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## Comparison of fermentation behaviors and properties of Naem-Hed supplemented with vegetables by spontaneous and controlled lactic acid fermentation

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**Abstract** Lactic acid bacteria (LAB) are widely used in the various fermentation processes with a positive impact on unique sensory characteristics of food products and growth inhibition of pathogens. Results described the application of *Lactobacillus pentosus* compared with spontaneous fermentation to ferment Naem-Hed and Naem-Hed supplemented with vegetables. The LAB growth kinetics, chemical characteristics and sensory evaluation were investigated. Acid production and LAB growth in each sample varied with the use of different fermentation treatments. Enhancement of bioactive properties was observed in supplementation of vegetables. Sensory evaluation of the fermented samples revealed that samples from Naem-Hed supplemented with vegetables featured a highly intense ‘appearance’, ‘color’, ‘flavor’ and ‘overall preference’ attributes. It is suggested that using *L. pentosus* as a starter with vegetable supplementation as a new effective fermentation strategy would improve the production of Naem-Hed.

**Keywords:** Lactic acid fermentation, Spontaneous fermentation, Fermented mushroom, Naem-Hed

### Introduction

Naem-Hed is a local preserved food of Thai traditional fermented mushroom with its unique odor and desirable taste. It is normally made of cooked mushroom, cooked rice and spices, tightly wrapped with banana leaves or stuffed into plastic bags and left to ferment at room temperature for 2–3 days until it becomes acidified by the lactic acid bacteria (LAB) present in the raw materials (Manowan *et al.*, 2020; Thai Industrial Standards Institute, 2004). Naem-Hed is typically fermented spontaneously with pH lowered to 4.6 after

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fermentation, thereby preventing the growth of unwanted microbes, particularly foodborne bacterial pathogens such as *Staphylococcus aureus*. Spontaneous fermentation is a complex community of microorganisms responsible for the degradation of substrates, such as protein and carbohydrate, and formation of fermented flavor compounds. However, the traditional spontaneous process may result in contamination and unstable quality of products. Inoculation of starter culture is a better control condition to improve the product in terms of consistency and microbiological safety of the product. Application of starter culture into fermented foods is advantage for shortening fermentation time, delaying spoilage, enhancing flavour, and improving product's quality (Bao *et al.*, 2018).

Lactic acid fermentation is an ancient process of food preservation where LAB transform carbohydrate substrates into organic acids and a wide range of metabolites enhancing shelf-life, pleasant sensory characteristics and nutritional value of foods. The organic acids from LAB, including propionic, formic, acetic acid, and lactic acid, create unfavorable conditions for the growth of spoilage and pathogenic microorganisms (Bangar *et al.*, 2022). LAB known as the starter culture for vegetable fermentation has been previously reported with various benefits including the decrease of biogenic amine levels (Li *et al.*, 2022). Variation of fermented fruit and vegetable products are produced by LAB such as fermented olive, pickled cucumber, sauerkraut, kimchi, fermented bamboo shoot. Salted conditions are important in these fermented products due to salting provides an appropriate environment for the growth of LAB which imparts acidic flavor (Ray and Didier, 2014). LAB consumes sugars, nonvolatile acids and volatile aroma compounds are formed. Their metabolites preserve products and create unique flavors, textures and enhanced nutrition. Lactic acid fermentations can be carried out by a controlled process through inoculation (Park *et al.*, 2019). This starter inoculation fermentation can ensure consistent product quality and safety (Capozzi *et al.*, 2017).

The *Pleurotus* spp., generally called the oyster mushroom, is a widely cultivated and consumed mushroom in Thailand due to its pleasant taste, well-balanced diet, and health-promoting. This mushroom has a high nutritional value and contains several bioactive compounds, including polysaccharides, peptides, dietary fiber, ergosterol, vitamins, minerals and antimicrobial agents (Tolera and Abera, 2017; Liu *et al.*, 2016, Pisoschi *et al.*, 2018). However, the *Pleurotus* mushroom is perishable and sensitive to deteriorate after harvest, thus its shelf life is limited to a few days (Tolera and Abera, 2017). A number of food preservation methods can be used to prolong the product shelf-life of mushroom. Fermentation is considered as an efficient approach to extend the shelf-life of this delicate mushroom. Moreover, the fermentation process

reduces the cooking time of food which is therefore economic benefits in reduced energy use (Steinkraus, 2002).

In this study, the fermentation kinetics, chemical and sensory properties of Naem-Hed with vegetable supplement using spontaneous fermentation and LAB inoculation were evaluated. The purpose was to improve the quality of product and shorten fermentation time by LAB starter.

## **Materials and methods**

### ***Starter culture preparation***

*L. pentosus* was provided by Faculty of Science, Maejo University and proliferated following the procedure indicated by Bao *et al.* (2018). *L. pentosus* was cultured in de Man, Rogosa and Sharpe (MRS) broth (Merck, Germany) at 37 °C for 24 h. Cell pellets were harvested by centrifuging at 12,000 x g for 2 min at 4 °C. Subsequently, they were washed twice with sterile 0.85% NaCl solution and re-suspended in 15-ml in the saline solution before use. A starter culture was inoculated at approximately 10<sup>9</sup> CFU/g of substrate.

### ***Mushroom and vegetable fermentation process***

A 2×2 factorial experiment in completely random (CRD) design was performed with three replications. Mushroom (*Pleurotus sajor-caju*) and fresh vegetables (Chinese cabbage, spring onion, carrot, red bell pepper and purple cabbage) were purchased from the market (Lampang, Thailand). The edible parts of mushroom and vegetable were selected, washed and sliced into 3 mm width. Mushroom was steamed for 15 minutes. Steamed mushroom, sliced vegetables, salt, garlic and rice were mixed together, inoculated with *L. pentosus* for controlled lactic acid fermentation and without *L. pentosus* inoculation for spontaneous fermentation then transferred to plastic bag (50 g) and tightly packed together to remove air. The experiment units were incubated at 32±2 °C for 72 hours.

### ***Microbiological analyses***

Microbiological analyses were carried out on fermented samples taken intervals from incubator (0, 3, 6, 9, 12, 24, 48 and 72 hours). Ten grams of each replicate were aseptically diluted in sterile Ringer's solution (Merck, Germany) and homogenized for 2 minutes. Appropriate dilutions were used for microbial enumerations using the pour plate technique. All plates were incubated at 30 °C

for 24-48 h. *Escherichia coli* was grown on EMB agar (Merck, Germany) plates and incubated at 37 °C for 24 h. Greenish metallic sheen colonies were counted.

#### ***Total soluble solid content and titratable acidity***

pH was measured by digital pH meter (Model C831, Belgium). Total acidity was determined by diluting each 5 g aliquot of sample in 50 mL distilled water and then titrating to pH 8.2 using 0.1 N NaOH (Nielsen, 2017). Titratable acidity was expressed as lactic acid percentage. Total soluble solid content was determined on an Atago hand-held refractometer. Free alpha amino nitrogen (FAN) was quantified by spectrophotometric method (Intaramoree and Chomsri, 2014). The modified methods of Bradford (1976) and Spínola *et al.*, (2015) were used to evaluate total phenolic content and total soluble protein content, respectively. The antioxidant activity was determined by modified method of Wongputtisin *et al.* (2007). The moisture content was determined by modified method of Kirk and Sawyer, (1991).

#### ***Total phenolic content***

The extraction was repeated three times under the same conditions. All supernatants were combined. After centrifugation at 15,000 x g for 3 min, the supernatant was transferred to a clean vial. The phenolic content of mushroom naem, conjugated and bound extracts was determined using the Folin–Ciocalteu method. Before the measurement, commercial Folin–Ciocalteu phenol reagent (Sigma-Aldrich, Poole Dorset) was diluted 1:10 (v/v) with deionised water. Gallic acid (GA) was used as the reference standard against which to assess the phenolic contents, which were then expressed as GA equivalents (GAE)/g of sample. Serial dilutions of a stock GA standard in deionised water were carried out to provide a calibration curve at 500, 250, 125, 62.5 and 31.25 µg/mL. 100 µL of GA standard solutions, blank or each extract was added into the test tube, followed by 7.65 mL of water. 250 µL of the diluted Folin–Ciocalteu phenol reagent and 2 mL of 20% sodium carbonate solution were added and the resulting solution thoroughly mixed. The absorbance values were measured at 765 nm using a spectrophotometer after incubation at 40 °C for 60 min. Final results were given as mg of GAE/kg sample.

### ***Antioxidant analysis***

Evaluation of antioxidant activity the ABTS•+ free radical scavenging activity was determined. ABTS•+ solution (2.50 mL) was mixed with 50 µL of the extract of Naem-Hed. A decrease in absorbance was determined at a wavelength of 734 nm after keeping the samples for 3 min in the dark box. The antioxidant activity of the ethanol extract of Naem-Hed was calculated from the ascorbic acid calibration curve and was expressed as mg ascorbic acid equivalent per one hundred grams.

### ***Determination of free $\alpha$ amino nitrogen, protein content and moisture content***

Free alpha amino nitrogen (FAN) was quantified by spectrophotometric method (Wylie and Johnson, 1961). The modified method of Bradford (1976) and Kirk and Sawyer (1991) were used to determine total soluble protein content and moisture content, respectively.

### ***Sensory analysis***

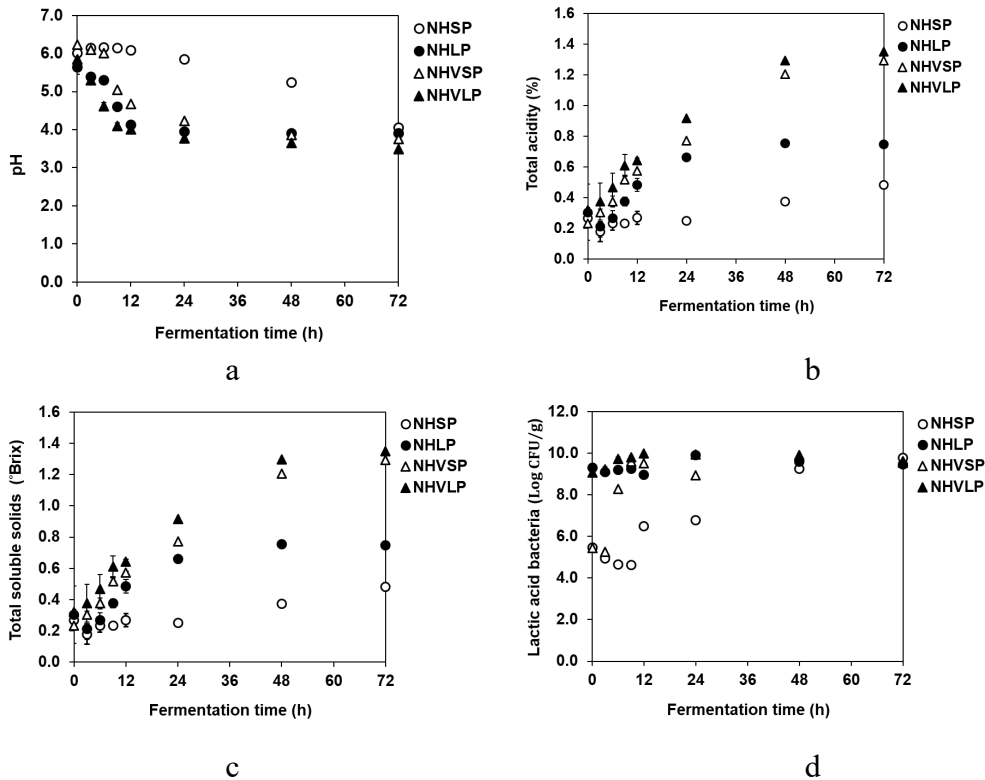
Fermented mushroom and vegetable product was evaluated for organoleptic quality. Assessors were experienced in fermented mushroom products. A 30- member panel took part in this study. Assessors were asked to rate the products for appearance, color, odor, flavor, and overall preference on a structured nine-point hedonic scale; 9 = like extremely; 8 = like very much; 7 = like moderately; 6 = like slightly; 5 = neither like nor dislike; 4 = dislike slightly; 3 = dislike moderately; 2 = dislike very much; 1 = dislike extremely and ranking test; ordering the four samples from “Like the most” to “Like the least” (Meilgaard *et al.*, 2016).

## **Results**

### ***Changes of pH, titratable acidity, total soluble solid content and lactic acid bacteria during the fermentation***

The initial pH ranged from 5.6 to 6.2 in the spontaneous and LAB-inoculated samples (Figure 1). During the fermentation process, pH values decreased to between 3.0-4.0 in all samples at the end of the fermentation. There was obviously significant difference between the inoculated and

vegetable supplement groups, indicating that inoculation of starter culture and supplementation of vegetables had a significant effect on pH values.



**Figure 1.** Changes of pH, titratable acidity, and lactic acid bacteria during the fermentation: NHSP; Naem-Hed with spontaneous fermentation, NHLP; Naem-Hed with *L. pentosus* inoculation, NHVSP; Naem-Hed supplemented vegetables with spontaneous fermentation and NHVLP; Naem-Hed supplemented vegetables with *L. pentosus* inoculation

The titratable acidity showed consistent rapid changes in Naem-Hed samples supplemented with vegetables during fermentation and there was a significant difference between the spontaneous and inoculation fermentation of mushroom without supplementation of vegetables. The acidity of samples supplemented with vegetables increased more sharply during the fermentation period than in the other two groups. Unlike pH and titratable acidity, changes of total soluble solid contents were fluctuated among sample groups which ranged between 5.50-6.60 °Brix. Total acidity values at the beginning were increased from 0.27-0.32 % to 0.48-1.35 % after 3 days of fermentation.

The largest increase in counts of LAB in spontaneous fermentation was observed in the sample supplemented with vegetables during the first 9 h fermentation, and then the growth reached a stationary phase (Figure 1). A noteworthy increase of 4 log CFU/g of LAB in generic fermentation of Naem-Hed was seen after 48 h fermentation. LAB starter inoculation was compared and consistently kept at the high levels of LAB counts throughout the fermentation period.

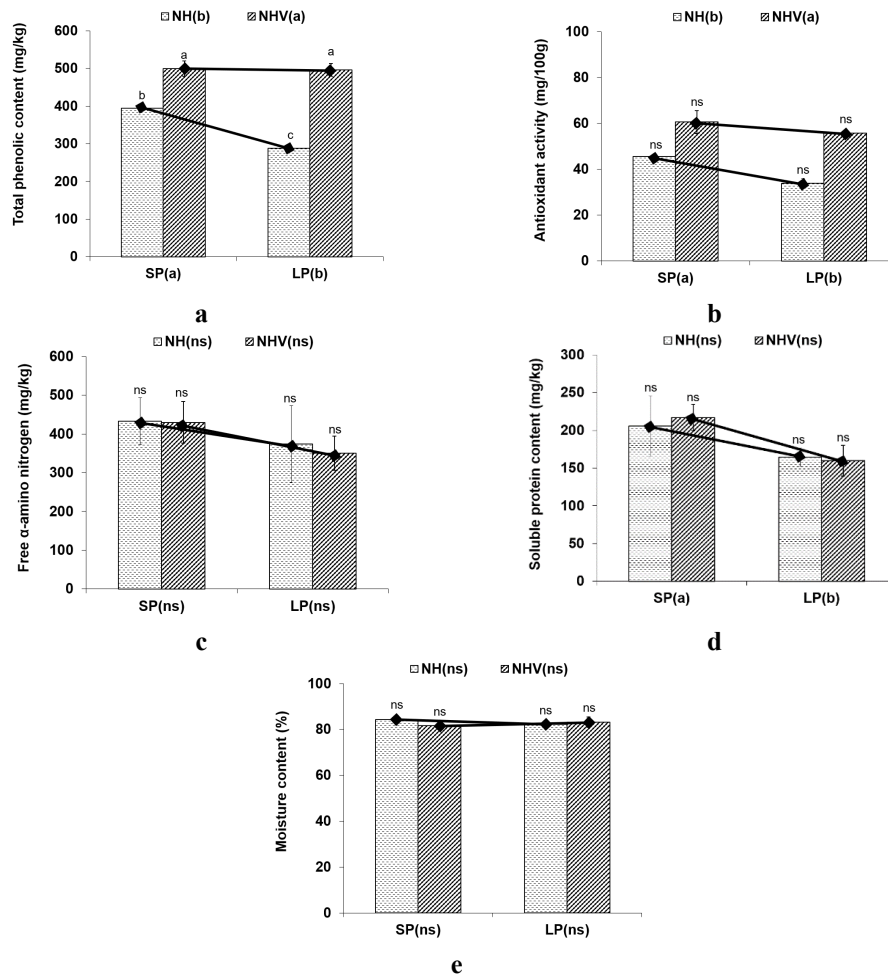
### ***Chemical properties of Naem-Hed products***

Chemical quality in the fermented product was determined and summarized in Figure 2. Total phenolic content, antioxidant activity, free alpha amino nitrogen, total soluble protein content and moisture content of the Naem-Hed products ranged between 288-499 mg/kg, 33.96-60.62 mg/kg ascorbic acid equivalent/100g, 350-433 mg/kg, 160-217 mg/kg and 81.41-83.18%, respectively. Supplementation of vegetables and LAB inoculation had significant effects on total phenolic content and antioxidant activity in Naem-Hed products ( $p \leq 0.05$ ). The trial found evidence of an interaction between supplementation of vegetables and LAB inoculation in treatment effect ( $p \leq 0.05$ ) for total phenolic content but no interaction existed for antioxidant activity. Higher total phenolic contents and antioxidant activity were obtained in the samples with vegetable supplement than no vegetable supplement while lower contents were detected in the samples with *L. pentosus* inoculation than with spontaneous fermentation. Vegetable supplementation had no significant effect on free alpha amino nitrogen contents, total soluble protein contents and moisture contents in Naem-Hed sample products. *L. pentosus* inoculation had also no significant effect on free alpha amino nitrogen contents and moisture contents in Naem-Hed sample products but the significant effect was found in total soluble protein contents. No interaction existed between the two factors for assessed properties of free alpha amino nitrogen contents, total soluble protein contents and moisture contents in Naem-Hed products.

### ***Sensory properties of Naem-Hed products***

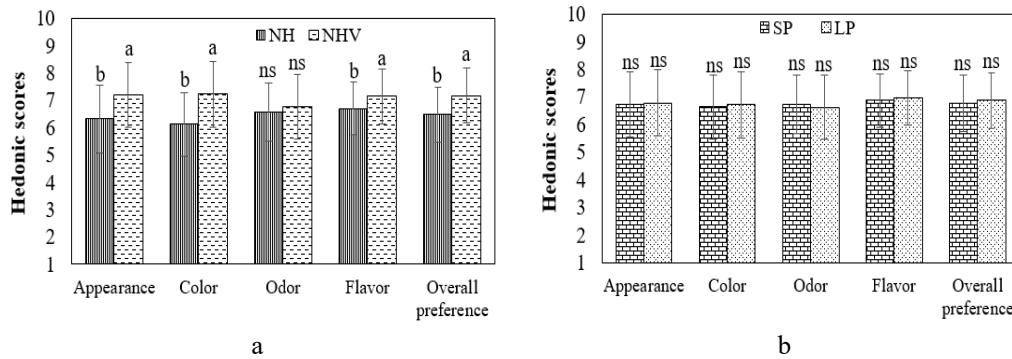
The organoleptic scores of all tested Naem-Hed products were evaluated by an experienced panel. The effects of vegetable supplement and LAB inoculation on sensory properties are given in Figure 3. The hedonic results of four attributes (appearance, color, flavor and overall preference) were significantly affected by vegetable supplement but no effect was detected by LAB inoculation on assessed sensory attributes. Characteristics of Naem-Hed

presented in Figure 4. Rating scale of like the most by ranking task was found in Naem-Hed produced by mushroom supplemented vegetables and fermented with *L. pentosus* inoculation.

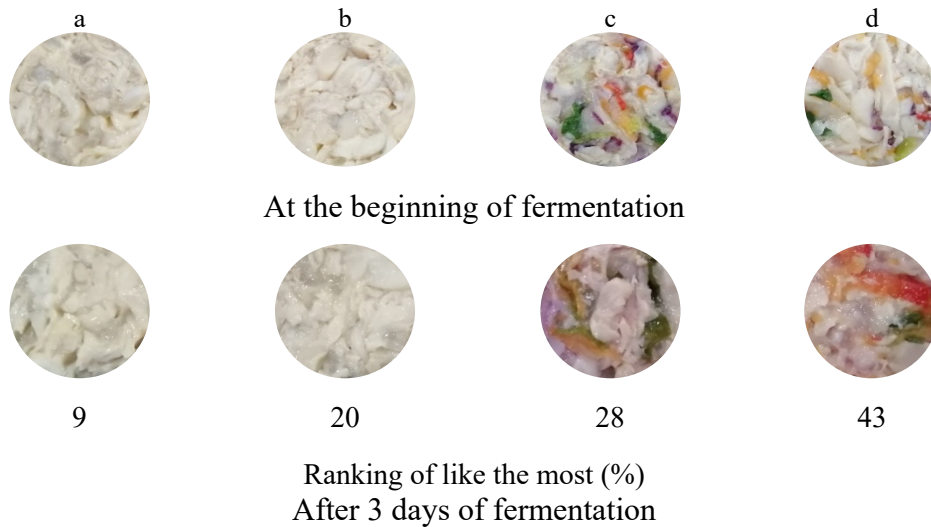


**Figure 2.** Interaction plots showing the effects of vegetable supplement and LAB inoculation on total phenolic content, antioxidant activity equivalent with ascorbic acid, free  $\alpha$  amino nitrogen, soluble protein content, moisture content: NHSP; Naem-Hed with spontaneous fermentation, NHLP; Naem-Hed with *L. pentosus* inoculation, NHVSP; Naem-Hed supplemented vegetables with spontaneous fermentation and NHVLP; Naem-Hed supplemented vegetables with *L. pentosus* inoculation: ns denotes means are not significantly different ( $p > 0.05$ ). Letters in parentheses shared in common indicate no significant difference between the respective groups ( $p \leq 0.05$ ). Bars with different letters represent significant differences ( $p \leq 0.05$ ).





**Figure 3.** Effect of vegetable supplement (a) and LAB inoculation (b) on sensory scores of Naem-Hed products



**Figure 4.** Characteristics of Naem-Hed samples at the beginning and after 3 days of fermentation, where NHSP= Naem-Hed with spontaneous fermentation (a), NHLP= Naem-Hed with *L. pentosus* inoculation (b), NHVSP= Naem-Hed supplemented vegetables with spontaneous fermentation (c) and NHVLP= Naem-Hed supplemented vegetables with *L. pentosus* inoculation (d)

## Discussion

The research findings are extended from the previously research in using LAB inoculation to produce Naem-Hed with enhancement of fermentation process and product quality (Chomsri and Manowan, 2020; Manowan *et al.*, 2020). The authors reported previously that different processes for Naem-Hed fermentation enhance its product quality. This study investigated whether vegetable supplement and LAB inoculation affected the fermentation behaviours and properties of Naem-Hed.

Spontaneous fermentation exhibited slowly to decrease pH rate which correlated to acidity, and LAB count increased during Naem-Hed fermentation when compared to *L. pentosus* inoculation. In particular, Naem-Hed with vegetable supplement showed similar acidity patterns and the maximum acidity generating was resulted from Naem-Hed with vegetable supplement and *L. pentosus* inoculation. Capability of LAB to lower pH in fermented vegetables was also reported in other fermented vegetables such as Kimchi (Lee *et al.*, 2019) and sauerkraut (Xiong *et al.*, 2014). An increase in LAB count results in a decrease in pH and an increase of titratable acidity in the product, finally affecting the shelf-life of Naem-Hed. These findings are consistent with previous studies, suggesting that the lactic acid fermentation can be controlled by through starter inoculation (Park *et al.*, 2019). Many studies have reported that LAB starter influenced changes during fermentation and product qualities (Jaichumjai *et al.*, 2010; Xiong *et al.*, 2014; Manowan *et al.*, 2020; Sharma *et al.*, 2021; Lee *et al.*, 2021). These LAB are reported to originate from the raw materials. Therefore, the proportions of the vegetable ingredients and seasonings employed in this study may all affect microbial diversity at the beginning of fermentation and also the product quality characteristics. The results from this study indicate that LAB can proliferate sufficiently without LAB starter inoculation. However, it is important to control fermentation process and shorten fermentation time by using LAB starter inoculation regarding quality, stability and safety (Park *et al.*, 2019; Capozzi *et al.*, 2017).

We hypothesized that LAB-starter inoculation could positively affect the fermentation state and microflora of the product as having efficient or adequate LAB and supplementation of vegetable as co-substrate in Naem-Hed production has an advantage in fermentation process because of increasing nutrient levels compared to generic Naem-Hed fermentation process. Moreover, phytochemical compounds from selected vegetable supplement are associated

with functional properties of Naem-Hed. The results suggested that pH and titratable acidity are important indicators of the degree of fermentation in Naem-Hed, which is closely correlated with LAB population. pH rapidly dropped at the first 24 hours of fermentation in this study. It was also observed in traditional fermented Naem-Hed (Chomsri and Manowan, 2020). Fermented fruits and vegetables usually have a low pH, such as sauerkraut and kimchi (Xiong *et al.*, 2014; Lee *et al.*, 2019), whose pH values varied between 3.1 and 4.4. However, the pH values of kimchi were higher than sauerkraut foods, mainly due to its fermentation condition, substrate difference, inoculation of LAB-starter.

Vegetable supplement as co-substrate of mushroom fermentation can significantly enhance total phenolic content and antioxidant activity of Naem-Hed. These phenomena are commonly explained by the phytochemical compounds containing in the vegetable used in this study (Rosa *et al.*, 2010). High moisture contents of Naem-Hed can easily cause food deterioration. However, a wide range of metabolites through fermentation of LAB such as propionic, formic, acetic acid, and lactic acid, create unfavorable conditions for the growth of spoilage and pathogenic microorganisms (Bangar *et al.*, 2022) leading to prolonging shelf-life, pleasant sensory characteristics and nutritional value of foods.

Vegetable supplement significantly showed the effect on the liking scores for sensory attributes of Naem-Hed products when compared to treatment of *L. pentosus* inoculation. This finding indicated that the use of vegetables mixed with mushroom has an advantage in delivering components. It was correlated with increased color and flavour compounds from vegetable supplement (Bozalan and Karadeniz, 2011; Chávez-Mendoza *et al.*, 2015). Although the treatment with *L. pentosus* inoculation for Naem-Hed fermentation had no effect on sensory properties, the control aspect of acid production leading to pH lowering is critical for food safety.

Vegetable supplement as co-substrate of mushroom fermentation could significantly enhance total phenolic content and antioxidant activity of Naem-Hed including sensory properties. Shortening fermentation time of LAB starter inoculation and quality improvement of vegetable supplementation in Naem-Hed production process demonstrated the potential application.

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