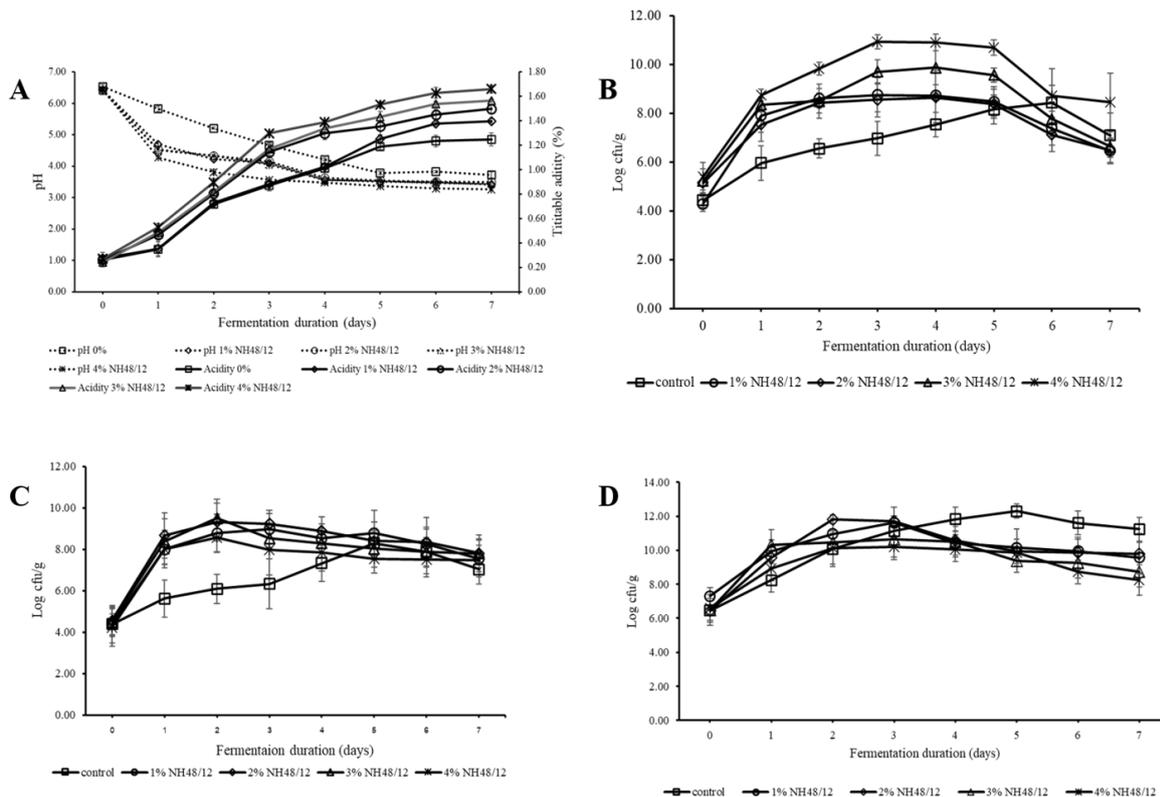

Figure 2 Changes in pH and microbial populations during the fermentation of Nham Hed with different concentrations of *L. plantarum* NH48/12: (A) pH, (B) lactic acid bacteria (LAB), (C) yeast, and (D) total plate count.

Optimization of co-starter cultures of L. plantarum NH48/12 and S. cerevisiae TISTR 5110

The optimization of co-starter cultures in Nham Hed production showed that varying the concentration of *S. cerevisiae* TISTR 5110 (0, 0.1, 0.2, 0.3% w/w), while maintaining *L. plantarum* NH48/12 at 4% w/w had no significant effect on pH and acidity (**Figure 3(A)**), LAB population (**Figure 3(B)**), or total plate count (**Figure 3(D)**) throughout the fermentation. However, yeast population dynamics (**Figure 3(C)**) differed significantly between the co-culture starter treatments and the single *L. plantarum* NH48/12 treatment. In the co-culture starter fermentations, yeast populations rapidly increased to 8 log CFU/g within 2 days, whereas in the single *L. plantarum* NH48/12

treatment, 4 days were required to reach the same level. Although no significant differences were observed among the co-culture starter treatments, the presence of *S. cerevisiae* TISTR 5110 accelerated yeast growth, potentially enhancing fermentation efficiency.

Free amino acid (FAA) of Nham Hed product analysis

The analysis of free amino acids in Nham Hed (**Table 1**) revealed distinct differences between spontaneous, *L. plantarum* NH48/12, and co-starter fermentations. In the co-starter fermentation, the levels of key flavor-enhancing amino acids, including alanine, glutamic acid, serine, proline, and leucine, showed an increasing trend, suggesting improved proteolysis and enhanced umami and sweet taste attributes. By contrast,

the histidine, lysine, and valine levels exhibited a declining trend, possibly reflecting selective microbial metabolism or differences in protein breakdown pathways. These findings suggest that co-starter fermentation not only influenced microbial activity and but also modulated FAA composition, potentially improving the sensory characteristics of Nham Hed.
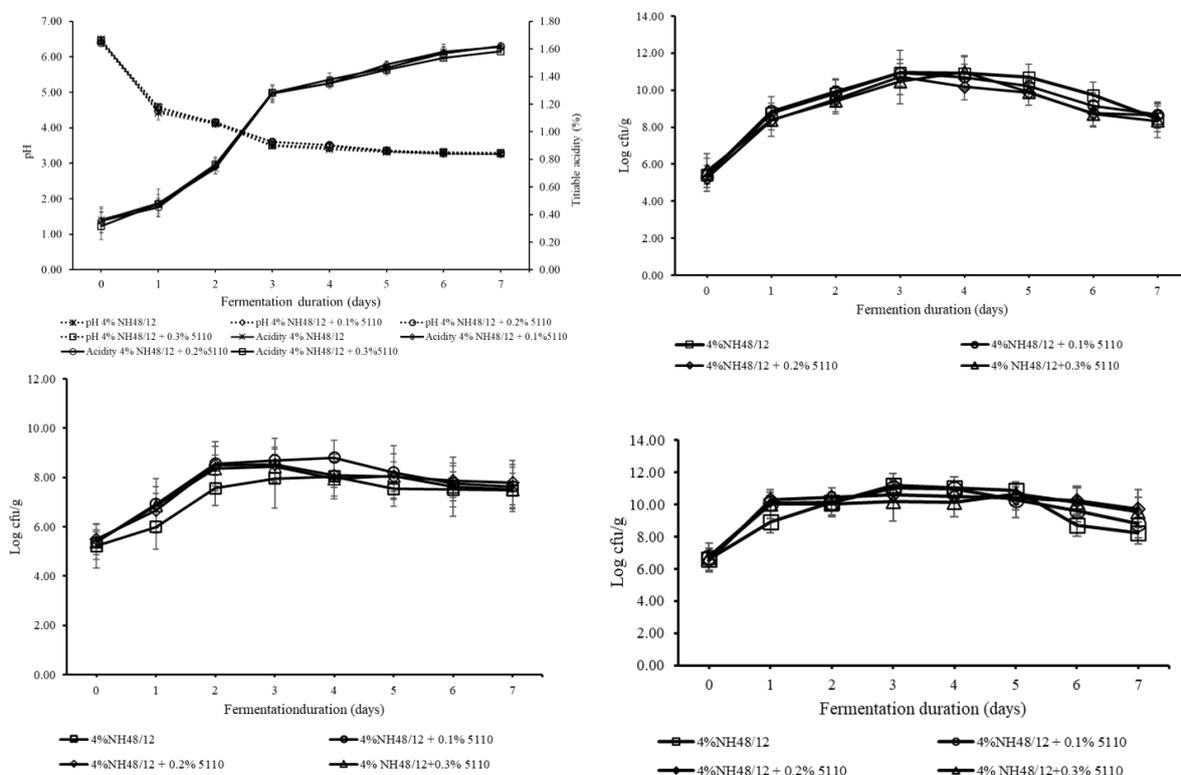

Figure 3 Changes in pH and microbial populations during the fermentation of Nham Hed with 4 % w/w *L. plantarum* NH48/12 and varying concentrations of *S. cerevisiae* TISTR 5110: (A) pH, (B) lactic acid bacteria (LAB), (C) yeast, and (D) total plate count.

Table 1 Amino acid profile of Nham Hed products fermented by spontaneous, *L. plantarum* starter, and co-culture starter fermentations.

Amino acid	Amino acid profile, mg/100 g sample		
	SF	LpF	CF
Non-essential			
Alanine	244.06	253.95	258.40
Arginine	10.55	13.21	12.42
Aspartic acid	211.69	224.12	214.71
Cysteine	8.76	9.98	10.12
Glutamic acid	516.24	523.34	547.56
Glycine	135.66	133.67	134.41
Proline	99.37	128.35	124.96
Serine	173.52	197.46	207.39
Tyrosine	163.78	170.71	178.61
Essential			
Histidine	164.37	132.51	107.72
Isoleucine	150.71	155.23	159.36
Leucine	358.13	374.46	391.30

Amino acid	Amino acid profile, mg/100 g sample		
	SF	LpF	CF
Lysine	389.07	331.89	357.33
Methionine	128.45	157.44	153.08
Phenylalanine	144.61	143.27	145.50
Threonine	166.10	163.12	170.96
Tryptophan	29.28	38.24	39.07
Tyrosine	163.78	189.39	178.61
Valine	157.49	154.25	142.31

SF: Spontaneous fermentation, LpF: *L. plantarum* NH48/12 fermentation, CF: Co-culture fermentation.

Volatile organic compound (VOC) analysis

The analysis of volatile organic compounds revealed distinct differences between spontaneous and co-starter fermentations. Fifteen VOCs (**Table 2**) were detected across all samples. Of these, 13 compounds were present in Nham Hed produced by spontaneous fermentation, whereas 15 were identified in samples fermented with the co-starter culture. Notably, butanoic acid, ethyl ester, and benzeneacetaldehyde were exclusively detected in the co-starter fermented samples.

Microbiological safety and sensory evaluation

The microbiological safety assessment of Nham Hed confirmed that both spontaneous and co-starter fermentations produced microbiologically safe products. Pathogenic bacteria including *E. coli*, *S. aureus*, *C. perfringens*, *B. cereus*, and *Salmonella* spp., were not detected in any samples (**Table 3**). These findings indicated that the fermentation conditions, whether spontaneous or controlled with co-starter cultures, effectively inhibited the growth of common foodborne pathogens. The absence of these harmful bacteria highlighted the safety of Nham Hed and supports the potential of co-starter fermentation as a reliable method for producing hygienic fermented mushroom products.

The sensory evaluation of Nham Hed products revealed significant differences in consumer acceptance between spontaneous fermentation and co-starter fermentation. The co-starter fermentation received significantly higher scores for odor, taste, and overall liking compared to spontaneous and single fermentations, suggesting that the combination *S. cerevisiae* TISTR 5110 and *L. plantarum* NH48/12 positively influenced the sensory attributes of the product. The enhanced aroma and flavor profile in the co-starter fermented Nham Hed was attributed to the production of specific volatile compounds such as benzeneacetaldehyde, as observed in earlier analyses. No significant differences were noted in appearance and texture; however, the improved sensory scores for odor, taste, and overall liking underscored the potential of co-starter cultures to enhance the consumer appeal of fermented mushroom products. These findings emphasize the importance of starter culture selection in optimizing the sensory quality of Nham Hed.

Discussion

The fermentation of Nham Hed was significantly influenced by the addition of *L. plantarum* NH48/12, which accelerated acidification and microbial stabilization [21,22]. The rapid pH declines within the first 24 h at 4% w/w starter concentration suggested enhanced lactic acid production, which is critical for inhibiting spoilage microorganisms and ensuring fermentation consistency [11]. In contrast, spontaneous fermentation exhibited a slower pH reduction, likely due to delayed LAB establishment. Microbiological analysis further confirmed that the 4% w/w *L. plantarum* NH48/12 culture supported robust LAB proliferation, reaching 8 - 10 log CFU/g within 3 days and stabilizing by day 7 [23]. These findings aligned with previous studies indicating that high LAB counts contribute to fermentation efficiency and microbial stability [22]. The slower LAB increase in spontaneous fermentation suggests a competitive microbial environment, leading to variability in fermentation outcomes [10].

The optimization of co-starter cultures demonstrated that varying *S. cerevisiae* TISTR 5110 concentrations (0 - 0.3% w/w) had no significant effect on pH, acidity, LAB population, or total plate count. This finding suggested that *L. plantarum* NH48/12 at 4% w/w provided sufficient acidification and bacterial

proliferation, consistent with studies showing LAB dominance in fermentation [10]. However, yeast population dynamics were significantly influenced, as co-culture treatments achieved 8 log CFU/g within 2 days compared to 4 days in the single *L. plantarum* NH48/12 treatment [11]. This indicates that *S. cerevisiae* TISTR 5110 accelerated yeast establishment,

enhancing fermentation efficiency and promoting microbial synergy [22]. Controlled microbial activity in co-starter fermentations contrasts with the variable microbial patterns observed in spontaneous fermentation, which can lead to inconsistent product quality [24].

Table 2 Volatile organic compounds (VOCs) detected in Nham Hed products fermented by spontaneous, *L. plantarum* starter, and co-culture starter fermentations.

No.	Retention time (min)	Volatile organic compound	Nham Hed products			Type
			SF	LpF	CF	
1	1.151	2-Butanol	/	/	/	Alcohol
2	1.376	Butanol, 3-methyl	/	/	/	Alcohol
4	2.818	Dimethyl disulfide	/	/	/	Disulfide
5	2.916	Heptadecane	/	/	/	Alkane
6	3.084	3-Octanone	/	/	/	Ketone
7	3.116	1-Octen-3-ol	/	/	/	Alcohol
8	3.202	Butanoic acid, ethyl ester	nd	nd	/	Ethyl ester
9	3.576	Acetic acid	/	/	/	Carboxylic acid
10	3.776	Hexanal	/	/	/	Aldehyde
11	4.945	2-Heptanal	/	/	/	Aldehyde
12	5.166	2-Octenal	/	/	/	Aldehyde
13	5.327	Toluene	/	/	/	Aromatic hydrocarbon
14	6.327	Butanoic acid	/	/	/	Carboxylic acid
15	9.291	Benzeneacetaldehyde	nd	nd	/	Aldehyde

/: detected, nd: Not detected, SF: Spontaneous fermentation, LpF: *L. plantarum* NH48/12 fermentation, CF: Co-culture fermentation.

Table 3 Sensory evaluation scores of Nham Hed products fermented by spontaneous, *L. plantarum* starter, and co-culture starter fermentations.

Sample	Appearance	Odor	Taste	Texture	Overall
SF	6.95 ± 0.64^a	5.33 ± 0.47^c	5.35 ± 0.48^b	7.10 ± 0.41^a	6.40 ± 0.50^b
LpF	7.10 ± 0.63^a	6.53 ± 0.51^b	6.68 ± 0.47^a	7.32 ± 0.51^a	6.23 ± 0.86^b
CF	7.13 ± 0.33^a	7.30 ± 0.46^a	6.58 ± 0.78^a	7.13 ± 0.75^a	7.23 ± 0.63^a

Values (mean ± SEM) with different superscripts in the same row indicate significant differences among treatments ($p < 0.05$). SF: Spontaneous fermentation, LpF: *L. plantarum* NH48/12 fermentation, CF: Co-culture fermentation.

Metabolite profiling revealed that co-starter fermentation enhanced the biochemical composition of Nham Hed. FAA analysis showed significant increases in alanine, glutamic acid, serine, proline, and leucine—amino acids associated with umami and sweet taste perception [25]. This indicates enhanced proteolysis

during co-fermentation, likely driven by the combined enzymatic activity of LAB and yeast [26, 27]. In contrast, the reduction in histidine, lysine, and valine levels suggests selective microbial metabolism, aligning with prior reports that LAB and yeast exhibit distinct amino acid utilization patterns [28]. These shifts in

amino acid profiles also suggest synergistic interactions between *L. plantarum* and *S. cerevisiae* at the metabolic level. LAB- derived peptides and free amino acids may serve as precursors or stimulants for yeast metabolism, enhancing volatile synthesis and amino acid conversion [29]. Conversely, ethanol and other yeast- derived metabolites may alter LAB activity and promote microbial cross- feeding, ultimately shaping the FAA composition [30]. Such interactions are known to influence metabolite profiles and contribute to enhanced flavor complexity in co- fermented foods [31], supporting the utility of co- starters in improving nutritional and sensory characteristics. Consistent with these findings, VOC analysis confirmed the impact of co- starter fermentation on aroma development. While 13 volatile compounds were detected in spontaneously fermented Nham Hed, 15 were identified in co- starter samples, with ethyl butanoate and benzeneacetaldehyde present exclusively in the latter. Ethyl butanoate, produced by *S. cerevisiae* via the esterification of butyric acid with ethanol, is known for its sweet, fruity aroma— imparting notes of strawberry, peach, and pineapple [18,19,32]. It has been identified as a major aroma- active compound in fermented products such as Chinese rice wine (OAV 38 - 59) [33], and its production can be enhanced through strain selection and co-culturing strategies [34,35]. Benzeneacetaldehyde, a floral and honey- scented aldehyde, is synthesized from phenylalanine via the Ehrlich pathway and is commonly associated with *Saccharomyces* metabolism [36- 38]. Despite its low concentration, it has a high odor activity value (OAV) and significantly influencing sensory perception in fermented foods such as chili peppers and soybean paste [36]. The exclusive presence of these compounds in the co- starter treatment highlights the contribution of *S. cerevisiae* to volatile generation through amino acid and lipid metabolism.

Moreover, the role of *L. plantarum* NH48/12 in lowering pH may facilitate precursor availability for *S. cerevisiae* TISTR 5110 metabolism, further reinforcing microbial synergy. Such interactions likely support cross- feeding and metabolic pathway activation, resulting in a richer and more complex aroma profile. Overall, these findings highlight how defined co- starter cultures improve both the biochemical and sensory quality of Nham Hed, offering a promising strategy for producing safe, flavorful, and consumer- acceptable fermented mushroom products.

The microbiological safety assessment confirmed that both spontaneous and co- starter fermentations produced Nham Hed free of common foodborne pathogens including *E. coli*, *S. aureus*, *C. perfringens*, *B. cereus*, and *Salmonella* spp.. The absence of these pathogens suggested that the fermentation conditions effectively inhibited microbial contaminants, likely due to the acidic environment and antimicrobial compounds produced by *L. plantarum* NH48/ 12 [7,38]. These findings concur with previous studies demonstrating the role of LAB in enhancing microbial safety in fermented foods [39] and support co- starter fermentation as a reliable approach for producing hygienic fermented mushroom products.

The sensory evaluation further highlighted the benefits of co- starter fermentation, with significantly higher scores for odor, taste, and overall liking compared to spontaneous and single fermentations. The improved sensory profile was attributed to the enhanced production of key volatile compounds such as benzeneacetaldehyde which imparts a pleasant aroma [36]. No significant differences were observed in appearance and texture, but the higher preference for co-starter fermented Nham Hed underscored the importance of optimizing microbial composition to enhance consumer appeal.

Co-starter fermented Nham Hed reflects a broader movement in food biotechnology toward controlled, functional fermentation, comparable to widely consumed products such as kimchi, sauerkraut, and tempeh. These foods rely on selected microbial cultures to enhance safety, flavor, and nutritional value. *L. plantarum*, a key strain in our study, plays similar roles in vegetable- based ferments by promoting acidification and microbial stability [40]. The incorporation of *S. cerevisiae* alongside LAB represents an innovative approach in mushroom fermentation, enriching aroma and amino acid profiles through microbial synergy. This strategy supports current trends in developing safe, consistent, and flavorful plant-based fermented foods.

Conclusions

This study demonstrates that the application of *L. plantarum* NH48/12 and *S. cerevisiae* TISTR 5110 as co- starter cultures offers a controlled and effective strategy for fermenting Nham Hed. Beyond improving acidification, microbial stability, and sensory quality, the co- starter approach supports a reproducible fermentation process that enhances product safety and consumer appeal. These insights advance the scientific understanding of microbial synergy in plant- based fermentations and provide a model for optimizing non-meat fermented foods. The findings also lay the groundwork for scaling co- starter technology in the commercial production of fermented mushroom products. Future research should explore the mechanistic basis of flavor development and refine process parameters for industrial application.

Acknowledgements

Declaration of Generative AI in Scientific Writing

The authors used ChatGPT-4.0 during manuscript preparation to assist with improving clarity and language quality.

CRediT author statement

Pramuan Saithong: Writing-review & editing, Investigation, Resource, Funding acquisition.

Jirawut Permpool: Investigation, Formal analysis, Writing-review & editing.

Wanida Tewaruth Chitisankul: Investigation, Formal analysis, Writing-review & editing.

Supachai Nitipan: Conceptualization, Methodology, Formal analysis, Writing-original draft, Resource, Funding acquisition, Visualization.

References

[1] D Bao, S Kinugasa and Y Kitamoto. The biological species of oyster mushrooms (*Pleurotus* spp.) from Asia based on mating compatibility tests. *Journal of Wood Science* 2004; **50**, 162-168.

[2] J Raman, KY Jang, YL Oh, M Oh, JH Im, H Lakshmanan and V Sabaratnam. Cultivation and nutritional value of prominent *Pleurotus* spp.: An overview. *Mycobiology* 2021; **49(1)**, 1-14.

[3] P Rangsinth, R Sharika, N Pattarachotanant, C Duangjan, C Wongwan, C Sillapachaiyaporn, S Nilkhet, N Wongsirojkul, A Prasansuklab, T Tencomnao, GP Leung and S Chuchawankul. Potential beneficial effects and pharmacological properties of ergosterol, a common bioactive compound in edible mushrooms. *Foods* 2023; **12(13)**, 2529.

[4] EI Eze, UM Ishiwu, CU Agbo, UP Chukwudi and JO Odo. Substrate effects on the yield, proximate, phytochemical and vitamin attributes of *Pleurotus pulmonarius* mushroom. *Tropical Journal of Natural Product Research* 2024; **8(10)**, 8819-8825.

[5] K Castellanos-Reyes, R Villalobos-Carvajal and T Beldarrain- Iznaga. Fresh mushroom preservation techniques. *Foods* 2021; **10(9)**, 2126.

[6] P Saithong, W Panthavee, M Boonyaratanakornkit and C Sikkhamondhol. Use of a starter culture of lactic acid bacteria in *plaa- som*, a Thai fermented fish. *Journal of Bioscience and Bioengineering* 2010; **110(5)**, 553-557.

[7] J García- Díez and C Saraiva. Use of starter cultures in foods from animal origin to improve their safety. *International Journal of Environmental Research and Public Health* 2021; **18(5)**, 2544.

[8] N Makhamrueang, S Sirilun, J Sirithunyalug, W Chaiyana, W Wangcharoen, S Peerajan and C Chaiyasut. *Lactobacillus plantarum* SK15 as a starter culture for prevention of biogenic amine accumulation in fermented beverage containing *Hericium erinaceus* mushroom. *Applied Sciences* 2021; **11(15)**, 6680.

[9] J Xu, S Peng, Y Xiong, Z Zheng, M Liu, J Xu, W Chen, M Liu, J Kong, C Wang, Y Wang, L Rao, L Zhoa and X Liao. A review on fermented vegetables: Microbial community and potential upgrading strategy via inoculated fermentation. *Comprehensive Reviews in Food Science and Food Safety* 2024; **23(3)**, e13362.

[10] Q Hua, Y Sun, Y Xu, P Gao and W Xia. Contribution of mixed commercial starter cultures to the quality improvement of fish- chili paste, a

Chinese traditional fermented condiment. *Food Bioscience* 2022; **46**, 101559.

[11] Y Liu, XX Xie, SA Ibrahim, SG Khaskheli, H Yang, YF Wang and W Huang. Characterization of *Lactobacillus pentosus* as a starter culture for the fermentation of edible oyster mushrooms (*Pleurotus* spp.). *LWT - Food Science and Technology* 2016; **68**, 21-26.

[12] J Zheng, S Wittouck, E Salvetti, CM Franz, HM Harris, P Mattarelli, PW O' Toole, B Pot, P Vandemme, J Walter, K Watanabe, S Wuyts, GE Felis, MG Ganzle and S Lebeer. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology* 2020; **70**, 2782-2858.

[13] N Garcia- Gonzalez, N Battista, R Prete and A Corsetti. Health- promoting role of *Lactiplantibacillus plantarum* isolated from fermented foods. *Microorganisms* 2021; **9(2)**, 349.

[14] S Nitipan, K Boonloy, H Da-oh, J Permpool and P Saithong. *In vitro* assessment of probiotic properties in *Lactobacillus* strains isolated from traditionally fermented Thai foods. *Naresuan University Journal: Science and Technology* 2022; **30(2)**, 11-23.

[15] B Yilmaz, SP Bangar, N Echegaray, S Suri, I Tomasevic, JM Lorenzo, E Melekoglu, JM Rocha and F Ozogul. The impacts of *Lactiplantibacillus plantarum* on the functional properties of fermented foods: A review of current knowledge. *Microorganisms* 2022; **10(4)**, 826.

[16] Y Liu, M Danial, L Liu, FA Sadiq, X Wei and G Zhang. Effects of co- fermentation of *Lactiplantibacillus plantarum* and *Saccharomyces cerevisiae* on digestive and quality properties of steamed bread. *Foods* 2023; **12(18)**, 3333.

[17] Z Que, S Wang, M Wei, Y Fang, T Ma, X Wang and X Sun. The powerful function of *Saccharomyces cerevisiae* in food science and other fields: A critical review. *Food Innovation and Advances* 2024; **3(2)**, 167-180.

[18] M Carpena, M Fraga- Corral, P Otero, RA Nogueira, P Garcia- Oliveira, MA Prieto and J Simal- Gandara. Secondary aroma: Influence of wine microorganisms in their aroma profile. *Foods* 2020; **10(1)**, 51.

[19] F Melini and V Melini. Role of microbial fermentation in the bio- production of food aroma compounds from vegetable waste. *Fermentation* 2024; **10(3)**, 132.

[20] R Abdulgani, CC Lau, N Abdullah and S Vikineswary. Morphological and molecular characterization of *Pleurotus pulmonarius* hybrids with improved sporophore features and higher biological efficacy. *International Journal of Agriculture and Biology* 2017; **19(4)**, 707-712.

[21] X Jin, W Chen, H Chen, W Chen and Q Zhong. Combination of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* DV10 as starter culture to produce mango slurry: Microbiological, chemical parameters and antioxidant activity. *Molecules* 2019; **24(23)**, 4349.

[22] H Wenkang, L Jingui, D Xuelu, R Yanjie, L Mingzhu, S Tingting, T Fuyao and Z Xuefeng. *Saccharomyces cerevisiae* strains L7 contribute to flavor and deacidification in Suanyu, a Chinese traditional fermented fish. *NPJ Science of Food* 2024; **8**, 92.

[23] M Zhang, L Wang, G Wu, X Wang, H Lv, J Chen, Y Liu, H Pang and Z Tan. Effects of *Lactobacillus plantarum* on the fermentation profile and microbiological composition of wheat fermented silage under the freezing and thawing low temperatures. *Frontiers in Microbiology* 2021; **12**, 671287.

[24] A Dysvik, SLL Rosa, KH Liland, KS Myhrer, HM Østlie, GD Rouck, EO Rakke, B Wasteren and T Wicklund. Co- fermentation involving *Saccharomyces cerevisiae* and *Lactobacillus* species tolerant to brewing- related stress factors for controlled and rapid production of sour beer. *Frontiers in Microbiology* 2020; **11**, 279.

[25] YN Li, Y Luo, ZM Lu, YL Dong, LJ Chai, JS Shi, XJ Zhang and ZH Xu. Metabolomic analysis of the effects of a mixed culture of *Saccharomyces cerevisiae* and *Lactiplantibacillus plantarum* on the physicochemical and quality characteristics of apple cider vinegar. *Frontiers in Nutrition* 2023; **10**, 1142517.

[26] S Tomita, J Watanabe, T Kuribayashi, S Tanaka and T Kawahara. Metabolomic evaluation of

different starter culture effects on water-soluble and volatile compound profiles in *nozawana* pickle fermentation. *Food Chemistry: Molecular Sciences* 2021; **2**, 100019.

[27] J Luo, S Xiao, J Wang, B Wang, Y Cai and W Hu. The metabolite profiling and microbial community dynamics during pineapple by-product fermentation using co-inoculation of lactic acid bacteria and yeast. *Fermentation* 2023; **9(2)**, 79.

[28] Z Tian, Q Zhu, Y Chen, Y Zhou, K Hu, H Li, K Lu, J Zhou, Y Liu and X Chen. Studies on flavor compounds and free amino acid dynamic characteristics of fermented pork loin ham with a complex starter. *Foods* 2022; **11(10)**, 1501.

[29] M Gobbetti, MD Angelis, RD Cagno, M Calasso, G Archetti and CG Rizzello. Novel insights on the functional/nutritional features of the sourdough fermentation. *International Journal of Food Microbiology* 2019; **302**, 103-113.

[30] LD Vuyst and F Leroy. Functional role of yeasts, lactic acid bacteria and acetic acid bacteria in cocoa fermentation processes. *FEMS Microbiology Reviews* 2020; **44(4)**, 432-453.

[31] O Ponomarova and KR Patil. Metabolic interactions in microbial communities: Untangling the Gordian knot. *Current Opinion in Microbiology* 2015; **27**, 37-44.

[32] Y Xu, KAHM Minhazul and X Li. The occurrence, enzymatic production, and application of ethyl butanoate, an important flavor constituent. *Flavour and Fragrance Journal* 2020; **35(6)**, 601-615.

[33] H Yu, Q Li, W Guo, L Ai, C Chen and H Tian. Unraveling the difference in flavor characteristics of *Huangjiu* fermented with different rice varieties using dynamic sensory evaluation and comprehensive two-dimensional gas chromatography-quadrupole mass spectrometry. *Frontiers in Nutrition* 2023; **10**, 1160954.

[34] S Wang, Y Lu, X Fu, M Wang, W Wang, J Wang, H Wang and Y Liu. Sequential fermentation with *Torulapora delbrueckii* and selected *Saccharomyces cerevisiae* for aroma enhancement of longyan dry white wine. *International Journal of Food Science and Technology* 2022; **57(1)**, 576-589.

[35] C Zeng, Y Mu, J Yuan, H Zhang, J Song and S Kang. Effects of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* co-fermentation on the physicochemical and flavor compounds of Huaniu apple cider. *Molecules* 2024; **29(8)**, 1750.

[36] Y Xiao, S Zhang, X Wang, X Zhao, Z Liu, C Chu, Y Wang, X Hu and J Yi. Characterization of key aroma-active compounds in fermented chili pepper (*Capsicum frutescens* L.) using instrumental and sensory techniques. *Food Chemistry: X* 2024; **23**, 101581.

[37] Z Shi, W Li, Z Chen, Z Peng, M Ma, J Zhang, D Wu, G Xie and J Lu. Exploring the impact of different *Saccharomyces cerevisiae* strains on the flavor profile of greengage alcoholic beverage using GC-E-Nose, HS-GC-IMS, and HS-SPME-GC-MS. *Foods* 2024; **13(24)**, 3984.

[38] D Zare, H Aryaee, S Mirdamadi and F Shirkhan. The benefits and applications of *Lactobacillus plantarum* in food and health: A narrative review. *Iranian Journal of Public Health* 2024; **53(10)**, 2201-2213.

[39] R Elsabagh, SM Nada and EM Abd-Elaaty. Controlling food poisoning bacteria in fermented chicken sausage using *Lactobacillus plantarum*. *World's Veterinary Journal* 2021; **11(3)**, 462-468.

[40] KY Park, JK Jeong, YE Lee and JW Daily. Health benefits of kimchi (Korean fermented vegetables) as a probiotic food. *Journal of Medical Food* 2014; **17(1)**, 6-20.