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EDITORIAL TEAM

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Welcome message from Editor-in-Chief

Dear authors, reviewers, and readers

We are honored to present the first issue of the fourth volume of the Journal of Science and Agricultural Technology (JSAT), the official journal of the Faculty of Science and Agricultural Technology, Rajamangala University of Technology Lanna (RMUTL), Thailand. This issue includes six research articles from various institutions that contributed to this issue. The JSAT has been published in Thai Journal Online (ThaiJO), indexed in Google Scholar, and Digital Object Identifier (DOI) under the National Research Council of Thailand and is in the process of Thai Citation Index (TCI). The journal will publish high-quality articles under an intense peer-review process with solid support from various educational institutions domestically and abroad.

As an Editor-in-Chief, I am so grateful for the support from our submitting authors, reviewers, and staff. I promise to move forwards to gain international recognition preparing for further higher index ranking. In addition, I strongly encourage researchers around the globe to submit manuscripts to share knowledge and promote the growing field of science and agricultural technology.

Kind regards,

Assoc. Prof. Dr. Suntorn Wittayakun

Editor-in-Chief Journal of Science and Agricultural Technology
Dean of the Faculty of Science and Agricultural Technology
Rajamangala University of Technology Lanna, Thailand.

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Journal of Science and Agricultural Technology (JSAT) publishes original research contributions covering science and agricultural technology such as:

• Natural and applied sciences: biology, chemistry, computer science, physics, material science and related fields. Papers in mathematics and statistics are also welcomed, but should be of an applied nature rather than purely theoretical.

• Agricultural technology: plant science, animal science, aquatic science, food science, biotechnology, applied microbiology, agricultural machinery, agricultural engineering and related fields.

Furthermore, the JSAT journal aims to span the whole range of researches from local to global application.

The JSAT is published two issues a year.
Issue 1: January - June
Issue 2: July - December

Submissions are welcomed from international and Thai institutions. All submissions must be original research not previously published or simultaneously submitted for publication or submitted to other journals. Manuscripts are peer reviewed using the double-blinded review system by at least 3 reviewers before acceptance. There is no publication or processing fee.

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Use of color sensor device with resazurin test for rapid screening of raw milk with low microbiological quality

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ABSTRACT

The microbiological quality of raw milk is important for the dairy industry because it can directly affect the quality and shelf life of dairy products. To evaluate the microbiologdical quality of raw milk, the resazurin test, a rapid dye reduction assay, was commonly used. However, resazurin test results are usually read by the eye, which can cause errors in judging the milk quality. In this study, a color sensor device based on the RGB system for measuring the color resulting from the resazurin test was developed. The device was embedded with VEML6040, an RGB-based color sensor, and operated using an Arduino microcontroller. The results showed that there is a reverse correlation between the percentages of the blue color and the number of microorganisms, both with the resazurin test performed in test tubes and in reduced-scale microtubes. A linear regression line with an R^2 of approximately 0.85 was obtained from the plot between the percentages of blue values from the microtube resazurin test and the corresponding numbers of microorganisms. This suggests that using the blue values to classify milk according to its microbiological quality is possible. With the present dataset, the blue value of 25% could be used as a cut-off value to separate raw milk with microbial loads of 7 log cfu/ml or higher from milk with lower microbial loads.

Keywords: milk quality, dye reduction assay, rapid method, colorimetric device

INTRODUCTION

The microbiological quality of raw milk is important for the dairy industry because it can directly affect the quality and shelf life of dairy products. Contamination of microorganisms in raw milk can occur during and after the milking process (Elmoslemany et al., 2009). The level of microbial contaminants is one of the crucial factors that affect milk quality because it directly affects the sensory qualities of raw milk and processed products and their shelf life (Murphy et al., 2021, Barbano et al., 2006). According to the Thai Agricultural Standard, raw milk can be divided into 3 quality categories based on the plate count values. The categories include premium quality milk, good quality milk, and standard quality milk, which have limits for microbial loads of less than 200,000 cfu/ml, between 200,000 and 400,000 cfu/ml, and between 400,000 and 600,000 cfu/ml, respectively (Thai Agricultural Commodity and Food Standard, 2005). Raw milk having microorganisms higher than 600,000 cfu/ml is considered to have a lower microbiological quality than the standard requirement or poor microbiological quality.

Analysis of standard plate count (sometimes referred to as total plate count, total bacterial count, or bacterial count), which reflects general microbial quality, is routinely performed in the dairy production chain (Mendonca et al., 2014). Nevertheless, the time taken to obtain the results for the standard plate count method is usually long, up to 48 hours (Rosmini et al., 2004, Duncan et al., 2016). Therefore, dye-reduction assays have been used in the dairy industry to indirectly evaluate milk microbiological quality (Braide et al., 2015). One of the most common methods based on the dye-reduction principle is the resazurin test. In this test, resazurin, a dye indicator for oxidation-reduction potential, was used. The color that changes after testing a milk sample, which correlates with the number of microorganisms in raw milk, is used to classify milk quality.

The principle of a dye reduction assay is the reduction of dye by substances in a sample, resulting in a change in the color of the dye, which is related to the metabolic rates of living cells in the sample. For the resazurin test, resazurin is reduced to resorufin and then to hydroresorufin, which turns the color from blue to colorless (Ibanez et al., 2019). A milk sample that still appears blue after the resazurin test is regarded as being of excellent quality. Milk samples that have turned mauve, pink, pale pink, or white are regarded as good, fair, poor, and bad quality, respectively (Al-shamary and Abdalali, 2011). For quality control purposes, it is important for the dairy industry or milk collection center to rapidly screen out milk that has poor microbiological quality before it enters the bulk tank or the processing lines.

Although the resazurin test is a quick (one-hour) test with great potential for screening milk quality, it can have errors due to individuals' color judgment, which can appear in a variety of shades. In addition, when there are many samples to analyze, it takes time to manually read and record the color results. Color sensors have been used in previous research for evaluating the quality of foods. Some examples include the use of sensors to screen the quality of tomatoes (Adamu and Shehu, 2018) and measure the color of flavored milk using an RGB camera-based image technique (Minz and Saini, 2021).

In this study, therefore, we aimed to develop a colorimetric system with an RGB-based color sensor for measuring the milk colors resulting from the resazurin test and for screening out milk with poor microbiological quality.

MATERIALS AND METHODS

Designing of colorimetric device

A box with a light-tight lid was designed using the program "360 Fusion" and printed using a 3D printer. The box was built to accommodate a six-well microplate, which was used to contain the sample for color measurement. An RGB color sensor VEML6040 was assembled into the lid of the box, with a white-light LED lamp installed next to the sensor as a light source. The sensor that is attached to the lid could be moved to the position above each well in the six-well microplate when the color measurement was taken.

Software Development

A software program to command the sensor was developed using an Arduino IDE program based on the RGB system. The original raw data obtained from the readings of red, green, and blue by the VEML6040 sensor was converted by the coding to percentages of red, green, and blue (%RGB), which, when combined, made up 100%. The reading was taken every second, 10 times for each sample.

Preparation of raw milk samples with different levels of microorganisms

Raw milk samples were collected from milk collection centers of different dairy cooperatives in Chiang Mai, Thailand. The samples were collected aseptically in a 1000-ml bottle and kept in an ice box while being transported to the laboratory for analysis. The original raw milk sample from each source was divided into subsamples with different levels of microorganisms. To prepare a set of subsamples for each original milk sample, a 30-ml portion of each sample was transferred to a 50-ml conical tube for 8–10 tubes in total. Each tube was then incubated at 37 °C in the water bath. The first tube was collected immediately (0 h, or identical to an original milk sample). The rest of the tubes were collected at the end of each hour. Plate counting was performed on each sample using the dilution-spread plate technique on Plate Count Agar (PCA).

Resazurin test

The resazurin solution was prepared in sterile distilled water at a concentration of 0.005% (w/v). The solution was kept from light and oxygen, stored at 4 °C, and used within 2–3 days. Each milk sample and the subsamples were subjected to the resazurin test in the traditional test tube and microtube configurations.

The traditional resazurin test was performed in glass test tubes according to the protocol described by Harrigan (1998) with a ratio of resazurin solution to the milk of 1:10. First, a 10-ml portion of a raw milk sample was transferred to a test tube. After that, one milliliter of the resazurin solution was added to the milk, and the tube was tightly closed and inverted twice. The tube was incubated for 1 h in a water bath at 37 °C. The modified resazurin test in the 1.5-ml microtube configuration was performed using the same ratio of resazurin solution to milk, but with a reduced volume of 1 ml. The milk-resazurin content was mixed by inverting the microtube 2–3 times, and the microtube was incubated for 1 h in the water bath set at 37 °C. For each replicate of the milk sample, the resazurin test was carried out in five microtubes. The resazurin test for each sample or subsample was carried out in triplicates.

Measuring color values from resazurin test using color sensor device

The results of the resazurin tests performed in two different configurations were read using the color sensor device installed with sensor VEML6040. After 1 h incubation of the milk samples subjected to the resazurin test, the contents of the test tubes and the microtubes were transferred into the wells of the six-well microplate. For the test performed in the test tubes, 5 ml of each replicate was transferred to each well. For the test performed in microtubes, the contents of 5 tubes (1 ml per tube) from each replicate were transferred to a well. The microplate was placed in the box of the color sensor device for color measurement. The digital data of the colors red, green, and blue were converted to percentages of red, green, and blue (%RGB), as described above.

Enumeration of microorganisms in raw milk samples.

To enumerate microorganisms in raw milk samples, a plate count was performed using the drop plating method on Plate Count Agar (PCA). Ten-fold serial dilution was carried out by diluting the raw milk samples in 0.1% (w/v) peptone water (mostly, dilutions 10^{-2} to 10^{-7} were used). Then, in triplicate, a 10-µl portion of each dilution was dropped on PCA. The plates were incubated for 48 h at 32 °C (De Silva et al., 2016)., and the average numbers of colonies from an appropriate dilution (dilution giving approximately 5–50 colonies) were calculated into colony-forming units per milliliter of milk.

Data analysis

The data from the sensors' digital colors were presented as mean values. The average numbers of microorganisms were converted to log colony-forming units per milliliter of milk. The linear regression model and the coefficient of determination (R^2) were generated using Microsoft Excel.

RESULTS AND DISCUSSION

Resazurin test color as observed by eye and color sensor

In this study, a color sensor device embedded with the VEML6040 color sensor was made. The device was used to measure the color resulting from the resazurin test of raw milk samples and subsamples from three different sources, which contained different levels of microorganisms.

After transferring the content in the test tubes and microtubes to the six-well microplate in order to read the RGB values, it was observed that each color that appeared to the eye corresponded to a wide range of digital color values of red, green, and blue, as well as a wide range of numbers of microorganisms (Table 1). This confirmed that the color sensor had a better capacity than the human eye to differentiate the colors. It also pointed out the limitations of manual color reading to accurately judge the microbiological quality of milk.

Table 1. Ranges of digital values of colors read by VEML6040 in relation to colors observed by eye resulted from the resazurin test performed using the traditional test tube and microtube formats

Config-uration	Value measured	Color observed by the eye				
		Blue	Mauve	Pink	Pale pink	White
Test tube	%R	33.25 – 36.36	34.27 – 39.73	37.40 – 38.54	36.31 -39.22	34.50 -38.61
	%G	36-76 – 39.84	35.37 – 39.41	36.88 – 37.57	35.96 – 39.26	36.52 – 42.70
	%B	24.21 – 27.98	24.90 – 26.95	24.57 – 25.04	23.50 – 26.19	21.85 – 24.87
Microtube	%R	33.17 – 36.69	34.67 – 39.00	36.51 – 38.60	36.09 – 39.07	34.50 – 39.06
	%G	37.75 – 39.71	36.08 – 39.16	36.70 – 37.85	36.18 – 39.15	36.01 – 42.27
	%B	25.32 – 27.53	24.92 – 26.80	24.53 – 25.63	23.49 – 26.20	21.89 – 24.93
MO (log cfu/ml)		5.08 – 6.60	5.03 - 7.20	6.28 – 7.60	7.19 – 8.61	7.11 – 9.90

MO: microorganisms

Colorimetric analysis of resazurin test

The digital values of the blue color (%B), measured by the color sensor device, showed a reverse correlation with the numbers of microorganisms, whereas those of the red and green colors (%R and %G) had inconsistent correlations with the numbers of microorganisms in raw milk. When the levels of microorganisms were approximately 7 to 8 log cfu/ml, a turning point in the red and green curves was observed (Figure 1) for every set of samples tested, regardless of the sources. The same pattern was observed for the resazurin test

performed in both configurations (test tube and microtube), and the %R, %G, and %B for the two configurations also showed similar trends (Figure 1). This indicated that it is possible to perform the resazurin test in the microtube configuration.

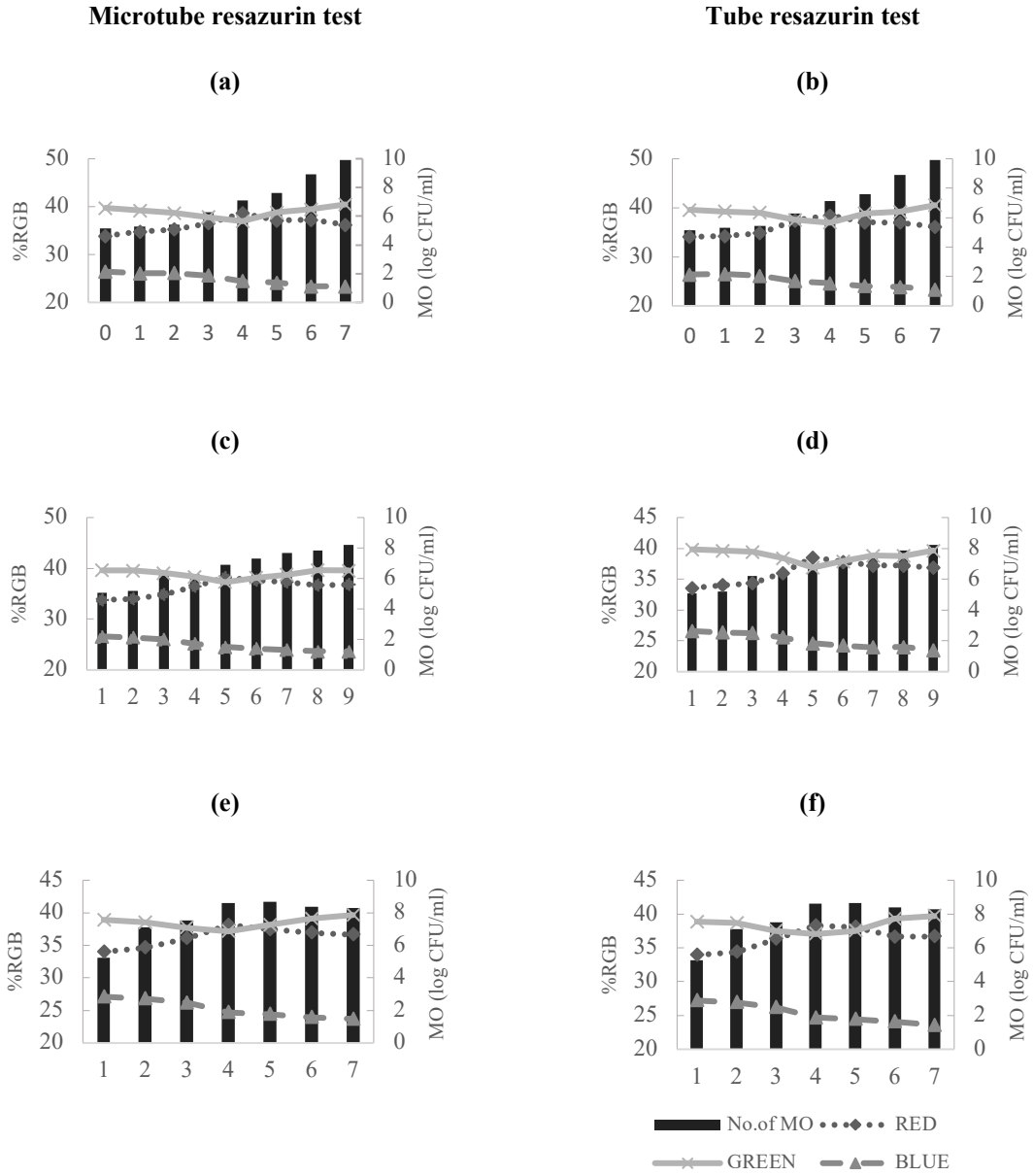

Figure 1. Results from the resazurin test carried out in the microtube and tube configurations. The digital color values of red, green, and blue (displayed in percentages; %RGB) measured using a color sensor device embedded with VEML6040 are presented together with the log number of microorganisms (MO) per milliliter. The results are presented by the source of raw milk: (a) and (b): Cooperative S, (c) and (d): cooperative W, (e) and (f): cooperative O.

Correlations between blue color values and numbers of microorganisms

As observed from the previous experiment, the blue color values obtained by the color sensor showed a reverse correlation to the number of microorganisms in raw milk. This correlation pattern was observed in milk sample sets from all three sources (three cooperatives). When plotting the digital blue values and the numbers of microorganisms using the combined data from all sources, a linear regression pattern can be seen for both microtubes and test tube configurations (R^2 of 0.8459 and 0.7313, respectively). As discussed above, it seemed to be possible to perform the resazurin test in the microtube configuration, which is a smaller scale, having 10 times fewer volumes of

both the sample and the resazurin reagent. However, it is suggested that a correlation curve to be used for the prediction of microorganism levels or for the classification of milk quality based on resazurin reduction should be constructed from the database of the resazurin test results performed in the same configuration.

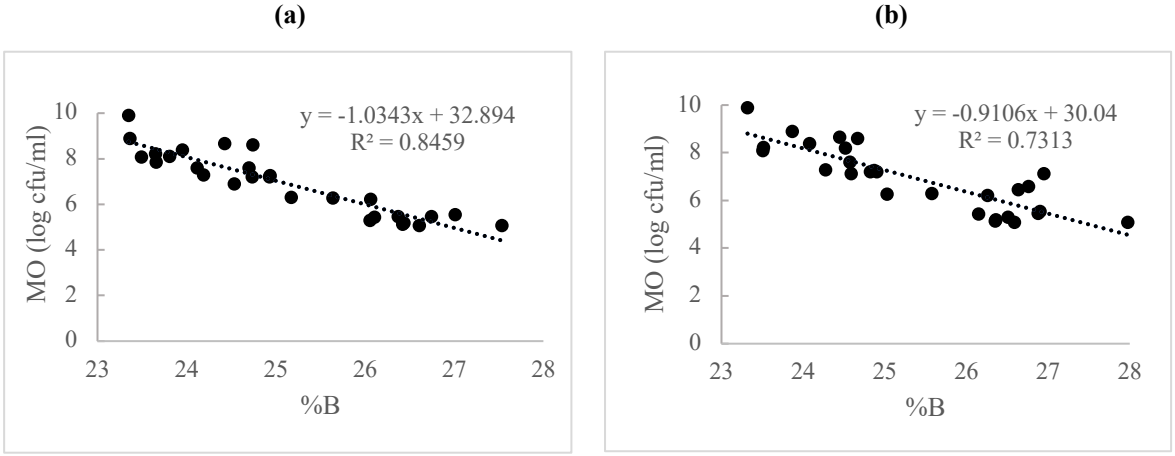

Figure 2. Correlation curves of %B values measured using the color sensor VEML6040 and the numbers of microorganisms (MO, displayed in log cfu/ml). The curves were constructed from the results obtained from the resazurin test performed in microtubes (a) and test tubes (b).

Potential application of correlations between blue values and numbers of microorganisms

The correlations between the percentages of B values from the color sensor and the numbers of microorganisms can theoretically be used to predict raw milk quality based on the level of microorganisms. With the available dataset, it is observed that when the levels of microorganisms were 7 log cfu/ml or above, the B values were almost always less than 25% (Figure 3). However, at the levels of microorganisms of 6 log cfu/ml or lower, the blue values appeared to cover a wider range.

Based on the observations above, we propose that a cut-off blue value (in this case, 25%) could be used to separate milk of low microbial quality (milk with extremely high microbial loads) from the milk of higher quality.

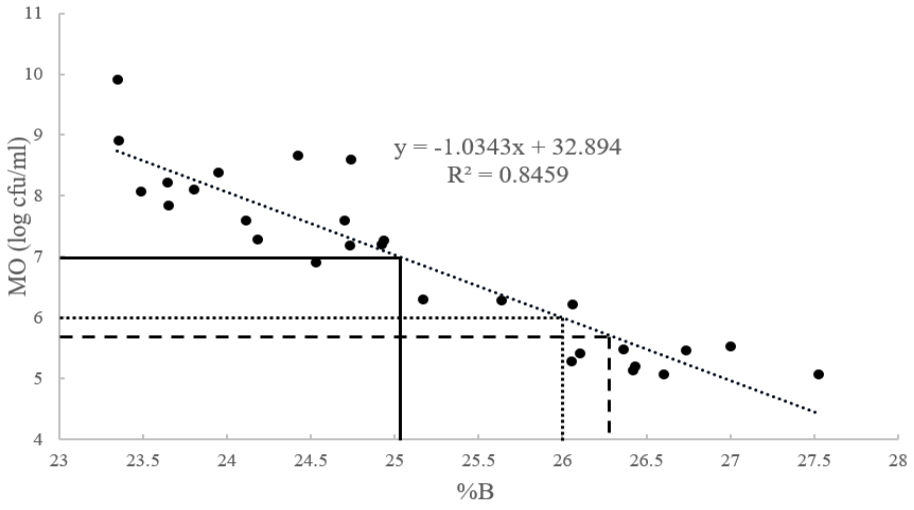

Figure 3. A demonstration of how the correlations between the blue values (%B) and the numbers of microorganisms in milk can be used. In this example, the blue value of 25% can be used as a cut-off value to separate raw milk with microbial loads of 10^7 cfu/ml (7 log cfu/ml) or higher from milk of higher quality (with lower microbial loads).

Color sensors are increasingly used to evaluate food quality. In this study, it is shown that an RGB color sensor could be used to measure the color resulting from the resazurin reduction test. This test is routinely used to evaluate raw milk quality entering the processing plants (O'Grady et al., 2020), and having a color sensor device as an alternative to the traditional manual reading would be of great benefit to the dairy industry. Researchers have long observed inconsistent correlations between the resazurin test colors and the numbers of microorganisms, which can be caused by the temperature of raw milk, the amount of cream, and the types of microorganisms in milk (Quigley et al., 2013). In this study, although with a limited amount of raw milk samples, we interestingly observed a reverse correlation between the blue values measured by the RGB-based VEML6040 color sensor and the numbers of microorganisms. Such correlation was consistent for the resazurin test results obtained in both a traditional glass test tube and a modified microtube. A linear regression model derived from this correlation would be particularly useful for the dairy industry and the milk collection units to improve the overall quality of bulk tank raw milk. It can also prevent milk with unacceptably high microbial loads from entering dairy processing plants. However, more samples are needed to construct an accurate predictive model. This point should be considered in future research work.

CONCLUSIONS

In this study, a color sensor device based on an RGB system for measuring the color resulting from the resazurin test was developed. The device was embedded with VEML6040, an RGB-based color sensor, and operated using an Arduino microcontroller. It was used to measure the color from the resazurin test of raw milk samples from three sources with different levels of microorganisms. The results showed that there is a reverse correlation between the blue values (expressed in percentages) and the numbers of microorganisms, both with the resazurin test performed in test tubes or in reduced-scale microtubes. The plot between the percentages of blue values from the resazurin test performed in microtubes and the numbers of microorganisms showed a linear regression pattern with an R^2 of approximately 0.85. This suggests that it is possible to use the blue values from the color sensor device to predict or screen milk quality. With the present limited dataset, the blue value of 25% could be used as a cut-off value to separate raw milk with microbial loads of 10^7 cfu/ml (7 log cfu/ml) or higher from the milk of better quality (milk with lower microbial loads). However, to increase the accuracy of prediction, more samples were needed to construct a linear regression model, and the color sensor may need to be further refined.

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Detection of *Bacillus cereus* group and emetic *Bacillus cereus* group strains in milk using multiplex polymerase chain reaction

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ABSTRACT

Bacillus cereus group is one of the most frequently contaminated foodborne bacterial pathogen groups in milk and dairy products. Members of this group can cause emetic foodborne illness because of their ability to produce the emetic toxin. In this study, we aimed to investigate the occurrence of *B. cereus* group in raw milk and to develop a multiplex PCR method for detecting emetic *B. cereus* group strains. A total of 286 raw milk samples were collected from various dairy farming areas, including Mae Jo, Mae Wang, Sankampaeng, and Mae On, in Chiang Mai province, Thailand. The presence of *B. cereus* group species was examined using Bacara chromogenic agar, through which *B. cereus* group was found in 34.27% of the milk samples from all farming areas. A duplex PCR that detected *its* (universal gene) and *motB* (gene specific for the *B. cereus* group) was also performed, through which 47.90% of the milk samples were detected positive for the *B. cereus* group. Furthermore, a multiplex PCR targeting the *motB, cesA*, and *its* genes was developed. The developed method was successfully used to identify emetic *B. cereus* isolates from raw milk.

Keywords: foodborne pathogen, emetic toxin, rapid method, milk quality, dairy industry

INTRODUCTION

Bacillus cereus group is a specific group of Gram-positive, endospore-forming, rod-shaped bacteria that are closely related at the *16S rRNA* gene level (Ehling-Schulz et al., 2019). *B. cereus* group is comprised of many closely related species, including *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. anthracis*, *B. weihenstephanensis*, *B. cytotoxicus*, *B. toyonensis*, and *B. wiedmannii* (Miller et al., 2018). *B. cereus* and some closely related species are known to cause foodborne illnesses due to their ability to produce toxins. The illnesses can be divided into two distinct types: diarrheal and emetic, which are associated with enterotoxins and emetic toxin, respectively. There are many types of diarrheal enterotoxins, including hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), and cytotoxin K (Sehoeni and Wong, 2005), all of which are heat-labile proteins (Wang et al., 2014). It is believed that enterotoxins are released in the small intestine after cells disintegrate (Jeßberger et al., 2017). Emetic toxin is a single-entity toxin, a cyclic peptide with a molecular weight of 1.2 KDa. It is also known as "Cereulide" and has a similar structure to the antibiotic Valinomycin (Granum and Lund, 1997). Cereulide is made up of alternating amino and hydroxy acids, which are synthesized by nonribosomal peptide synthetases (NRPS), encoded by the genes in the cereulide synthetase (*ces*) cluster (Marxen et al. 2015). The toxin is highly resistant to heat, extreme pH, and proteolytic enzymes and is released in food by living cells (Rajkovic et al., 2008).

Because *B. cereus* group bacteria are widely distributed in the environment, they can easily contaminate many types of food (Stenfors Arnesen et al., 2008). Dairy products are among the types of food that are most frequently contaminated by the *B. cereus* group, which mainly comes from raw milk (Vidal et al., 2015; Coorevits et al., 2008). Maintaining the quality of raw milk is crucial as it may harbor *B. cereus* and serve as a source of contamination at milk processing plants. However, the data on the prevalence of *B. cereus* in raw milk in Thailand was very limited. Furthermore, the spores of *B. cereus* can survive pasteurization and can germinate and multiply in dairy products, producing toxins under favorable conditions. The heat-stable emetic toxin, once produced in dairy products, is difficult to eliminate and can pose health risks to consumers. Therefore, rapid methods for detection of *B. cereus* group species and its emetic toxins are needed for the dairy industry.

In this study, we aimed to investigate the occurrence of *B. cereus* in raw milk from local dairy farms in four dairy cooperatives located in different parts of Chiang Mai province, one of the most important dairy production areas in the northern part of Thailand. Also, because there is no simple, industrially applicable assay for detection of emetic

B. cereus group species, we also aimed to develop a multiplex PCR assay for the rapid detection of emetic *B. cereus*.

MATERIALS AND METHODS

Collection of raw milk samples

Samples of raw milk from dairy farms affiliated with dairy cooperatives in different areas of Chiang Mai, Thailand, which included the areas of Mae Jo, Mae Wang, Sankampaeng, and Mae On, were collected from January to July 2020. In total, 286 samples were collected from individual farms from the receiving tank at the dairy cooperatives with which the dairy farms were affiliated. Duplicate milk samples (50 ml) were taken from the receiving tank immediately after all the milk from each individual farm was combined. The samples were stored on ice after collection and during transportation, kept at 4 °C in the laboratory, and analyzed within 24 h.

Isolation on a chromogenic agar plate

B. cereus group was isolated from raw milk samples according to the method described in the Bacteriological Analytical Manual (BAM, US FDA). In brief, a 1-ml portion of each sample was transferred into 9 ml of Butterfield's phosphate buffer dilution water and mixed. The homogenate was serially diluted (10-fold) in the same buffer until the desired dilutions were obtained. A 100-µl portion of each dilution was plated on Bacara agar (BioMérieux, Marcy-l'Étoile, France) using the spread plate method. Plating was performed in duplicate. The plates were incubated aerobically at 30 °C for 24 h. Bacterial colonies that were pink or orange surrounded by an opaque zone of egg yolk hydrolysis, which were typical *B. cereus* group colonies, were selected from each plate and subcultured on Tryptone soya agar (TSA) (Oxoid, Basingstoke, UK) to obtain pure cultures. The pure cultures were stored at 4 °C and as frozen glycerol stock cultures.

Duplex PCR for detection of B. cereus group in raw milk

Extraction of Bacterial DNA from Raw Milk

Bacterial DNA was extracted from raw milk using the cetyltrimethylammonium bromide (CTAB) extraction method, modified from Worden (2009). Raw milk samples were centrifuged at 7000×g for 10 min. The pellet from each sample was resuspended in 600 µl of CTAB buffer [1 M Tris (pH 8.5), 5 M NaCl, 0.5 M EDTA, and 0.2% (w/v) CTAB]. Proteinase K (15 µl) was added to each sample, vortexed, and incubated at 60 °C for 1 h. Then, an equal volume of chloroform was added to the mixture. The content was mixed and centrifuged at 12,000×g for 15 min at 4 °C. The supernatant was transferred to a new tube, an equal volume of isopropanol was added, and the microtube was incubated at -20 °C for 1 h or overnight. The DNA pellet was collected after centrifugation at 12,000×g for 15 min, then washed twice with 70% (v/v) ethanol, and centrifuged at 12,000×g for 15 min. The supernatant was discarded, and the DNA pellet was air-dried before being resuspended in 50 µl of TE buffer. The DNA samples were stored at -20 °C. Throughout the preparation, filtered pipette tips were used.

Detection of the B. cereus group in raw milk samples using duplex PCR

A duplex PCR was used for the detection of *B. cereus* group species, targeting the *motB* gene (encoding the flagellar motor protein specific to *B. cereus* group species) and the *its* gene (internal transcribed spacer, used as an internal control for the presence of bacterial DNA). The primers used for the amplification of these genes are shown in Table 1. The 25 µl PCR reaction mixture contained 1 µl of DNA template (0.5–5 µg/µl), 0.2 µM of each primer, 0.2 mM of each dNTP, 3 mM of $MgCl_2$, 0.4 units of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and 2.5 µl of 10×Taq polymerase buffer. PCR was performed in the Eppendorf Mastercycler (Eppendorf, Framingham, MA, USA). The amplification was operated with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min. The amplified PCR products were analyzed using gel electrophoresis on a 1.5% agarose gel alongside a 1 Kb DNA ladder (Meridian Bioscience, Cincinnati, OH, USA). Agarose gel electrophoresis was performed in 1×TAE buffer for 80 min at 80 V. Following electrophoresis, the agarose gels, which were prestained with Safe Green dye (Vivantis, Selangor Darul Ehsan, Malaysia), were visualized under a blue light transilluminator.

Development of multiplex PCR for detection of emetic *B. cereus*

Bacterial strains and isolates

The reference bacterial strains used in this study included *B. cereus* F4810/72 (also known as B0358, an emetic strain) and *B. cereus* DSM 4384 (also known as B–4ac, a diarrheagenic strain). *B. cereus* group isolates used in this study were obtained from raw milk samples. They were isolated in this

study (see above) or in a previous study (Sinchao, 2021; received from Chanita Sinchao, an associate member of the Microbial Resource and Technology Laboratory, SCB2806, Chiang Mai University).

DNA extraction from bacterial isolates

Chromosomal DNA was prepared from *B. cereus* strains and isolates grown in Tryptone soy broth (TSB) (Oxoid, Basingstoke, UK) supplemented with 0.6% yeast extract for 24 h at 37 °C. The cell pellet from a 3-ml culture was washed twice with 500 µl of phosphate buffer saline (pH 7.2), resuspended in 600 µl of extraction buffer [200 mM Tris (pH 8.5), 250 mM NaCl, 25 mM EDTA, and 0.5% (w/v) SDS], followed by extraction with 300 µl of phenol: chloroform: isoamyl alcohol (25:24:1). After that, the mixture was centrifuged at 14,000 g for 10 minutes at 4 °C, and the upper layer was transferred to a new tube. DNA was precipitated by adding the same volume of isopropanol and incubated at -20 °C for 20 min. The DNA pellet was collected after centrifugation at 14,000×g for 10 min. The DNA pellet was washed with 70% (v/v) ethanol and centrifuged at 14,000×g for 10 min. The supernatant was removed, the DNA pellet was air dried, then resuspended in 50 µl of TE buffer, and DNA samples were stored at -20 °C. Throughout the preparation, filtered pipette tips were used.

Development of multiplex PCR

A multiplex PCR targeting *motB* (gene encoding the flagellar motor protein that is specific to the *B. cereus* group), *cesA* (the *cereulide synthetase* gene specific to emetic *B. cereus*), and *its* (universal for bacteria, used as an internal control) was developed with primers listed in Table 1. To optimize the multiplex PCR conditions, the annealing temperatures were tested from 55 to 70 °C. The multiplex PCR was performed in a 25 µl reaction volume, containing 2.5 µl of 10× buffer, 0.2 µM of each primer, 0.2 mM of each dNTP, 3 mM of $MgCl_2$, 1 unit of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and 1 µl of DNA template (0.5–5 µg/µl). The amplification was operated with an initial denaturation step (5 min at 95 °C), 35 amplification cycles (30 s at 95 °C, 45 s at 55 °C to 70 °C, 1 min at 72 °C), followed by a final extension (10 min at 72 °C). The developed multiplex PCR was tested with *B. cereus* group isolates from raw milk, *B. cereus* F4810/72, *B. cereus* DSM 4384, *B. subtilis*, and *Escherichia coli*.

Table 1. PCR primers for detection of the *B. cereus* group and emetic *B. cereus*

Target gene	Primer	Expected PCR product size (bp)	Primer sequence	T_m (°C)	Reference
its	*its* F	185	5' AATTTGTATGGGCCTATAGCTCAGCT 3'	64.7	Yang et al., 2005
	its R		5' TTTAAAATAGCTTTTTGGTGGAGCCT 3'	61.6	
mot B	*motB* F	577	5' ATCGCCTCGTTGGATGACGA 3'	60.5	Oliwa-Stasiak et al., 2010
	motB R		5' CTGCATATCCTACCGCAGCTA 3'	61.3	
ces A	*cesA* F	996	5' CCGCCAGCTAGATGAAAAAGA 3'	55.0	Sinchao, 2021
	cesA R		5' ATCACTTTCGGCGTGATACC 3'	55.0	

RESULTS AND DISCUSSION

Occurrence of B. cereus group in milk by chromogenic agar plate

From the analysis of 286 raw milk samples from four dairy farm areas, the *B. cereus* group was found in 34.27% of the milk samples tested. A parallel duplex PCR assay for detecting the occurrence of *B. cereus* group species through the presence of the *motB* gene, was also performed. It appeared that *B. cereus* group species were found in 47.90% of the samples tested. The results from this study suggest that the duplex PCR assay was more sensitive than the chromogenic agar method for detecting *B. cereus* group in milk. Due to the low levels of *B. cereus* contamination that can occur in milk, the PCR method, which is faster and more sensitive than traditional, culture-based methods, is often preferred. Even at low concentrations, it is difficult to culture them in the laboratory. The PCR assay has been suggested as a convenient method for detecting enterotoxigenic *B. cereus* isolates. (Wehrle et al. 2009). With the target of PCR detection being a species-specific gene and an emetic-toxin-related gene, performing a duplex or a multiplex PCR offers

a more rapid detection than performing multiple single reactions (Kalyan Kumar et al., 2010). Chelliah et al. (2017) found that *motB* was very specific to the *B. cereus* group, and detection of this gene could be an efficient way to identify the presence of this bacterial group. Previous studies found contamination of the *B. cereus* group in 9%–43% of raw milk samples by selective plating methods (Ahmed et al., 1983, Mosso et al., 1989, and Yobouet et al. (2014), and in 62% by a PCR method (Aksoy et al., 2021). In our study, *B. cereus* group was found in raw milk from farms in all areas examined (26.09%–36.59% by chromogenic Bacara isolation and 46.26%–53.85% by the PCR method) (Table 2), which, although within the range previously reported, indicated that the proportion of milk samples contaminated with *B. cereus* was high. Milk from some areas, such as Sankampaeng, was found to be less frequently contaminated by the *B. cereus* group species than milk from the other areas, using the Bacara isolation method. This could be because the bacteria were absent or present at a lower level than the detection limit. The contamination of the *B. cereus* group in milk from the individual farms in all the areas examined indicates that *B. cereus* group species would also be present in the bulk tank milk, where milk from individual farms in each location was combined. Since the raw milk was immediately sent through the pipeline to the bulk storage tank, where it is cooled down, the presence of *B. cereus* in the milk in the bulk tank was unavoidable. *B. cereus* in the bulk tank milk, which is used as raw material for the dairy industry, would survive pasteurization and contaminate the pasteurized dairy products. Therefore, it is important to keep the level of *B. cereus* group low through the proper storage of raw milk before processing and of pasteurized milk products to prevent the production of toxins, especially the heat-stable emetic toxin. In addition, proper cleaning and sanitizing of milking equipment, regular testing and monitoring, and maintaining milk storage temperature are necessary for ensuring milk quality and safety (Burke et al., 2018).

Table 2. Occurrence of the *B. cereus* group in milk from farms in four dairy farming areas in Chiang Mai

Area	No. of samples positive on Bacara agar (%)	No. of *motB*-positive samples (%)
Mae Jo	15/41 (36.59%)	19/41 (46.34%)
Mae Wang	18/52 (34.62%)	28/52 (53.85%)
Sankampaeng	12/46 (26.09%)	22/46 (47.83%)
Mae On	53/147 (36.05%)	68/147 (46.26%)
Total	98/286 (34.27%)	137/286 (47.90%)

Optimization of multiplex PCR for detecting B. cereus group

Annealing temperatures for a multiplex PCR designed to detect the *B. cereus* group and emetic *Bacillus* strains were tested from 55 °C to 70 °C. This temperature range was chosen from the melting temperatures of each primer (Table 1). The target genes in the multiplex PCR included *motB* and *cesA*, which are specific for *B. cereus* group and emetic *B. cereus* strains, respectively. The *its* gene was an additional target gene included in the multiplex PCR, which served as an internal control. Each primer set was first tested individually and later in the multiplex PCR, using the DNA templates from the reference strains: *B. cereus* F4810/72 (an emetic strain, *cesA*-positive) and *B. cereus* DSM 4384 (a non-emetic strain, *cesA*-negative). The PCR products from *B. cereus* F4810/72 showed clear bands for *its*, *motB*, and *cesA* at 185 bp, 577 bp, and 996 bp, respectively. The annealing temperatures at which three bands were clearly seen ranged from 56.1 °C to 63.4 °C (Figure 1 (a)). Therefore, the annealing temperature of 59 °C was chosen for the multiplex PCR analysis for the detection of emetic *B. cereus*.

The developed multiplex PCR was then used to detect emetic *B. cereus* isolates obtained from raw milk samples. The results showed that the multiplex PCR could successfully detect emetic *B. cereus* group isolates (Figure 1 (b), lanes 5–10). Many researchers had previously confirmed the specificity of *motB* for the *B. cereus* group and other species that are closely related to *B. cereus* (Oliwa-Stasiak *et al*. (2010); Chelliah *et al*. (2017)). Detection of *cesA*, the *cereulide synthetase* gene that is specific to emetic *B. cereus*, gives more information on the potential of the *B. cereus* group present in a raw milk sample to produce the emetic toxin. The internal control (*its* gene), a gene found in all bacteria, was included as a universal target gene to ensure that the PCR reaction was free of any PCR inhibitors and was correctly prepared (Yang *et al.* 2005). These primers and the optimized conditions

for the multiplex PCR could also be further applied for the detection of *B. cereus* group and emetic *B. cereus* in dairy products.

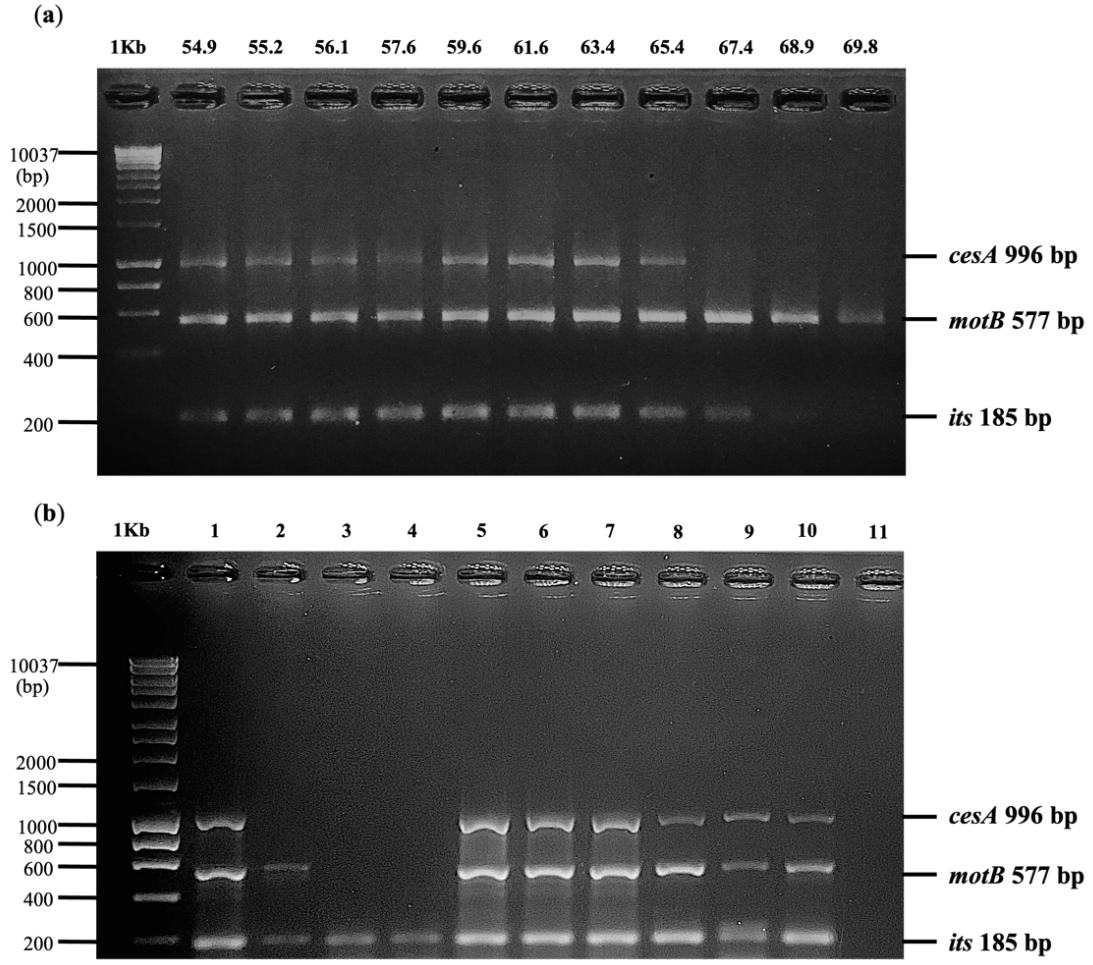

Figure 1. The amplified fragments obtained from the multiplex PCR designed for detection of the *its*, *motB*, and *cesA* genes. **(a)** Different annealing temperatures were tested to find an optimum annealing temperature, using the DNA from *B. cereus* B358 (F4810/72) (an emetic strain, *cesA*-positive) as a template. **(b)** Examples of multiplex PCR performed at an annealing temperature of 59 °C, using DNA from emetic *B. cereus* reference strains isolates from raw milk samples. Lane 1 = DNA from *B. cereus* F4810/72 (reference emetic strain), Lane 2 = DNA from *B. cereus* DSM 4384, non-emetic strain), Lane 3 = DNA from *B. subtilis (Bacillus species outside the B. cereus group)*, Lane 4 = DNA from *E. coli,* Lanes 5–10 = DNA from emetic *B. cereus* isolates from raw milk, and Lane 11 = negative reaction control (distilled water, no DNA). 1Kb = 1 kb DNA ladder (Meridian Bioscience, Cincinnati, OH, USA).

CONCLUSIONS

B. cereus group species were detected in raw milk samples from four dairy farming areas in Chiang Mai with a frequency of 34.27% and 47.90% by the Bacara chromogenic agar method and the PCR method, respectively. The duplex PCR targeting the *its* and *motB* genes was more sensitive than the chromogenic agar method and could be adopted as a method for rapid screening of the *B. cereus* group in milk. A multiplex PCR for detecting emetic *B. cereus,* which amplifies the *its, motB,* and *cesA* genes, was also developed. The PCR reaction was optimized using the DNA of *B. cereus* F4810/72, a reference emetic strain. As a result, an optimum condition was found with an annealing temperature of 59 °C. This multiplex PCR was tested with raw milk isolates of *B. cereus* and could successfully identify emetic isolates.

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Spray-dried powder of *Bacillus amyloliquefaciens* strain C2-1 for control of rice diseases

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ABSTRACT

Rice cultivation in Thailand faces challenges in achieving high yields due to diseases. This research aims to develop a spray-dried powder containing *Bacillus amyloliquefaciens* strain C2-1 for control economically damaging rice diseases caused by *Xanthomonas oryzae*, *Fusarium moniliforme, Pyricularia grisea*, and *Bipolaris oryzae*. The *B. amyloliquefaciens* strain C2-1 was cultured in 3 different media, including 1) medium contained nutrient broth with glucose (NB), 2) medium contained beef extract, molasses, K_2HPO_4 and KH_2PO_4 (BM), and 3) medium contained yeast extract, molasses, K_2HPO_4, and KH_2PO_4 (YM). The results showed that YM medium gave the highest concentration of bacteria at $1.30x10^{16}$ cfu/ml, followed by BM medium at $9.88x10^{12}$ cfu/ml, and NB medium at $5.3x10^{12}$cfu/ml. Therefore, YM medium was selected as the optimal culture medium. Subsequently, the *B. amyloliquefaciens* strain C2-1 was produced into a spray-dried powder using the spray-drying technique. Tapioca starch and carboxymethyl cellulose were used as spray-drying carriers in a ratio of 10:1. The optimal spray-drying conditions included a hot air inlet temperature of 90°C, a hot air outlet temperature of 70°C, and a feed rate of 10-15 ml/min, resulting in the highest survival rate at 2.8×10^{12} CFU/g^{-1}. The efficacy of *B. amyloliquefacien* strain C2-1 powder was then tested against rice diseases. The inhibition of *X. oryzae* was assessed using the paper disc diffusion method, while the inhibitions of *F. moniliforme*, *P. grisae*, and *B. oryzea* were evaluated using dual culture assay. The results demonstrated that *B.amyloliquefacien* strain C2-1) powder exhibited strong inhibitory effects against *X. oryzae,* with clear zone measuring 5.5, 5.7, and 5.7 mm at 24, 48, and 72 h, respectively. Regarding inhibiting *F. moniliforme*, *P. grisae*, and *B. oryzea*, *B. amyloliquefaciens* strain C2-1 demonstrated effective control of these rice pathogenic fungi, resulting in inhibitions of 73.0%, 68.72%, and 48.42% at 7 days, respectively. These findings strongly suggest that the *B.amyloliquefacien* strain C2-1 obtained from spray drying serves as a promising biocontrol agent against bacterial leaf blight disease, bacterial blight, bakanae disease, rice blast disease, and brown spot disease caused by *F. moniliforme*, *P. grisae*, and *B. oryzea*, respectively.

Keywords: Antagonist bacteria, *B.amyloliquefacien*, biological control, rice disease, spray drying

INTRODUCTION

Rice (*Oryza sativa L.*) is the crucial main food crop belonging to the family Poaceae and is widely cultivated in most tropical and subtropical regions (Ezuka and Kaku, 2000). Nowadays, rice cultivation in Thailand still faces problems that affect the growth and yield of rice caused by weeds, soil, water, and environmental changes. One primary disease of rice is the bacterial leaf blight disease caused by *Xanthomonas oryzae* which can infect rice at any growth stage causing production losses depending on climate, growing season, rice variety, and growth; if the disease is severe, the yield can be reduced by 50 percent (Chintaganon et al., 2022). *Pyricularia grisea* is a fungus that causes blast disease that damages rice. Rice blast disease is favored by a number of factors such as high relative humidity (>80 percent), low temperature (15°C - 26°C), cloudy weather, more wet or rainy days, and excessive doses of nitrogen fertilizers (Saleh et al., 2020). *Bipolaris oryzae* causes brown spot disease that affects both quantity and quality of rice grains. This pathogen, under favorable epidemic conditions, can cause reduction in grain yield of up to 90 percent (Dorneles et al., 2020).

Strains of *B. amyloliquefaciens* strain C2-1 have been reported as a potential biological agent for controlling other crop diseases (Shrestha et al., 2016). This is accompanied by Srivastava et al. (2016), who reported that *B. amyloliquefaciens* (SN13) acts as a biocontrol agent and enhance the immune response against *R. solani*,

a necrotrophic fungus causing sheath blight in rice, by modulating various physiological, metabolic, and molecular functions. Moreover, Prabhukarthikeyan et al. (2019) also reported that *Bacillus amyloliquefaciens* strain BS5 effectively reduced the brown spot disease in rice under *in vitro*, glasshouse, and field conditions.

The spray-drying technique has been routinely used for the preservation and concentration of microorganisms due to its gentle protocols of drying by exposing substances to only a short burst of extreme temperature, then providing a cooling effect via the evaporation process, which is able to maintain a high survival rate of microorganisms and low production cost (Desmond et al., 2001). In order to prolong shelf life, and increase efficacy, it is important to keep the bacterium in a dormant state, while maintaining its viability. The efficacy of bio-product was tested in a greenhouse. The result showed that the disease incidence was reduced by 100 and 61.54% when treated the rhizome with bio-products MTR13 and PS6, respectively, before planting and application of their suspension was done one hour after the pathogen inoculation when compared with the control. (Thano and Akarapisan, 2018). The aim of this study was to develop bio-based products based on *B. amyloliquefaciens* strain C2-1 under optimal spray drying conditions for controlling rice disease. Therefore, the information on efficacy testing may be useful and able to apply or recommended to farmers or those who are interested in controlling diseases in rice fields.

MATERIALS AND METHODS

Microorganism and inoculum preparation

The antagonist bacterium *B. amyloliquefacien* strain C2-1 was isolated from food waste compost obtained from the Center for Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. The rice pathogen, namely *X. oryzae, F. moniliforme, P. grisea, B. oryzae.* The mass losses may be attributed to the effect of the different parameters of spray drying, such as inlet temperature and airflow, as well as to the low Tg of carrying agents obtained from Phattalung Rice Research Center, Phattalung, Thailand. The *B. amyloliquefaciens strain C2-1 and X. oryzae* were cultured in nutrient broth (NB) (nutrient broth 13 g/L; glucose 10 g/L) and glucose broth medium (NGB) (13 g of nutrient broth and 12.5 g of glucose anhydrous per 1 L of distilled water), respectively at 37°C for 24 h on 200 rpm of the rotary shaker to prepare the inoculum. The *F. moniliforme, P. grisea, B. oryzae* were grown on potato dextrose agar (PDA) at 28°C for 7 days to prepare the inoculum.

Substrates and chemicals

All chemicals were of analytical grade, including nutrient broth (NB) (Himedia, Nashik, India) and KH_2PO_4 (anhydrous, Loba Chemie, Mumbai, India). Yeast extract powder was obtained from TM Mida (Rajasthan, India), beef extract from Srichem (Mumbai, India); KH_2PO_4 was obtained from Cloisters (Cherrybrook, Australia); glucose was purchased from Utopian Co., Ltd., Samuthprakarn, Thailand; and molasses was purchased from local markets (Songkla, Thailand).

Effect of culture media on antagonist bacterium growth

The antagonist bacterium *B. amyloliquefaciens strain C2-1* was cultured in different culture media, including nutrient broth (NB), beef extract molasses broth (BM) (beef extract 3 g/L; molasses 20 g/L; K_2HPO_4 0.05 g/L; KH_2PO_4 0.5 g/L) and yeast extract molasses broth (YM) (Yeast extract 0.5 g/L; Molasses 20 g/L; K_2HPO_4 0.05 g/L; KH_2PO_4 0.5 g/L) were prepared in each 250 mL flask with 100 mL medium containing. Each flask was cultured at 37°C for 24 h on 200 rpm of the rotary shaker before checking the colony forming unit (CFU/mL) using the serial dilution method on the NA agar medium. This experiment was maintained with three replications.

Preparation of antagonist bacterium in the spray-dried powder using spray-drying technique

The antagonist bacterium was cultured in YM, NB, and BM at 37 °C for 24 h on 200 rpm of the rotary shaker. The cell suspension was adjusted to 0.5 optical density (O.D. 600 nm) using the spectrophotometer (Thermo Fisher Scientific, USA) before mixing with tapioca starch and carboxymethyl cellulose (CMC). The mixing solution consisted of 30 mL cell suspension, 18.18 g tapioca starch, and 1.82 g CMC (Teera-Arunsiri et al., 2003) Spray drying was carried out in a Mini Spray Dryer B290 (BÜCHI, Labortechnik AG, Flawil, Switzerland) at the following operation conditions: feed pump of 5 ml/min °C, aspiration of 100% and pressure of 1.5 bar. The inlet and outlet temperatures were set as 90 and 70 °C, respectively. The obtained spray-dried powder was calculated as a percentage yield as following Eq. (1) that those reported by (Chumthong et al., 2016). Three replicates were performed for each formulation.

$$\% \text{ Yield} = [\text{outlet weight/inlet weight}] \times 100 \quad (1)$$

Viable bacterial counts in the formulations were tested against the bacterium before drying using the drop-on plate method (Zuberer, 1994) with bef. 0.1g/mL suspension of spray-dried powder was prepared in sterile distilled water and was serially diluted from 1×10^{-1} to 1×10^{-12}. The viable bacteria were cultured on NA medium at 37 °C for 24 h, after which the CFU was counted. The number of colony-forming unit per gram (CFU/g) was the average of six drops per dilution. The cell concentrations exhibited between 30 and 300 CFU (1×10^{-4} and 1×10^{-5}) were selected (Daniel et al., 2020). Equation (2) was employed for the quantification of culturability. All experiments were conducted in triplicate, and the reported values represented the average of the calculated values. Formulations with the concentrations of highest were chosen for further studies.

$$\text{CFU/g} = \frac{\text{[N°plate colonies x dilution factor]}}{\text{mL sample seeded}} \quad (2)$$

When N° is the initial population of *B. amyloliquefaciens* strain C2-1 (cfumL^{-1})

Antagonistic activities of B. amyloliquefacien strain C2-1 for pathogenic bacteria inhibition assays

Rice pathogenic bacteria, *X. oryzae* was cultivated in an NGB medium. 100 mL working volume of the NGB medium was conducted in each 250 ml Erlenmeyer flask for cultivation. The optical density of cell suspension was measured at 600 nm (OD600) with a UV-vis spectrophotometer (Thermo Fisher Scientific, USA). The culture flasks were inoculated with 10% (v/v) inoculums (initial cell suspension equal to 0.2 at OD600) at 25°C for 24 h on 200 rpm of the rotary shaker. The inhibition assays of spray-dried powder of *B. amyloliquefacien*s strain C2-1 testing were done using the agar well diffusion method (Yang et al., 2012). Glucose agar medium was prepared by pouring 10 mL glucose agar (NGA) medium into a petri dish, and let and let it set in a laminar flow hood as a basal layer. After that, use a cork borer, a diameter of 5 mm, drill 5 holes, and then using a cotton swab sterile dip the pathogens that have been prepared and make a swab over the entire surface of the culture medium. In this method, the agar plate was inoculated with a powder of *B. amyloliquefaciens strain C2-1*- dissolved in water by dropping each hole 10 ul of and placed on the four corners of the seed layer. At the same time,10 uL of sterile water was dropped into one hole in the center of the same petri dish (control). The petri dishes were then incubated at 37 °C for 24, 48, 72 h, and 1 week, and then measured the diameters of inhibition growth zones. The inhibition growth zones can be calculated from Equation (3).

$$R_a = (D_c - D_s) / 2 \quad (3)$$

where R_a is the inhibition radius (mm); D_c is the diameter of the clear area (mm), and D_s is the diameter of the specimen (mm)

Antagonistic activities of B. amyloliquefaciens strain C2-1 for pathogen fungi inhibition assays

Three fungal pathogens, *F. moniliforme, P. grisea*, and *B. oryzae* were cultured on PDA at 28°C for 7 days to test the inhibition of pathogen mycelial growth. The pathogen fungi inhibition assays of spray-dried powder of *B. amyloliquefaciens strain C2-1 spray-dried* was dissolved in 1 mL of sterile water. Each treatment was conducted in 3 replications. Mycelial inhibition of pathogen fungi was assessed as a percentage of mycelial inhibition at 3, 5, and 7 days after culturing pathogen fungi. The inhibition growth zones can be calculated from Equation (4) (Morton and Stroube, 1995).

$$\text{Percentage of pathogen mycelial inhibition} = [(R_1 - R_2) / R_1] \times 100 \quad (4)$$

Where R_1 is the colony radius of pathogen fungi on PDA incorporated powder *B. amyloliquefaciens* strain C2-1 solution of bacterial formulation, and R_2 is the colony radius of pathogen fungi control on PDA.

RESULTS AND DISCUSSION

Cultural media basic suitable for growth

The antagonist bacterium *B. amyloliquefaciens* strain C2-1 grown in YM medium performs the highest cell viability, followed by BM and NB, respectively (Table 1). As a result, YM and BM media contained a higher nutrient composition than the NB medium. It also can be seen that YM used molasses as a major ingredient which is a cheap carbon source, and yeast extract is a good source of nitrogen for growth. The buffered K_2HPO_4 and KH_2PO_4 can also help the infection grow better. It may help to adjust the pH condition so that it does not change according to the metabolites produced by the germ during growth.

Table 1. Viability of *B. amyloliquefaciens* C2-1 grown in different media

Formulation	cfu/g*		
	1st	2nd	3rd
NB	$1.8\pm0.0 \times 10^{12}$	$2.8\pm0.1 \times 10^{12}$	$2.6\pm0.0 \times 10^{12}$
BM	$3.6\pm0.1 \times 10^{12}$	$3.8\pm0.2 \times 10^{12}$	$5.5\pm0.2 \times 10^{12}$
YM	$4.9\pm 0.0 \times 10^{15}$	$5.2\pm 0.0 \times 10^{15}$	$4.7\pm0.1 \times 10^{15}$

Antagonist bacterium in the spray-dried powder using spray-drying

The initial amounts of powder dry matter of *B. amyloliquefaciens* strain C2-1 that grew in all three media after spray drying at a constant 90 °C inlet temperature were similar. From the initial amount of dry matter, 20 grams, after drying, the remaining 13 grams, thus the calculated average yield was 65%. The mass losses may be attributed to the effect of the different parameters of spray drying, such as inlet temperature and airflow, as well as to the low Tg of carrying agents (León-Martínez et al., 2010). Although the spray-dried product of *Bacillus* sp., especially *B*. subtilis (Yánez et al., 2012), has been devised and tested to control insect pests and plant disease, in this study, a spray-dried product of *B. amyloliquefaciens* strain C2-1 had been formulated and tested for the control of rice disease in the laboratory. The characteristic of spray-dried powder can be seen in Fig.1. The powder is fine and slightly yellowish-white. The viability of spray-dried powder of *B. amyloliquefaciens* strain C2-1 grown in different media is shown in Table 2. The result found that *B. amyloliquefaciens* strain C2-1 grown in YM medium performed the highest cell viability followed by BM and NB, respectively, which gave the same experiment result as the effect of culture media on antagonist bacterium growth. Therefore, the spray-dry powder of *B. amyloliquefaciens* strain C2-1 with YM medium was further studied for the pathogen inhibition assays.

Figure 1. Characteristic of powder of *B. amyloliquefaciens* strain C2-1 after spray drying at constant 90 °C inlet temperature.

Table 2. Viability of spray-dried powder of *B. amyloliquefaciens* strain C2-1 grown in different media

Formulation	cfu/g*		
	1st	2nd	3rd
NB	$3.4\pm0.0 \times 10^{8}$	$1.8\pm0.1 \times 10^{7}$	$1.7\pm0.1 \times 10^{7}$
BM	$4.2\pm0.2 \times 10^{10}$	$5.7\pm0.1 \times 10^{10}$	$5.8\pm0.0 \times 10^{10}$
YM	$2.6\pm0.1 \times 10^{12}$	$3.4\pm0.1 \times 10^{12}$	$2.8\pm0.1 \times 10^{12}$

Biocontrol activities of antagonistic B. amyloliquefacien strain C2-1

The test results of the efficacy in inhibiting the rice pathogen *X. oryzae* by the spray-dried powder of *B. amyloliquefaciens* strain C2-1 can be seen in Figure 2. The inhibition zone showed that the spray-dried powder was still effective against *X. oryzae* rice pathogens at 24, having clear zones of 1.65. This result shows that *B. amyloliquefaciens* strain C2-1 gave the inhibition of the causative agent of rice leaf blight. This inhibition may be caused by volatile substances from *B. amyloliquefaciens* strain C2-1, according to Wu et al. (2015), who reported that *B. amyloliquefacien* FZB42 had antibacterial activity against *Xoo* rice pathogens.

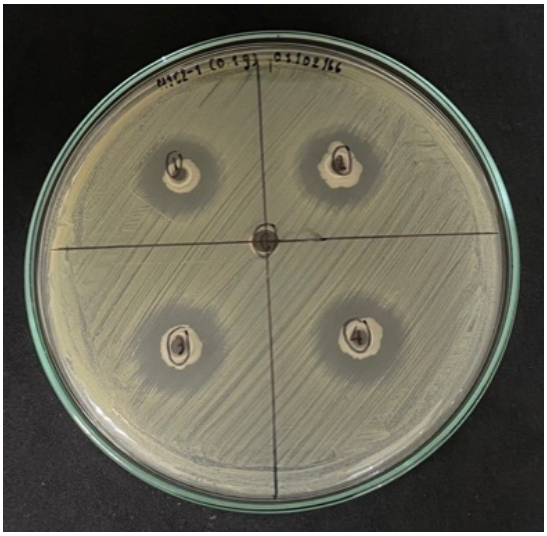

Figure 2. Reaction of the *X. oryzae* in agar well diffusion assay after inoculation for 24 hr. at 37 °C temperature, when inhibited by Powder *B. amyloliquefacien* strain C2-1.

The spray-dried powder of *B. amyloliquefacien* strain C2-1 showed antifungal activity against all 3 rice pathogenic fungi in dual culture tests incubated for 7 days. The spray-dried powder showed the strongest inhibition, as seen in Fig. 3, mycelial growth was reduced compared to control plate 3, 5, and 7 days. The inhibition percentages *F. moniliforme* were 0.78, 52.51, and 73.0%, respectively. For *B. oryzae*, the inhibition percentages by powder *B. amyloliquefaciens* strain C2-1 were 0.65, 22.86, and 48.42%, respectively. The mycelial growth of the pathogen *P. grisea*, and the inhibition levels by powder *B. amyloliquefaciens* strain C2-1 were 0.76, 56.99, and 68.72 %, respectively. Thus, powder *B. amyloliquefaciens* strain C2-1 had comparatively high effectiveness in suppressing the pathogens, after incubation for 3, 5, and 7 days.

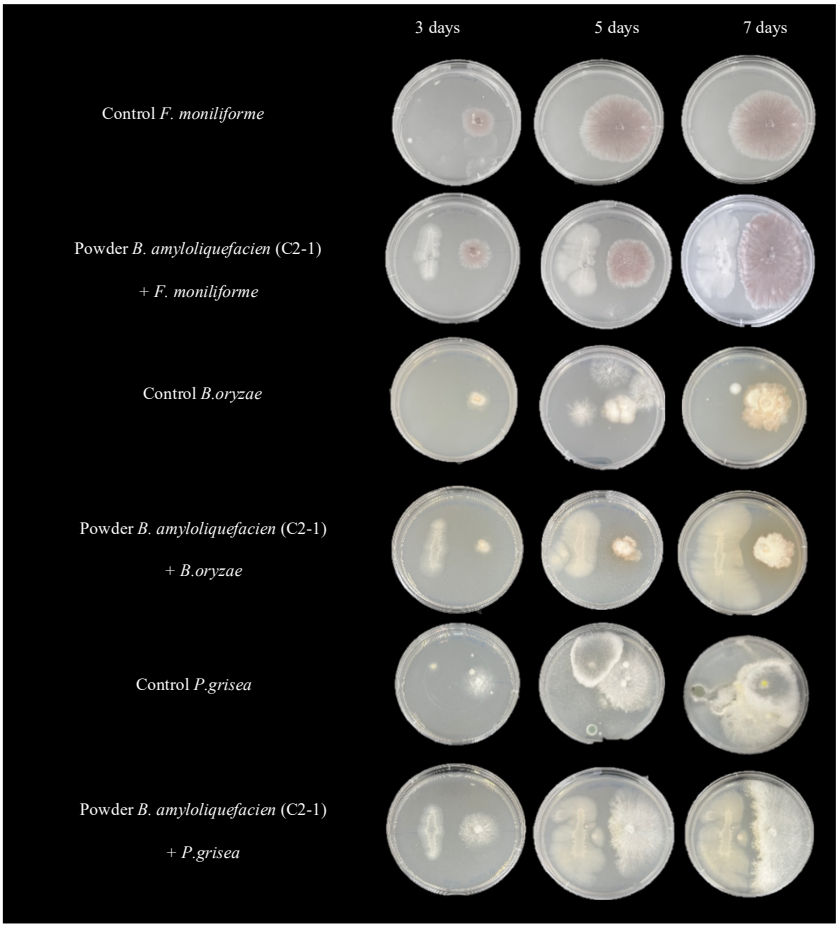

Figure 3. Effects of spray-dried powder of *B. amyloliquefaciens* strain C2-1 against *F. moniliforme, P. grisea,* and *B. oryzae* on dual culture plates incubated for 3, 5, and 7 days at room temperature.

CONCLUSIONS

In this study, *B. amyloliquefacien* strain C2-1 isolated from food waste compost, effectively inhibiting the growth of *X. oryzae* as well as the mycelial growth of *F. moniliforme, P. grisae,* and *B. oryzea* strain. Among the three general media tested, YM was selected as the optimum culture media for cell growth and viability after spray drying due to its cost-effectiveness and highest cell concentration.-The inhibition of *Xoo B. amyloliquefacien*s strain C2-1 powder against *X. oryzae* gave the widest clear zones. Regarding inhibiting *F. moniliforme*, *P. grisea*, and *B. oryzae*, it was found that *B. amyloliquefacien* strain C2-1 was able to control the growth of all these pathogenic rice fungi with 73.0%, 68.72 and 48.42 % inhibitions, at 7 days respectively. Thus, our results highlight the potential of spray-dried powder of *B. amyloliquefacien*s strain C2-1 as a promising biocontrol agent against the prevalent rice diseases currently affecting crops.

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Effect of cross-inbred lines and supplementation of brewed ground corn on the productivity and carcass quality of black pig

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ABSTRACT

The study intended to compare the growth performance and carcass quality of black pigs fed four different supplements of brewed ground corn (BGC) as an appetizer before feeding commercial feed. The breeding program was assigned using crossing inbred lines of L_1 male to L_1, L_2, and L_3 female to generate L_1L_1, L_1L_2, and L_1L_3 piglets, respectively. Then, healthy weaning piglets (n = 8 of each cross-inbred line) with nearly the same body weight were randomly allocated to the pen for two piglets each (one male and one female each). The BGC was given daily as an appetizer at 0, 0.5, 1.0, and 1.5 percent of body weight. Pigs were raised in twelve experimental pens of 2 x 3 meters in size and fed with a commercial diet to meet nutritional requirements according to their age up to 16 weeks. The analysis of variance was used to evaluate the datasets, and the means were compared using Duncan's new multiple-range test. Data indicated that pigs had substantially higher body weight gain but lower feed conversion and feed cost per gain than L_1L_1 and L_1L_3. Carcass percentage and some characteristics such as pH, temperature, pork color, and drip loss were not substantially different between the three black pig cross-inbred lines ($P>0.05$). The LSQ index and back fat thickness showed that three sets of black pigs had moderate lean and fat percentages. The BGC supplementation may increase feed intake. On the other hand, it has no discernible effect on growth rate, carcass characteristics, or quality. The L_1L_3 black pig demonstrated excellent growth efficiency, decent quality pork, and carcass percentage. However, BGC supplementation has no discernible effect on growth rate, carcass characteristics, or quality. The L_1L_3 black pig demonstrated great efficiency of growth performance, acceptable quality pork, and carcass percentage. The L_1L_3 can be the priority option and can be implied for improving the pig output of small farmers in Nan Province. The BGC supplementation at the rate of 1.0 percent of body weight/day may increase the feed intake while not affecting black pig growth or carcass.

Keywords: black pigs, small farm, growth performance, carcass characteristics

INTRODUCTION

Thailand's pig industry has been transforming and proliferating since the 1980s by importing pig breeds from European countries and the United States. The climate control house technologies and quality feed and feeding management were induced, supporting the potential genetic increase. The higher the genetics potential, the higher the nutritional feed and good management were needed (Rocadembosch et al., 2016; Homma et al., 2021). Industry pig production is entirely produced under the closed housing system with controlled environmental conditions. These led to increasing the cost of production (Rocadembosch et al., 2016). This was why the pig industry belonged to some of the big companies in Thailand. Since then, pig production in the village has declined because farmers have inadequate financial support for raising the high genetics potential pigs breed (Charoensook et al., 2019). The small farmers have some choices only to the contact farming system. So, the new alternative pig breed may need to fulfill supporting pig production in the village. Pig production for the rural may, sometimes, require a lower growth rate and lower lean percentage than commercial breeds. The alternative pig breeds need less intensive management and low cost for production. Moreover, the lifestyle and culture of villagers may be a consideration for creating the breeding plan. The breeding plan may introduce Thai Native Pigs (TNP) due to Raad, Kra Done, Puang, Hailum, and Kwai breeds for crossing with the commercial breed. Although the TNP is very low in production performance and carcass quality but is more suitable in the case of climate endurance, the scarcity of feed, tropical disease, and poor management. There were three pig breeding plans, which were constructed due to cross-breeding between Duroc Jersey (D) x Meishan (M), called DM (L_1), Pietrain (P) x TNP, called PN (L_2), and (Duroc Jersey-Pietrain) x (Large White-Meishan), called DPYM (L_3). The DM and PN

were improved by the Bureau of Animal Husbandry and Genetics Improvement (BAHG, 2005). The DPYM was proposed by the Department of Animal Science and Fishery, Rajamangala University of Technology Lanna Nan (RMUTLN). All kinds of crossbred pigs were selected for the black-coated color that the local people in Northern Thailand required. In case a new pig breed is released, the production and feeding systems are needed to test, support, and be suitable for the new breed. Some feedstuff was residual leftover from alcoholic production in the rural. Villagers always utilize residual leftover feeding pigs and find evidence of good performance for growth and tasty pork. Ano et al. (2020) reported that feeding distillation remnants of Shochu, a traditional Japanese liquor, can reduce stress and improve the sirloin tenderness, juiciness, umami, and fat tastiness of fillets. Therefore, this research aimed to test growth performance and carcass quality of the crossing between the inbred lines of black pigs due to L_1L_1, L_1L_2, and L_1L_3 with different substitution levels of fermented corn gain with alcoholic yeast in commercial feed.

MATERIALS AND METHODS

Breeding plan of produced black pig

There were three inbred lines of the black pig called DM, PN, and DPYM. DM or Line 1 (L_1) was a crossbreed between Duroc Jersey x Meishan. PN or Line 2 (L_2) was a crossbreed between Pietrain x Thai Native pig. DPYM or Line 3 (L_3) was a crossbreed between Duroc Jersey- Pietrain x Large White- Meishan. The inter-se mating system produced an inbred line of each black pig for the three generations. Then, the selected L_1 male was crossed to the L_1, L_2, and L_3 females using the artificial insemination techniques. So, there are three crossings of inbred lines of piglets due to L_1L_1, L_1L_2, and L_1L_3. After weaning, the piglets were selected based on good health and have nearly the same body weight for piglets per crossed inbred lines breed (4 males and 4 females each). After that, the male and female piglet within the same crossed inbred line was randomly matched as a couple for testing on growth performance and carcass quality.

Preparation of brewed ground corn

The grain corn was roughly crushed using the hammer mill. Then, the ground corn was packed into the container to produce brewed ground corn (BGC). The composition of BGC consisted of 100 kg of ground corn, 1.5 kg of brown sugar, 1.5 kg of molasses, 150 g of dry alcoholic yeast, and 50 liters of clean water. Preparation of yeast was processed by taking clean water of about 10 liters along with brown sugar, and molasses into the same bucket. Then, the mixer was stirred using a wooden paddle until all was completely dissolved. After that, the alcoholic yeast was added and gently stir until white foam happens. The solution after that was poured to mix with the remaining water in another bucket and added air using an air pump for about two hours. The ground corn was packed into the container by allowing air space above about 12 inches (about 50 kg per container) and then poured with the solution that was prepared before until the solution level was higher than the ground corn level by about three centimeters. The containers were enclosed using the lid or plastic bag and stored at room temperature for at least three weeks before taking to feed the pigs as an appetizer before feeding the main commercial diet during the experiment.

Experimental design

Weaning piglets of the same cross-inbred line were carried and mixed together in the same pen. After that, the healthiest piglet at the same body weight was inspected and selected for testing on growth performance. One female and one male on the same cross-inbred line were randomly matched and taken into the experimental pen. There were 12 pens where the same cross-inbred line breed piglets belonged to 4 pens. So, there were 24 piglets in the total for this study due to 3 types of cross-inbred lines piglets. The pen size was 2 x 3 meters, including a water basin size of 1 x 2 meters for reducing body temperature. All experimental pens were installed in the same facility for raising. Pigs were made full-feeding with a commercial diet following the nutrient requirement depending on age periods and body weight (10-30, 30-60, and 60-100 kg). The brewed ground corn was supplemented at four levels due to 0, 0.5, 1.0, and 1.5 percent of pig body weight as a fed basis. Pigs were fed three times daily at 07.00 am, 12.00 am, and 4.00 pm. All pigs were allowed access to the water supply as needed using the nipple. Pigs were weighed every week simultaneously through 16 weeks of an experiment. Feed intake was recorded and summarized every week. Data, after that, were modified and conversed to an average daily gain (ADG), feed conversion ratio (FCR), and feed cost per gain (FCG), respectively.

Carcass characteristics and evaluation

After finishing, all twelve male fattening pigs were taken to study the carcass characteristics and evaluation for quality. All were carried to Nan municipal slaughterhouse and skipped feeding for 12 hrs except for the water that allowed free drinking

as needed. Pigs were weighed before slaughter. The carcass was separated into left and right sides using an electric splitting saw. The hot left and right carcasses were weighting separately. Back fat thicknesses were measured at the angle base of the gluteus medius muscle (bf3), at the peak of the gluteus medius muscle (bf4), and at the top angle of the gluteus medius (bf5). The Lenden-Speck Quotient (LSQ) index (Pfeiffer and Falkenberg, 1972) were measured as a proportion of backfat thickness to the wide of longissimus dorsi muscle (b) as shown in Figure 1.

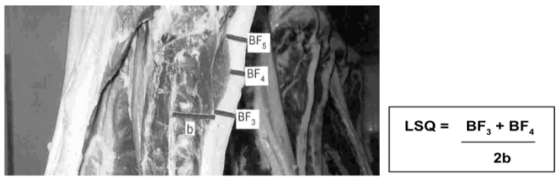

Figure 1. The location to measure the lean and backfat thickness of pig carcass (left) and the formula to calculate LSQ index (right). Source: Sethakul et al. (2010)

Carcass evaluations were measured for pH and temperature 45 minutes after slaughter and 24 hours of chilling at 4°C. The portable spectrophotometer (HunterLab MiniScan EZ 4500) measured the lean colors. The marbling score was measured from the longissimus-dorsi muscle following the Thai agricultural commodity and food standard method: 6000-2004 (TACFS, 2004).

Data analysis

Growth performance, carcass characteristics, and evaluation were analyzed using the analysis of variance (ANOVA) following the statistical model below.

$$Y_{ijk} = \mu + \propto_i + \beta_j + \varepsilon_{ijk} \quad \ldots\ldots. [1]$$

Where, Y_{ijk} = observation data

μ = an overall mean

$\propto_i$ = effect of cross-inbred lines of black pig (i = L_1L_1, L_1L_2, and L_1L_3)

$\beta j.$ = effect of supplementation of corn fermented (j = 0, 0.5, 1.0, and 1.5% of body weight as fed basis)

εijk = random residual effect

The statistics of the model [1] were an arithmetic mean and standard deviation. The mean comparison was tested against Duncan's new multiple range test (DMRT). Those can be done using the procedure of the Statistical Analysis System (SAS) software (SAS, 1999).

RESULTS AND DISCUSSION

Growth performance of black pig

The effect of a cross-inbred line of black pigs on growth performance is shown in Table l. The result found the final body weight (FBW) and body weight gain (BWG) was high for those of L_1L_3, and differed significantly from L_1L_2, and L_1L_1, respectively. These may become from a fractional of the breed that L_1L_3 consisted higher the European pig breed than that of L_1L_1 and L_1L_2. The three patterns of the cross-inbred line affected an average daily gain (ADG) and feed intake (FI) but were not significantly different. The ADG of this research was a bit lower than that of the pure breed Large White (816 g/d), Land Race (814 g/d), and Duroc (779 g/d) under the testing on pig performance of Kamphaeng Saen Central Testing Station (KPSCTS) reported by Chomchai et al. (1998). However, the feed conversion ratio (FCR) differed significantly between cross-inbred lines of black pigs. The FCR of L_1L_3 was the lowest and differed from that of L_1L_1, and L_1L_2 cross-inbred lines. This study showed better growth performance than that of the crossbred pigs between Duroc Jersey-Meishan (DM) tested by BAHG (2005). They reported ADG equal to 524 and 542 g/d, and FCR equal to 2.71 and 2.86 for DM crossbred in filial 1 and 2, respectively. The performance resulted in the range of three breeds cross fattening pigs (Chomchai et al., 1998; Srikanchai et al., 2005), GPP pure breed (Tungtrakoolsub, 2013) under hot-humid conditions testing.

Table 1. Effect of cross-inbred lines on production efficiency of black pig

Items	Types of cross-inbred lines of black pig			Pr > F
	L_1L_1	L_1L_2	L_1L_3	
	Mean ± SD	Mean ± SD	Mean ± SD	
FBW (kg)	90.13 ± 5.50^{ab}	84.25 ± 2.06^{b}	99.75 ± 4.70^{a}	0.014
BWG (kg)	77.08 ± 5.48^{b}	72.33 ± 2.40^{b}	85.25 ± 3.54^{a}	0.018
FIPD (kgDM)	1.84 ± 0.11	1.81 ± 0.11	1.95 ± 0.16	0.214
ADG (kg)	0.69 ± 0.05	0.65 ± 0.02	0.76 ± 0.03	0.769
FCR (kgDM)	2.67 ± 0.11^{ab}	2.79 ± 0.08^{a}	2.56 ± 0.11^{b}	0.003
TFC (baht)	3,141.56 ± 182.53	3,088.80 ± 184.81	3,345.57 ± 268.40	0.208
FCG (baht)	40.81 ± 1.67^{b}	42.68 ± 1.17^{a}	39.20 ± 1.73^{c}	0.003

FBW = final body weight, BWG = body weight gain, FI = feed intake per day, ADG = average daily gain, TFC = total feed cost, FCG = feed cost per gain.

^{abc}The different superscripts on the same row indicated significant differences ($P<0.05$).

There were no significant differences in feed intake per day (FIPD) and total feed cost (TFC) between the three types of cross-inbred lines of black pigs. However, feed conversion ratio (FCR) and feed cost per gain (FCG) indicated significantly different ($P<0.05$) between the three types of black pigs. The L_1L_3 showed lower FCR and FCG than those of L_1L_1 and L_1L_2, respectively. These may become from breed fractions of black pigs affecting growth performance efficacy. The fraction breed of L_1L_3 consisted 37.5%Duroc Jersey: 12.5%Pietrain: 12.5%Large White: 37.5%Meishan that was 62.5%European pig breed: 37.5%Asiatic pig breed. That was higher than that of L_1L_1, and L_1L_2, which have a proportion of 50%European pig breeds: 50%Asiatic pig breeds. The L_1L_2 showed less performance efficacy than those of others. This may be the effect of the Thai native pig breed that was included as a fractional line breed. However, the main objective of the breeding plan needed black coated color of pig and endured to climate and scaring management of small farmers in the rural area. So, the Asiatic breed was induced to breeding plans like Meishan, Thai native breeds such as Kra Done, Puang, and Kwai.

The effect of the level of brewed ground corn (BGC) on the growth performance of black pigs is shown in Table 2. The result found that the final body weight (FBW), body weight gain (BWG), feed intake per day (FIPD), and average daily gain (ADG) was not significantly different between the treatment that supplemented BGC to the control. Supplementation of BGC for 1.5% body weight of pigs can affect the feed conversion ratio (FCR) of black pigs. However, supplementation BGC for 0.5 and 1.0% body weight can affect FCR not significantly different compared to control treatment. Supplementation BGC for about 1.5% can affect the total feed cost (TFC) and feed cost per gain (FCG) higher than these of the others.

This indicated that BGC could be supplemented to commercial feed with about 1.0% body weight and may enhance the FIPD and ADG of black pigs. The properties of brewed ground corn with yeast have 4.18±0.05 in pH, 5.99±0.36 in salinity, 4.98±0.27 percentage in alcohol, 59.86 in dry matter percentage, and 9.7% in crude protein contents. Many researchers reported that nutrients in leftover fermented gain like corn and barley (Ano et al., 2020), can reduce stress and plasma cortisol (Ano et al., 2020), improve feed intake and growth rate (Suzuki et al., 2018), and resulted in better-tasting sirloin and fillets (Suzuki et al., 2018; Ano et al., 2020). By observation, black pigs with supplementation BGC consumed more time lying down and sleeping after feeding (Figure 2). Pig behavior in this study looked like the previous experiment that was supplementation of L-tryptophane in pig diets (Kuha et al., 2012). These because alcohol may affect the pig to decrease their activity and can help to preserve energy for growth. The result was also familiar to many farmers who fed residual leftovers from liquor production, called Nam joe, in Nan province (personal communication). Since the pigs were less stressed, they produced higher-quality meat, especially sirloin and filet cuts.

Table 2. Effect of supplementation levels of brewed ground corn on production efficiency of black pig

Items	Levels of supplementation of brewed ground corn				Pr > F
	0%	0.5%	1.0%	1.5%	
	Mean± SD	Mean± SD	Mean± SDS	Mean± SD	
FBW (kg)	89.67 ± 7.22	91.50 ± 8.05	91.50 ± 11.53	92.83 ± 8.50	0.894
BWG (kg)	76.13 ± 6.06	78.73 ± 7.75	78.00 ± 9.30	80.00 ± 6.96	0.698
FIPD (kgDM)	1.81 ± 0.11	1.80 ± 0.10	1.85 ± 0.16	2.00 ± 0.12	0.211
ADG (kg)	0.68 ± 0.05	0.70 ± 0.07	0.70 ± 0.08	0.71 ± 0.06	0.768
FCR (kgDM)	2.67 ± 0.14^{ab}	2.57 ± 0.16^{a}	2.66 ± 0.09^{ab}	2.80 ± 0.09^{b}	0.014
TFC (baht)	3,102.86 ± 179.01	3,087.43 ± 181.27	3,165.20 ± 271.96	3,412.42 ± 204.16	0.229
FCG (baht)	40.83 ± 2.17^{b}	39.35 ± 2.46^{b}	40.68 ± 1.33^{b}	42.72 ± 1.29^{a}	0.015

FBW = final body weight, BWG = body weight gain, FI = feed intake, ADG = average daily gain, TFC = total feed cost, FCG = feed cost per gain.
The different superscripts on the same row indicated a significant difference (P<0.05).

Figure 2. Black pigs always laying down to sleep after feeding.

Some carcass characteristics and quality are shown in Table 3. Hot carcass weight and hot carcass percentage were not significantly different between the three groups of different line cross breeds of black pig. An average back fat thickness significantly differed between three cross-bred lines of black pigs. The L_1L_3 was the thinnest differed from L_1L_1 but not L_1L_2. The LSQ index was not different between the three groups of black pigs. The LSQ index following the criteria graded by Sethakul et al. (2003) reported that the LSQ indexes equal to ≤ 0.20, 0.21-0.26, 0.27-0.32, 0.33-0.38, 0.39-0.44, and ≥ 0.45 had the lean percentage equal to 48.47, 46.88, 45.05, 43.37, 42.00, and 40.31 and the fat percentage equal to 14.39, 16.34, 18.07, 19.49, 20.62 and 22.11, respectively. The correlation between the LSQ index and lean percentage, and fat percentage was about 0.69 and 0.67, which can be used LSQ index to predict lean and fat percentages with a high confidence level (Sethakul et al., 2003). So, the L_1L_3 black pig was a more lean percentage and less fat percentage than others. The other items observed for pork quality due to marbling score, carcass pH and temperature, drip loss, and pork colorization were significant differences between the 3 groups of black pigs. The pork of L_1L_1 trended to be more red color (a*) than that of L_1L_3.

The supplementation levels of BGC on some carcass characteristics and quality of black pigs are shown in Table 4. The hot carcass weight and hot carcass percentage were not different between the different levels of supplementation of BGC. Backfat thickness was sequentially increased if the levels of supplementation BGC increased. This can imply that more supplementation of BGC can affect the high backfat thickness. There were no significant differences due to the LSQ index, marbling score, carcass pH and temperature, drip losses, and colorized of pork. The LSQ indexes were low in the case of supplementation BGC 0.5 and 1.0 percent body weight that, related to low backfat thickness.

Table 3. Effect of cross-inbred lines of the black pig on carcass characteristics and quality

Items	Types of cross-inbred lines of black pig			Pr > F
	L_1L_1	L_1L_2	L_1L_3	
	Mean ± SD	Mean ± SD	Mean ± SD	
Fasting body weight (kg)	99.33 ± 5.51	103.00 ± 4.11	106.75 ± 4.11	0.384
Hot carcass weight (kg)	79.70 ± 5.00	77.80 ± 4.60	82.40 ± 4.60	0.841
Hot carcass percentage (%)	80.30 ± 4.30	75.50 ± 1.70	77.10 ± 1.70	0.641
AVG. back fat (cm)	3.10 ± 0.60[a]	2.70 ± 0.60[b]	2.50 ± 0.60[b]	0.059
LSQ index	0.40 ± 0.10	0.50 ± 0.10	0.30 ± 0.10	0.296
Marbling score	1.90 ± 0.85	3.00 ± 0.47	2.68 ± 0.47	0.975
Carcass pH				
30 min post-mortem	5.47 ± 0.33	5.91 ± 0.25	5.44 ± 0.25	0.333
24 hr chilled	5.57 ± 0.04	5.43 ± 0.62	5.89 ± 0.62	0.356
Carcass temperature				
30 min post-mortem	37.98 ± 1.37	37.85 ± 1.22	37.76 ± 1.22	0.626
24 hr chilled	12.57 ± 2.18	13.00 ± 1.47	11.63 ± 1.47	0.860
Drip loss (%)	3.50 ± 2.08	2.23 ± 1.26	3.20 ± 1.26	0.384
Lean color				
L*	56.05 ± 4.32	55.82 ± 1.71	60.18 ± 4.92	0.105
a*	8.03 ± 1.43	7.20 ± 0.46	6.60 ± 1.46	0.090
b*	16.23 ± 1.03	15.59 ± 0.37	15.29 ± 1.07	0.127

[abc]The different superscripts on the same row indicated a significant difference (P<0.05).

Table 4. Effect of supplementation levels of brewed ground corn on carcass characteristics and quality of black pig

Items	Levels of supplementation of brewed ground corn				Pr > F
	0%	0.5%	1.0%	1.5%	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Fasting body weight (kg)	103.00 ± 0.00	104.50 ± 0.71	101.00 ± 9.90	105.50 ± 9.19	0.776
Hot carcass weight (kg)	78.90 ± 1.60	81.50 ± 4.90	81.10 ± 2.60	81.80 ± 9.60	0.981
Hot carcass percentage (%)	76.60 ± 1.50	78.00 ± 4.20	80.50 ± 5.30	77.40 ± 2.40	0.879
AVG. back fat (cm)	2.60 ± 0.10[bc]	2.10 ± 0.60[c]	3.00 ± 0.40[ab]	3.30 ± 0.40[a]	0.038
LSQ index	0.50 ± 0.10	0.30 ± 0.10	0.30 ± 0.00	0.40 ± 0.10	0.439
Marbling score	2.50 ± 0.71	2.70 ± 0.00	2.00 ± 1.41	2.50 ± 0.71	0.230
Carcass pH					
30 min post-mortem	5.50 ± 0.58	5.38 ± 0.41	5.55 ± 0.16	5.62 ± 0.08	0.614
24 hr chilled	5.48 ± 0.07	5.52 ± 0.02	5.66 ± 0.07	6.19 ± 0.86	0.610
Carcass temperature					
30 min post-mortem	37.18 ± 0.95	37.90 ± 1.98	38.18 ± 1.45	38.18 ± 0.11	0.550
24 hr chilled	11.60 ± 1.98	11.90 ± 2.40	11.95 ± 1.48	13.15 ± 1.91	0.859
Drip loss (%)	2.10 ± 0.19	2.22 ± 0.11	3.21 ± 0.98	3.25 ± 1.10	0.776
Lean color					
L*	58.20 ± 4.40	57.80 ± 3.20	60.20 ± 7.40	56.10 ± 3.20	0.489
a*	6.50 ± 1.10	7.40 ± 1.30	7.20 ± 2.20	7.80 ± 1.10	0.417
b*	15.10 ± 1.10	15.90 ± 1.10	16.20 ± 1.20	15.60 ± 0.90	0.288

[abc]The different superscripts on the same row indicated a significant difference (P<0.05).

CONCLUSIONS

There was no significant between the growth performance of the three types of cross-bred black pigs due to the feed intake per day and total feed cost. The L1L3 black pigs showed significantly high body weight gain, but less in feed conversion, and feed cost per gain compared to L1L1. The carcass percentage, and some properties due to pH, temperature, pork color, and drip loss, were not significantly different between the three cross-inbred lines of black pigs. The back fat thickness and LSQ index indicated that three groups of black pigs have moderate lean and fat percentages. Supplementation of CGF may improve the feed intake of black pigs and reduce stress due to more sleep. Black pigs can consume CGF about 1.0 percent of body weight daily by not adversely affecting growth performance and carcass quality.

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The effects of energy and protein content in maize forage-based complete diet on *in vitro* ruminal fermentation, gas production, and feed degradability

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ABSTRACT

This research aimed to evaluate the effects of energy and protein contents in a complete diet on *in vitro* gas production, rumen fermentation, and feed degradability. The experiment used a 3x3 factorial arrangement in a randomized block design with two factors and three replications. The first factor was energy content in the complete diets; E_1=12.5MJ/kg DM, E_2=13.5MJ/kg DM, and E_3=14.5 MJ/kg DM. The second factor was protein content in the complete diets; P_1=10.5%, P_2=13.5%, and P_3=16.5%. The complete diet was composed of maize forage silage 37.5 % w/w DM, elephant grass (*Pennisetum purpureum*) 12.5 % w/w DM, and concentrate 50.0 % w/w DM. The concentrate was composed of commercial dairy concentrate feed produced by SAE local dairy cooperative, cassava waste, soybean meal, rice bran, and coffee husk. All of the treatment diets were tested using *in vitro* gas production test. The variables were total, potential, and rate of *in vitro* gas production, NH_3 concentration, efficiency of microbial protein synthesis (EMPS), dry matter degradability (DMD), and organic matter degradability (OMD). Either energy or protein content of the treatment diets had a highly significant effect ($P<0.01$) on the total, potential, and rate of *in vitro* gas production, NH_3 concentration, EMPS, DMD, and OMD, but not for the treatment combination. An increase in energy and protein content in the treatment diets increased the value of all parameters but decreased EMPS.

Keywords: energy, protein, complete diets, degradability, gas production, microbial growth

INTRODUCTION

Protein and energy are the most nutrients required by all organisms after water requirement (McDonald et al., 2010). Upon consumption by ruminants, all of the feed nutrients enter firstly into the reticulo-rumen and are firstly digested or utilized by rumen microbes. The feed consumed by ruminants can directly affect the condition of the reticulo-rumen condition, rumen microbial growth, and feed digestion. Energy in the diet is mostly from carbohydrates, either fiber carbohydrates or mainly non-fiber carbohydrates. The carbohydrates also directly affect rumen condition, especially pH. Fiber carbohydrates increase rumen pH effectively, while non-fiber carbohydrates decrease rumen pH. Carbohydrates also function as a source of carbon skeletons for amino acid synthesis by rumen microbes during their growth. Protein in the diet is in the form of true protein and non-protein nitrogen. Both proteins are also firstly digested and utilized by rumen microbes. Dietary protein is the most nitrogen source for microbial growth in the rumen. All rumen conditions and nutrients affect sensitively rumen microbial growth and population. The rumen microbes are importantly responsible for feed digestion in the rumen, especially for dietary fiber into VFAs which are the main source of energy for host ruminants. Some rumen microbes flow into the abomasum and small intestine and function as the main source of protein for host ruminants.

In addition to the quantity of feed, the balance of the available nutrients, mainly carbohydrates or energy, and protein, is also very important for rumen microbial growth. The availability of protein for rumen microbes in balance with the availability of energy, either in their quantity and time of availability must increase the rumen microbial growth and population as well as their activities in degrading feed, especially fiber carbohydrates. Upon their degradation in the rumen, carbohydrates supply energy and carbon skeletons, and protein supply nitrogen to rumen microbes for synthesis of microbial cells amino acids for

the growth. Leng (1991) stated that balancing energy and protein in the ration will affect the efficiency of nutrient utilization for production.

Feeding a complete diet to ruminants is an important strategy in ruminant feeding management. The complete diet is well formulated and mixed forages and concentrates for ruminants. Feeding a complete diet to ruminants must create better rumen conditions and supply adequate and balanced nutrients for rumen microbial growth than separate feeding of forages and concentrate (Beigh et al., 2017). However, availability of forages is one constraint in ruminant production in the tropics, such as in Indonesia (Hartutik et al., 2022). Most farmers have very limited land, and the focus of its utilization is on growing food or cash crop, not on fodder plants. Farmers mostly collect forages from rangelands. In addition, two different seasons in this area also affect the stability of forage availability, grasses mostly grow better during the rainy season and are scarcely available during the dry season (Achmadi, 2007). Maize (*Zea mays* L.) forage is a common source of forage for ruminants. Heuzé et al. (2017) stated that maize green forage consists of stems, leaves, and ears with high energy for ruminants. Maize forage contains CP 10.9%, fat 2.17%, crude fiber 33.21%, NFE 46.05%, and gross energy 3791 kcal/kg, which is good nutrition for ruminants (Binol et al., 2020). A hectare of maize plantation produces 3 to 7 ton maize forages and can be fed to ruminants as fresh or conserved as silage to maintain nutrition (Zaidi et al., 2013). Wang et al. (2021) stated that ensiling is an important method for keeping the forage nutrient, and it can supply feedstuff throughout the year. Achmadi et al. (2020) mentioned that to achieve feed availability for ruminant production, processing by-product of agroindustry is necessary to be used during the dry season when the availability of roughage feed is low. Maize silage feeding can be in the form of a single feed or mixed with other feed material into a complete diet. Wibisono et al. (2020) reported that formulating a complete diet with 30% maize silage and 70% commercial concentrate showed crude protein content of 12.99%, CF of 14.22%, and NFE of 46.42%. For those based on the review, this study was done to evaluate the effect of energy and protein contents in maize forage silage-based complete diet on *in vitro* ruminal feed fermentation, gas production, and feed degradability.

MATERIALS AND METHODS

Location and Time

This study was conducted from August 2020 to March 2021 in the Faculty of Animal Science, Brawijaya University Sumber Sekar Field Laboratory for making the maize forage silage and in Animal Feed and Nutrition Laboratory to evaluate the *in vitro* rumen fermentation, gas production, and degradability and samples analysis.

Method

This experiment used a 3x3 factorial arrangement in a randomized block design with two factors. The first factor was energy contents in the complete diets as the second priority nutrients required by the body after water, i.e. E_1=12.5MJ/kg DM, E_2=13.5MJ/kg DM, and E_3=14.5 MJ/kg DM. The second factor was protein contents in the complete diets as the third priority nutrients required by the body, i.e. P_1=10.5% DM, P_2=13.5% DM, and P_3=16.5% DM. Thus, in total, there were nine treatment combinations. Each treatment ration was evaluated using *in vitro* gas production test according to the procedure of Makkar et al. (1995). The evaluation was done three times using rumen fluids collected at three different times as replication.

The complete diet was composed of maize forage silage 37.5 % w/w DM, elephant grass (*Pennisetum purpureum*) 12.5 % w/w DM, and concentrate 50.0 % w/w DM. The concentrate was composed of commercial dairy concentrate feed produced by SAE local dairy cooperative, cassava waste, soybean meal, rice bran, and coffee husk. The silage was made of maize forage harvested 65 days after planting, molasses, and *Lactobacillus plantarum* 1 x 10^6 CFU/mg. The maize forage was wilted for a day and chopped into 2-5cm particle size. The forage was then properly mixed with a mixture of molasses and *Lactobacillus plantarum* 1x10^6 cfu/g (10: 1 ratio) as much as 6% of maize forage weight, put in airtight plastic bag silo and incubated for 14 days.

The nutrient content of each feed ingredient as analyzed using proximate analysis (AOAC, 2005) and the energy content as estimated using the procedure of Menke et al. (1979) is presented in Table 1, and feed composition in each treatment diet is presented in Table 2.

Table 1. Nutrient content of feedstuffs used in each treatment diet

Feedstuff	DM (%)	OM (% DM)	Ash (% DM)	CF (% DM)	Fat (% DM)	CP (% DM)	ME** (MJ/kg DM)
SAE dairy concentrate feed	97.60	90.06	9.94	14.97	4.70	18.38	18.39
Cassava waste	92.59	82.87	17.13	25.39	0.44	1.76	20.57
Soybean meal (SBM)	93.53	91.62	8.38	4.04	2.57	47.53	15.14
Rice bran	90.63	87.40	12.60	16.20	13.00	10.15	12.49
Coffee husk	94.14	89.42	10.58	34.00	1.49	10.11	9.74
Maize forage silage	94.54	89.36	10.64	22.45	0.94	7.80	12.39
Elephant grass	96.12	85.95	14.05	31.99	2.35	12.08	11.81

** Metabolizable Energy content as estimated using the procedure of Menke et al. (1979).

Table 2. Composition of ingredients in each treatment diet on DM basis

Treatment	ME content (MJ/kg DM)	Protein Content (%)	Maize silage (%)	E. grass (%)	Concentrate (%)	Rice bran (%)	Cassava waste (%)	SBM (%)	Coffee husk (%)
E_1P_1	12.5	10.5	37.5	12.5	18	12	4.5	-	15.5
E_1P_2	12.5	13.5	37.5	12.5	23	7	-	5.5	14.5
E_1P_3	12.5	16.5	37.5	12.5	18	-	-	14.5	17.5
E_2P_1	13.5	10.5	37.5	12.5	24	6	11.5	-	8.5
E_2P_2	13.5	13.5	37.5	12.5	50	-	-	-	-
E_2P_3	13.5	16.5	37.5	12.5	36.5	-	2.3	11.2	-
E_3P_1	14.5	10.5	37.5	12.5	30	-	19.5	0.5	-
E_3P_2	14.5	13.5	37.5	12.5	23	-	17.6	9.4	-
E_3P_3	14.5	16.5	37.5	12.5	13.5	-	17	19.5	-

All of the treatment diets were evaluated using *in vitro* gas production test according to the procedure of Makkar et al. (1995) three times as replication using rumen liquid collected from a rumen fistulated cow feed on fresh elephant grass and concentrate at 60%:40% DM weight ratio in three different days.

Variable

The variables measured in this study were total gas production, gas production potential, and gas production rate based on the difference in gas volume in the syringe after 48 hours of sample incubation and the initial volume, pH, temperature, NH_3 concentration of supernatant, dry matter and organic matter degradability, the efficiency of microbial protein synthesis (EMPS) measured after 48 hours sample incubation according to the procedure of Blümmel et al. (1997).

Data Analysis

The data were analyzed using ANOVA of a 3x3 factorial arrangement in a randomized block design with two factors. If the treatment showed a significant effect ($P<0.5$), a mean comparison was continued with Duncan Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Nutrient contents of the treatment diets

The nutrient contents of all treatment diets are presented in Table 3. DM content ranged from 94.59 (E_3P_3) to 96.25% (E_2P_2), OM content from 87.89 (E_3P_1) to 89.40% DM (E_1P_3), ash from 10.60 (E_1P_3) to 12.11% DM (E_3P_1), CF from 18.92 (E_2P_3) to 23.47% DM (E_1P_1), fat from 1,86 (E_3P_3) to 3.3% DM (E_1P_1, CP content from 10.52% (E_2P_1) to 16.51% DM (E_2P_3) and ME from 12.52 (E_1P_2) to 14.50 MJ/kg DM (E_3P_3).

To adjust crude protein and energy content of the treatment diets that were as treatments in this experiment was used mainly SBM as a protein source and cassava waste as an energy source for major adjustment, and rice bran and coffee pulp for minor adjustment (Table 2). The energy content of the treatment diets increased using cassava waste, while the protein content of the treatment diets increased by using soybean meal replacing concentrate feed.

Cassava waste contains high energy in respect of its protein content (20.57% DM vs 1.76 MJ/kg DM), while SBM contains high protein in respect of its energy content (47.53% DM vs 15.14 MJ/kg DM). MLA (2015) stated that the major nutrient components of feed that contribute to energy content are carbohydrates, fat, and protein. The different components of feed provide different amounts of energy to the animal and will be used in different ways by the animal. Lukuyu et al. (2014) mentioned that cassava waste provides great energy

for ruminants. Hartutik et al. (2020) stated that SBM could increase the value of CP content and total digestible nutrient (TDN).

Table 3. Nutrient content of treatment diets

Treatment diets	DM* (%)	OM* (% DM)	Ash* (% DM)	CF* (% DM)	Fat* (% DM)	CP* (% DM)	ME** (MJ/Kg DM)
E_1P_1	94.62	88.54	11.46	23.47	3.30	10.61	12.62
E_1P_2	95.01	89.09	10.91	22.15	3.00	13.45	12.52
E_1P_3	95.04	89.40	10.60	21.65	2.13	16.41	12.59
E_2P_1	94.94	88.24	11.76	22.79	2.73	10.52	13.49
E_2P_2	96.25	89.28	10.72	19.90	3.00	13.63	13.25
E_2P_3	95.67	89.29	10.71	18.92	2.66	16.51	13.50
E_3P_1	95.24	87.89	12.11	21.88	2.16	10.54	14.49
E_3P_2	94.97	88.17	11.83	20.71	2.05	13.44	14.45
E_3P_3	94.59	88.37	11.63	19.54	1.86	16.48	14.50

*Proximate analysis in Animal Feed and Nutrition Lab. Faculty of Animal Science, Brawijaya University (2021).
**Calculated according to Menke et al. (1979).

The effect of treatment diets on the parameters of in vitro gas production test

In vitro gas production tests for all of the treatment diets were done according to the procedure of Makkar et al. (1995). Based on the average pH and temperature of the substrate in the syringes after 48 hours of incubation time were 6.87±0.03 and 37.59±0.29° C (Table 4), respectively, were not significantly different between the treatments and volume of gas production in the syringes that increased steadily during the tests (Figure 1), so that it convinced that feed fermentation processes by microbes during the tests took place properly. The results of the tests are presented in Table 4 and are discussed below. Owen and Goetsch (1988) stated that to achieve maximum microbial growth, rumen conditions must have a pH in the range of 5.5-7.2 and a temperature between 38-41^{0}C.

According to Guo et al. (2022), rumen pH generally ranges from 6 to 7 and can be used to judge the ruminal environment and health. Rumen pH is affected by VFA interaction in chyme with buffer salt in saliva, and the absorption of VFAs by the rumen epithelium and outflow with chyme. A diet with high non-fiber carbohydrates decreased pH value along with decreased acetate: propionate ratio.

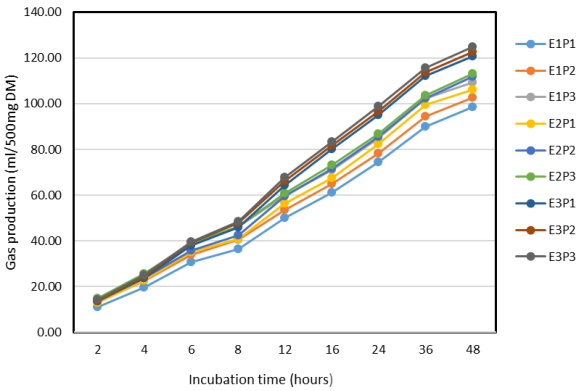

Figure 1. *In vitro* gas production curve of each treatment diet

Data in Table 4 show that the content of energy and protein in the diet, each gave a very significant effect (P<0.01) on all parameters, including the total and the potential of *in vitro* gas production, degradability of DM (DMD) and OM (OMD) as well as NH_3 concentration and EMPS. Protein content in the diets gave also significant effect (P<0.5) on the rate of *in vitro* gas production, but not for energy content in the diets. The value of all parameters increased due to the increase of energy as well as protein content in the diet, except the value of EMPS decreased due to the increase of energy as well as protein content in the diet. However, the treatment combination between energy and protein contents in the treatment diets did not give a significant effect (P>0.05) on all parameters, although all of the data increased consistently due to the increase of energy and protein contents in the diet.

As shown in Table 4, DMD and OMD, the total gas production and its rate of production as one of the products of DM and OM digestion increased as the energy and protein content in the treatment diets

increased where the highest values were in the treatment diets with highest energy (E3) and protein (P3) contents. The data were in line with the research of Sultan et al. (2010), who did an evaluation on nutrient digestibility and feedlot performance of lambs fed diets varying protein and energy contents. Zheng et al. (2020) mentioned that the larger the amount of gas produced, the better the feed fermentation.

Ammonia or NH_3 is the final product of crude protein degradation in the rumen, which is mostly from feed protein and, to a lesser extent, from lysis rumen microbes that release their cell protein. Ammonia is the main nitrogen source for rumen microbial growth, especially for rumen bacteria and fungi. With the availability of energy and other nutrients, ammonia is incorporated with carbon skeletons to synthesize cell protein during rumen microbial growth. Hence in *in vitro* digestibility test, the ammonia pool in the samples is directly affected by protein content and quantity of feed sample, as well as its degradation in the rumen liquid minus ammonia utilization for microbial growth. McDonald et al. (2002) stated that the concentration of NH_3 in the rumen is influenced by the protein content of the feed, rumen pH, the solubility of protein feed ingredients, and the time after feeding. Data in Table 4 show that the concentration of NH_3 in the treatment diets ranges from 4.29-7.75 mMol. Some of the NH_3 concentrations in this research were less than ideal as the optimal NH_3 concentration in the rumen, according to McDonald et al. (2010), ranges from 6 to 21 mMol. The concentration increased as the protein and energy content, as well as DM and OM degradability increased, and microbial biomass, as well as the efficiency of microbial protein synthesis decreased.

EMPS data in Table 4 ranged from 34.06-53.77 g microbial N/kg FOM. The optimal EMPS ranges from 30-40 g N/kg FOM but normally ranges from 10-70 g N/kg FOM (Karsli and Russell, 2001). The EMPS values decreased as the energy and protein content in the treatment diets increased, which were in contrast with the ammonia concentration, gas production as well as DMD and OMD that increased as the energy and protein content in the treatment diets increased. Karsli and Russell (2001) reported that microbial protein synthesis is highly dependent on the adequacy of nutrients available for their growth, especially energy in the form of ATP as a result of degradation of organic matter and N as a result of degradation of protein in the rumen.

In a closed cell or microbial cultures such as *in vitro* digestibility test, the growth of cell or microbe is usually divided into lag, exponential, stationary, and death phases (Prescott et al., 2002; Peleg and Corradini, 2011). During the lag phase, cells or microbes undergo intracellular changes to adjust to a new environment, and little or no cell reproduction takes place. During the exponential phase, cells reproduce at a rate proportional to the number of cells leading to an exponential increase in the number of cells. The stationary phase follows when nutrients are limited, or other environmental conditions restrict the number of cells that can be supported. Finally, cellular death and a declining population occur when the surroundings cannot maintain the population.

Thus, based on the typical microbial growth pattern in the closed cultures, the microbial growth and biomass decrease, or the microbes reach the death phase when the availability of nutrients is depleted. Thus, feed degradability and gas production in the rumen reflects the intensity of the rumen microbe's activities in digesting feed nutrients and their growth or population. The rate of feed digestion and gas production must be determined by the amount of feed available and its degradability as well as the microbe population, kind, and their activities. The nutrient depletion and then death phase of microbial growth happen earlier when the substrates are easier to be degraded by the microbes. In this experiment, the phenomenon was confirmed by the total gas production and its production rate (Figure 1 and Table 4) as well as DMD and OMD (Table 4). As shown in Table 4, the total gas production and its rate of production, as well as DMD and OMD, were highest in the treatment diets with the highest energy (E_3) and protein (P_3) contents that were in line with the research of Sultan et al. (2010). However, E_3 and P_3 treatments showed the lowest EMPS or microbial biomass. The highest DMD and OMD in the E_3 and P_3 treatments, as shown also by their highest total gas production and its rate of production, resulted in nutrient depletion in E_3 and P_3 treatments that took place earlier than those in the other treatments. Consequently, the microbes reached the death phase earlier and then the EMPS or microbial biomass decreased faster than in the other treatments. Feed degradability and gas production in the rumen reflects the intensity of the rumen microbe's activities in digesting and utilizing feed nutrients for their

growth. In other words, the rate of feed digestion and gas production must be determined by the feed itself (its availability and degradability) and the microbe population, kind, and their activities. In addition, Sauvant and Van Milgen. (1995) reported that microbial protein synthesis would be optimal if the release of N precursors and carbon skeletons in the rumen needed by microbes is aligned or synchronized. EMPS value is influenced by the availability of energy and amino acids used by rumen microbes. Insufficient energy will cause the deamination of amino acids and available carbon chains will ferment into VFAs. Conversely, an excess of amino acids in the rumen will only become NH_3, because some microbes cannot produce amino acids (Bach et al., 2005).

Table 4. The effects of treatment diets on parameters of *in vitro* gas production test

Treatment diets	pH	Temp (ºC)	Total gas prod. (ml/0.5g DM)	Potential of gas prod. (ml/0.5 g DM)	Rate of gas prod. (ml/hour)	DMD (%)	OMD (%)	NH_3 (mM)	EMPS (g N/kg FOM)
Effect of energy content (E)									
E_1	6.89	37.33	104.16^a	109.91^a	0.055	54.67^a	56.22^a	5.44^a	49.50^b
E_2	6.84	37.67	110.62^b	116.77^b	0.057	57.87^a	59.58^b	5.80^{ab}	45.57^{ab}
E_3	6.86	37.78	123.56^c	131.19^c	0.059	61.30^b	63.85^c	6.66^b	39.59^a
SEM	0.03	0.29	9.33	9.74	0.08	4.28	4.50	1.12	5.89
Sign.	n	n	**	**	n	**	**	**	**
Effect of protein content (P)									
P_1	6.89	37.39	108.74^a	116.71^a	0.057^{ab}	54.67^a	56.93^a	4.83^a	48.69^b
P_2	6.84	37.61	112.91^b	119.30^{ab}	0.055^a	57.54^a	59.14^a	6.04^b	46.06^{ab}
P_3	6.86	37.78	116.68^c	121.85^b	0.059^b	61.63^b	63.58^b	7.03^c	39.92^a
SEM	0.03	0.29	9.33	9.74	0.003	4.28	4.50	1.12	5.89
Sign.	n	n	**	**	*	**	**	**	**
Effect of energy and protein content (EP)									
E_1P_1	6.95	37.00	98.93	106.65	0.052	51.25	53.00	4.57	53.77
E_1P_2	6.85	37.50	103.29	108.68	0.055	53.06	54.43	5.45	49.73
E_1P_3	6.87	37.50	110.25	114.39	0.060	59.69	61.23	6.29	45.01
E_2P_1	6.85	37.50	105.95	114.01	0.055	53.97	56.34	4.29	49.98
E_2P_2	6.83	37.50	112.10	118.45	0.056	58.85	59.83	6.06	46.04
E_2P_3	6.85	38.00	113.83	117.85	0.059	60.79	62.57	7.05	40.69
E_3P_1	6.87	37.67	121.34	129.48	0.058	58.79	61.45	5.62	42.31
E_3P_2	6.85	37.83	123.36	130.77	0.060	60.71	63.15	6.62	42.40
E_3P_3	6.85	37.83	125.97	133.32	0.060	64.39	66.96	7.75	34.06
SEM	0.02	0.11	1.91	1.93	0.001	0.99	0.99	0.24	1.35
Sign.	n	n	n	n	n	n	n	n	n

CONCLUSIONS

Based on the results of this research, it can be concluded that the increase of either energy or protein content in the diets increased the rate of feed DM and OM digestibility as well as the rate and total gas production in the rumen *in vitro*, but fastened the decrease of the EMPS or microbial biomass.

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Effect of storage on physicochemical, microbiological, and sensory properties of Thawai Dueankao mango jelly

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ABSTRACT

Observing the quality changes of food products during storage can assist in determining their shelf life. The Thawai Dueankao mango (TM) jelly was evaluated during 4 weeks of storage at 25±5 ℃ for the physicochemical characteristics, sensory evaluation, and microbiological characteristics. The TM jelly maintained physicochemical characteristics without modification over time storage ($P>0.05$). The storage period did not affect its acceptance, and microbiological qualities were safe for human consumption during storage. Furthermore, the TM jelly presented potential functional properties: total phenolic content (TPC), vitamin C content, and antioxidant activities (2,2-Diphenyl-1-picryhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) were 81.53±0.68 mg GAE/100g, 5.51±0.37 mg/100g, 36.49±1.51 and 2,365.17±9.43 µmol TE/g, respectively. The results suggest that jelly preparation from TM could improve the shelf life of fruit and make it a promising option in the jellies market due to its health advantages.

Keywords: Thawai Dueankao mango, jelly, gelatin, shelf life

INTRODUCTION

Mango (*Mangifera indica* L.) is a tropical fruit highly valued and widely consumed worldwide because of its appealing texture, flavor, taste, and abundant nutritional content. It contains a variety of beneficial substances, such as carotenoids, vitamin C, vitamin E, phenolic compounds, minerals, and fiber, which have potent antioxidant properties (Charles et al., 2013; Asif et al., 2016). Mango consumption can supply the body with significant quantities of bioactive compounds to help prevent degenerative conditions such as cancer and cardiovascular disease when included in the daily diet (Derese et al., 2017; Alothman et al., 2010). Thawai Dueankao mango (TM) is a local variety of mango in Chachoengsao; its simplicity in cultivation and production yields large fruit. Its sour taste and commonly consumed raw and semi-ripe; when ripened, it becomes sweet but has an unpleasant odor that makes it less popular for consumption. However, the fruit is produced considerably during the season, so the selling price of natural products may fall due to increased supply in the market. Therefore, farmers or producers prefer to process the fruit into products rather than selling the raw fruits for consumption which can create added value and generate additional income.

Gelatinous confectionery, especially jelly candy, has an elastic texture and glossy appearance and comes in various shapes and colors, making it attractive to people of all ages and genders. These sweets are usually made by gelatinizing a sugar mixture containing sucrose and corn syrup and adding flavorings, acids, and colorants using pectin, agar, or gelatin. However, their excessive sugar content and poor nutritional value can lead to dental cavities, obesity, and hyperglycemia when consumed in large quantities (Gallo et al., 2020). The addition of fruit to jelly can reduce the amount of added sugar needed in the recipe, as fruit naturally contains sugar. It can also add natural flavor and color to the jelly, reducing or eliminating the need for artificial flavoring and dyes. Moreover, fruit contains fiber which can slow down the absorption of carbohydrates, further helping to regulate blood sugar levels; using natural ingredients can provide additional nutritional benefits. Thus, this study developed the Thawai Dueankao mango flavored jelly (TM jelly), produced from gelatin, sucrose, glucose syrup, citric acid, and the addition of fresh TM juice to their formulation. Nutritional compositions, antioxidant activities, total phenolic and vitamin C contents were evaluated initially. The sensory evaluation, physicochemical and

microbiological characteristics were analyzed over 4 weeks of storage.

MATERIALS AND METHODS

Preparation of TM juice

Raw TM was bought directly from the farmer in Bangkla, Chachoengsao, Thailand. TM was washed under running water and manually peeled, sliced into small pieces, and pulps mixed with water (1:1 w/w), blended, and filled. The diluted juice was bottled and stored at 4±1 ℃ before jelly preparation.

Jelly preparation

For the preparation of TM jelly, 60% TM juice, 17% sucrose, 12.8% glucose syrup, 10% gelatin, and 0.2% citric acid were used. At first, gelatin and part of TM juice were mixed, and the mixture sat for 10 minutes to hydrate and soften before adding. Sugar and glucose syrup was added to the remaining TM juice, mixed and heated to its boiling point, and cooled down by 85-90 ℃. After that, the hydrated gelatin and citric acid were added to the juice sugar and mixed until dissolved and clarified. The hot solution was poured into silicone molds and refrigerated (4±1 ℃) for 24 hours for the jelly to settle; after that, the jellies were taken out of the molds and packed in polyethylene bags.

Nutritional compositions

The TM jelly was determined total energy, total fat, protein, dietary fiber, moisture, ash, total sugar, and sodium contents according to AOAC (2012). Carbohydrate content were calculated by difference (100 - (%moisture + %protein + %fat + %ash)).

Total phenolic and vitamin C contents

The total phenolic content of the TM jelly was determined using the Folin-Ciocalteu method as modified by Amarowicz et al. (2004). The results were expressed as mg gallic acid equivalents (GAE) per 100g of sample (mg GAE/100 g).

Vitamin C content of the TM jelly was evaluated by 2,4-Dinitrophenylhydrazine (2,4-DNPH) method, according to Duais et al. (2009). The results were expressed as mg per 100 g of product.

Antioxidant activities

DPPH assay (1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity) was determined according to the method described by Katsube et al. (2004). The results were expressed as µM Trolox equivalent per 100 g of sample (µmoles TE/100 g).

ORAC assay (Oxygen radical absorbance capacity) was determined according to Ou et al. (2001) method. The final ORAC values were calculated using the differences in area under the fluorescence decay curve (AUC) between the blank and the sample. The area under the fluorescence decay curve (AUC) was calculated according to the following equation:

$$AUC = 0.5 + f_1/f_0 + f_i/f_0 + \ldots + f_{89}/f_0 + 0.5(f_{90}/f_0)$$

Where $f0$ = initial fluorescence reading at 0 min and fi= fluorescence reading at the time i min. Final ORAC values were calculated as follows and expressed as µmol TE/100g of sample (dry basis):

$$\text{ORAC value} = [(AUC_{sample} - AUC_{blank})/(AUC_{trolox} - AUC_{blank}) \times \text{dilution}]$$

Storage test

The TM jellies were packed in polyethylene bags and stored at ambient temperature (approximately 25±5 ℃) for 4 weeks. The samples were determined the physicochemical, microbiological, and sensory properties immediately after production (time zero) and after 1, 2, 3, and 4 weeks.

pH measurement

The pH values were measured at room temperature (25±1 °C) using a pH meter (Mettler, Mettler-Toledo International Inc., Greifensee, Switzerland). Three replicate readings (three different samples) were taken for each pH of the samples.

Water activity (Aw)

It was determined using a water activity meter (MIS-Aw, Nobasina Aair Ltd, Zurich, Switzerland) in triplicate at room temperature (25±1 °C). The samples were placed in a plastic sample holding container and put inside the meter.

Color values

The measurement of color in the CIE (L*, a*, b*) system and hue angle (ho) were evaluated using a spectrophotometer (ColorFlex EZ, Hunter Associates Laboratory Inc., Virginia, USA). In this color system, L* value is a measure of lightness to darkness (0 = black and 100 = white); a* is a measure of redness (+) to greenness (-); and b* is a measure of yellowness (+) to blueness (-).

Texture profile analysis

The texture profile of TM jelly was evaluated by Texture Analyzer (TA.XT plus, Stable Micro Systems Ltd, YL, UK) with a cylinder probe (50 mm diameter). The analysis was conducted at room temperature (25±1 °C) and set in compression mode with a pre-test speed of 2 mm/s, test speed of 2 mm/s, post-test speed of 2 mm/s, distance between probe and sample of 10 mm, trigger force of 5 g and the delay between two compressions was 2s. The data were analyzed using Texture Expert Version 1.22 Software (Stable Micro System Ltd, Scarsdale, NY) to measure jelly hardness, cohesiveness, and springiness as described by Bourne (1978).

Sensory evaluation

Fifty volunteers participated in the sensory analysis, evaluating the samples using a nine-point hedonic scale described by Stone et al. (2012). The scale ranged from a score of 9 for "like extremely" to a score of 1 for "dislike extremely." The attributes assessed by the volunteers included color, flavor, taste, texture, and overall acceptability.

Microbiological analysis

The total plate count, yeast, and molds were determined according to BAM (2001). The results were reported in CFU/g.

Statistical analysis

A completely randomized design (CRD) with three replications was conducted for physicochemical properties, antioxidant content, and antioxidant activities. The statistical program, SPSS software for window version 18.0 (SPSS Inc., Illinois, USA), was used to perform the statistical analysis. Means comparison was performed using Duncan's new multiple range tests at the 95% significance level.

A randomized complete block design (RCBD) with three replications was conducted for sensory properties. The statistical program, SPSS software for window version 18.0 (SPSS Inc, Illinois, USA), was used to perform the statistical analysis. Means comparison was performed using Duncan's new multiple range tests at the 95% significance level.

RESULTS AND DISCUSSION

Compositions of TM jelly

The total energy of TM jelly per serving size (30 g) was 60 kcal. The protein, fat, carbohydrate, dietary fiber, and total sugar contents per serving size were 22.15%, <0.01%, 28.91%, 1.35%, and 19.68%, respectively (Table 1). The data indicated that TM jelly exhibited antioxidant activity and contained phenolic compounds and vitamin C (Table 1). The results were consistent with the other potentially functional jelly candies: Cappa et al. (2015) reported total anthocyanin content of 430.1-564.0 mg Mal eq/kg, total flavonol content of 140.1-176.5 mg Que eq/kg and antioxidant activity (FRAP): 16-45 mmol Fe(II)/kg for fruit candies enriched with grape skin. Also, Novelina et al. (2016) showed lycopene content: 18.32-18.76 mg/100 g, vitamin C content: 9.51-17.70 mg/100 g, and antioxidant activity (DPPH): 24.55-54.59%. While De Oliveria Nishiyama-Hortense et al. (2022) found total anthocyanins of 686.78 mg malvidin-3,5-glucoside/kg for jelly candy enriched with BRS Violeta grape juice. Recently, Piechowiak et al. (2023) indicated that the addition of young pine shoots in the jellies generated an increase of 150% in ascorbic acid level, 98% in total phenolics level, and 173-178% in antioxidant activity (DPPH-ABTS). Mangoes are known as a rich source of dietary antioxidants, including ascorbic acid, carotenoids, and phenolic compounds (Riberio et al., 2007). Hence, adding mango to jelly can contribute to its antioxidant capacity.

Table 1. Nutritional compositions, total phenolic, vitamin C contents, and antioxidant activity of TM jelly

Compositions	Contents
Total energy (kcal/100g)	204.28±0.53
Protein (%)	22.15±0.11
Total carbohydrate (%)	28.91±0.25
Total dietary fiber (%)	1.35±0.03
Total fat (%)	<0.01
Moisture (%)	48.74±0.15
Ash (%)	0.19±0.02
Total sugar (%)	19.68±0.06
Sodium (mg/100g)	27.28±0.72
Total phenolic (mg GAE/100g)	81.53±0.68
Vitamin C (mg/100 g)	5.51±0.37
[1]DPPH (µmol TE/g)	36.49±1.51
[2]ORAC (µmol TE/g)	2,365.17±9.43

[1]DPPH (2,2-diphenyl-l-picrylhygrazyl) and [2]ORAC (Oxygen radical absorbance capacity)

Physicochemical characteristics of TM jelly

As shown in Table 2, the pH, water activity (A_w), and color values of TM jelly showed no significant difference (P>0.05) over the storage period. The TM jelly produced, which had an acidic pH level ranging from 3.41 to 3.47 and an intermediate water activity between 0.70 and 0.73, exhibited significant microbiological stability at room temperature. A previous study reported pH values and water activity of jelly candies as 3.42-3.54 and 0.67-0.68, respectively (De Moura et al., 2019). In another study, the pH values and water activity of jelly candies were reported as 3.81-3.87 and 0.71-0.73, respectively (Miranda et al., 2020).

All values of color parameters (L*, a*, and b*) did not differ significantly (P>0.05) over time of storage. Muzzaffar et al. (2016) also reported non-significant differences in chromatic parameters L*, a*, and b* during two months of storage for pumpkin candy. Miranda et al. (2020) suggested that adding jucara and passion fruit pulp to the jelly candies can substitute synthetic dyes once they have color stability during storage.

Table 2. pH, water activity (Aw), and color values of TM jelly during storage at 25±5 °C for 4 weeks

Weeks	pH^{ns}	A_w^{ns}	Color		
			L*^{ns}	a*^{ns}	b*^{ns}
0	3.43±0.02	0.70±0.01	52.32±0.46	4.35±0.25	29.25±1.29
1	3.41±0.01	0.72±0.06	50.97±0.84	3.93±0.34	29.80±0.87
2	3.43±0.04	0.71±0.03	51.08±1.12	4.12±0.56	31.07±1.51
3	3.47±0.01	0.73±0.04	49.93±1.33	3.97±0.22	29.90±1.03
4	3.45±0.03	0.73±0.02	49.95±0.70	4.07±0.16	30.54±0.76

^{ns} Means values in the same column are not significantly different (P>0.05). Values are means of triplicate ± standard deviation.

Texture profile analysis

The hardness, cohesiveness, and springiness of TM jelly did not present significant (P>0.05) changes during storage for 4 weeks at ambient temperature (25±5 °C) (Table 3). However, the hardness was slightly increased along the shelf life; this behavior in the texture variable could be due to the decrease in humidity. The increase in the hardness of jelly during storage time is in agreement with Rubio-Arraez et al. (2018), Seremet et al. (2020), and Rivero et al. (2021), who reported that the hardness was changed with a significant influence on time storage. Furthermore, it is essential to reduce the amount of moisture lost from gelatin-based candies to preserve the structural quality of these products (Kopjar et al., 2016).

Table 3. Texture parameters of TM jelly during storage at 25±5 °C for 4 weeks

Weeks	Hardness (g)^{ns}	Cohesiveness^{ns}	Springiness^{ns}
0	1796.77±80.71	0.67±0.05	1.01±0.03
1	1802.19±96.35	0.68±0.02	0.98±0.04
2	1854.51±91.66	0.71±0.07	0.98±0.01
3	1911.23±75.43	0.70±0.04	1.03±0.02
4	1930.38±83.04	0.73±0.03	1.05±0.01

^{ns} Means values in the same column are not significantly different (P>0.05). Values are means of triplicate ± standard deviation.

Sensory evaluation

The use of sensory assessment proved crucial in examining the effects of adding fruit juice as natural colorants and flavor and evaluating the impact of storage duration on TM jelly. According to Table 4, all the sensory attributes of the sample obtained scores above 7.0 (like moderately), and there was no significant (P>0.05) influence of the duration of storage; they were assessed as sensorial acceptable.

Table 4. Sensory attribute scores of TM jelly during storage at 25±5 °C for 4 weeks

Weeks	Color^{ns}	Flavor^{ns}	Taste^{ns}	Texture^{ns}	Overall acceptability^{ns}
0	7.38±0.89	7.43±1.14	7.55±1.03	7.67±0.95	7.53±0.48
1	7.40±1.02	7.33±0.96	7.45±1.21	7.68±0.72	7.48±0.99
2	7.35±0.72	7.26±0.88	7.48±0.75	7.70±1.24	7.62±0.85
3	7.44±1.13	7.30±0.65	7.52±0.68	7.50±0.97	7.55±1.08
4	7.29±1.11	7.34±1.05	7.50±0.42	7.51±0.86	7.47±0.79

^{ns} Means values in the same column are not significantly different (P>0.05). Values are means of triplicate ± standard deviation.

Microbiological analysis

The total plate count, yeast, and molds of TM jelly during storage are presented in Table 5. The finding demonstrated that the sample kept at ambient temperature (25±5 ℃) for 0 weeks met the acceptable limit according to the Thai community product standard: liquid jelly (Thai Industrial Standard Institute, 518-2547). After 2 weeks of storage, the total plate count of the sample was slightly increased but still within the acceptable limit according to the Thai community product standard: liquid jelly ($1x10^4$ CFU/g) (TISI, 2004). While, yeast and molds of the sample after storage for 1, 2, 3, and 4 weeks at ambient temperature (25±5 ℃) were within the acceptable limit according to the Thai community product standard: liquid jelly (100 CFU/g) (TISI, 2004). Similar results were reported by Thongsook et al. (2008) and Yuenyongputtakal et al. (2018), which confirmed that the assessment of the total plate count, yeast, and molds verified the safety of the jelly for consumption during the entire storage period. These results were probably because the concentration of citric acid was lower the pH of TM jelly to 3.4, and microbiological stability of jelly depended on the high concentration of their sugar (Pilgrim et al., 1991).

Table 5. Total plate count, yeast, and molds of TM jelly during storage at 25±5 °C for 4 weeks

Weeks	Total plate count (CFU/g)	Yeast and Molds (CFU/g)
0	<10	<10
1	<10	<10
2	27	<10
3	35	<10
4	46	<10

CONCLUSIONS

TM juice can serve as a natural additive in jelly and provide flavor, color, and antioxidant properties to the product without altering the sensory acceptable, physicochemical, and microbiological characteristics throughout the storage period.

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Jackson, M.B. 1982. Ethylene as a growth promoting hormone under flooded conditions. In: Wareing, P.F. (ed) Plant growth substance. Academic Press, London. p.291-301.

• Proceeding, symposia etc.

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3. Equation, figure, table and unit of measurement

Equation

All equations presented in the text can be prepared by Equation editor from Microsoft word or Math Type.

Figure

Data presented in figure must be written in English. Color or gray scale figure should be prepared as TIFF format (.tif) with at least 600 dpi. Photograph should be prepared as TIFF (.tif) or JPEG (.jpg) format with at least 600 dpi. Figure with caption should be placed next to the relevant text, rather than at the bottom of the file.

Table

Data presented in table must be written in English. Table with caption and footnote should be placed next to the relevant text, rather than at the bottom of the file.

Unit

Author should use the International System of Units (S.I.)

4. Structure of manuscript

- Title
- Author names and affiliations
- ABSTRACT
- Keywords
- INTRODUCTION
- MATERIALS AND METHODS
- RESULTS AND DISCUSSION (The results and discussion can be combined or separated depends on author design)
- CONCLUSIONS
- ACKNOWLEDGMENTS (if any)
- REFERENCES

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