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EFFECTS OF WHEY PROTEIN ISOLATE ON BLACK PEPPER ESSENTIAL OIL ULTRASOUND-ASSISTED EMULSION ON PHYSICAL CHARACTERISTICS, ANTIOXIDANT ACTIVITY, AND ANTIBACTERIAL PROPERTIES

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ABSTRACT

The purpose of this research was to determine the best ratio of whey protein isolate (WPI) for encapsulation of black pepper (*Piper nigrum*) essential oil (BPEO) on ultrasound-assisted emulsion. O/W emulsion was made from the oil phase from BPEO and corn oil (30:70) and the aqueous phase was made from the liquid modified glucomannan. The effect of WPI was investigated at different ratios (T_0= 0%, T_1= 2%, T_2= 4%, T_3= 6%,). BPEO emulsion was prepared using an ultrasonication method with the intention for produce nanoemulsion. The method of this research was an experiment with a Completely Randomized Design (CRD) by using four treatments and three replications, then continued by Duncan Multiple Range Test (DMRT). The physical characteristics were observed by viscosity and optical microscope. DPPH scavenging activity was used to know the effect of encapsulation on antioxidant activities. The Antibacterial activity was evaluated by the Kirby Bauer method with *E. coli* as the pathogen bacteria and *L. casei* as the probiotic bacteria. The increase ratio of WPI influences morphology. Different ratio of WPI gave a highly significant effects ($p<0.01$) on the viscosity and antioxidant, and gave no significant effects ($p>0.05$) on the inhibition diameter zone for *E. coli* and L. *casei*. Based on its physical characteristic, antioxidant activity, and antibacterial properties, it can be concluded that WPI can protect the BPEO by stabilizing O/W emulsion and may have the potential to be used as an alternative natural antioxidant and antibacterial additive in many food applications.

Keywords: Encapsulation; emulsion; essential oil; whey protein isolate; antibacterial; antioxidant

INTRODUCTION

The demand for healthy food leads the producer to use natural additives in food industries (Esmaeili *et al.,* 2020). One of the natural additives applications which need to be explored is essential oil (EO). Several studies have shown that EO can increase the shelf life of both raw and processed food due to its and antioxidant activity and antibacterial properties. Essential oils (EO) are aromatic compounds that have many bioactive properties (Zhu *et al*., 2021).

One type of essential oil is black pepper (*Piper nigrum* L) which has the potential for food preservation because of its inhibition of many pathogens. BPEO have at least twenty bioactive compounds, the most bioactive are the monoterpene and sesquiterpene groups (Hien & Dao, 2022). Those bioactive compounds are very sensitive to external factors, such as light, temperature, and oxygen (Rehman *et al.,* 2021). Another disadvantage of the application of essential oils in food products is their hydrophobic behavior (Farshi *et al.,* 2017). One way to protect these compounds is by encapsulation techniques with emulsification using biopolymers (Fernandes *et al.,* 2016). Emulsification generates oily or aqueous droplets commonly named capsules of a wide range of sizes (Abd El-Salam, & El-Shibiny, 2015).

Nanoemulsions are considered for food applications because of their food compatibility, good physical stability, and ability to enhance the antimicrobial activity of essential oils (Farshi *et al.,* 2019). Moreover, Hebishy *et al.* (2017) explained that oil-in-water (O/W) nanoemulsions have been researched in order to encapsulate lipophilic molecules in the pharmaceutical, food, and cosmetic industries. McClements & Rao (2011) explained that the most important ingredient in nanoemulsion is the emulsifier. Emulsifiers can be made from small-molecule surfactants, phospholipids, proteins, and polysaccharides. Proteins and polysaccharides have advantages as natural ingredients rather than artificial emulsifiers, but the manufacture of emulsions with these materials must use high energy method. Ultrasound emulsification is one of the high-energy methods which has some benefits such as easily manipulating systems, energy efficiency, and more efficient in retaining volatile compounds (Fernandes *et al.,* 2016). The ultrasonic method uses high-intensity ultrasonic waves to disturb the oil and the water phase so both phases will break up and it will become small droplets (McClements & Rao, 2011).

The composition of the emulsion will influence the physical characteristics of the nanoemulsion. Farshi *et al.* (2019) explained that one of the nanoemulsion approaches is combining protein emulsifiers with polysaccharides as a co-emulsifier. Polysaccharides can increase the viscosity of emulsions in the continuous phase through the formation of hydrogen bonds with water molecules. Campelo *et al.,* (2017) successfully combined biopolymer (GA and WPI) as wall material for microencapsulation to protect lime essential oil.

Glucomannan has been chosen as one of the ingredients for emulsions because it has the potential as an emulsifier, stabilizer, and microcapsule material (Li *et al*., 2018). According to Manab *et al.* (2016), modification of glucomannan liquid can upgrade the physicochemical properties of

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glucomannan. The modification of glucomannan through a combination process of thermal or irradiation and lactic acid could be increasing the binding and interaction between glucomannan and milk protein.

Protein can be a good emulsifier in the emulsion system because it has hydrophobic and hydrophilic properties (Adjonu *et al.*, 2014). The use of whey protein (whey protein isolate, whey protein concentrates, and beta-lactoglobulin) as nano-emulsifying agents has received much attention, with most studies concentrating on the use of native proteins rather than hydrolysates. Milk protein is also a good moisture binder when used in meat processing. Based on Fuentes *et al.* (2021), the hydrophilic-lipophilic balance (HLB) of WPI is 9.1 which means the WPI is compatible as an emulsifier for oil in water (O/W) emulsion. Moreover, Hebishy *et al.* (2017) in their research proved that whey protein isolates (WPI) enhance the formation and stability of (O/W) emulsions by ultra-high pressure homogenization.

This study is focusing on the effectiveness of whey protein isolate as an emulsifier for black pepper essential oil (BPEO) nanoemulsion combined with modified glucomannan. The ability of whey protein isolate as a natural emulsifier can be investigated from the physical stability through viscosity, morphology through an optical microscope, antioxidant activity, and antibacterial activity after the emulsification process. This research goal is to determine the best ratio of whey protein isolate (WPI) for encapsulation of black pepper (*Piper nigrum*) essential oil (BPEO) on the ultrasound-assisted emulsion.

MATERIALS AND METHODS

Materials

Black pepper (*Piper nigrum* L.) essential oil (DDistillers® from PT. Syailendra Bumi Investama, Central Java). *Whey Protein Isolate* (95%) (*Milk Specialties Global*, WI, USA), Konjac glucomannan flour (CV. Nura Jaya). Lactic acid (PT. Brataco), corn oil (Tropicana Slim®), DPPH (Smartlab®), methanol PA , Eosin Methylene Blue (EMB) agar (Hi-Media Ltd., Mumbai, India), de Man Rogosa Sharpe agar (Hi-Media Ltd., Mumbai, India), *Escherichia coli* bacteria, *Lactobacillus casei* bacteria, *Magnetic stirrer* (SBS-A06), *microwave* (SHARP R 222Y), Centrifuge (Universal 32R, Hettich, Tuttlingen, Germany) ultra-turrax (IKA® T25 digital), ultrasonic homogenizer (Lawson®), viscometer NDJ-8S, calipers (Sigmat Digital), centrifuge tube, optical microscope Olympus®, sterile filter paper disks (Whatman No. 3, 6mm, Sigma-Aldrich), Spectrophotometer UV-VIS, cuvette, micropipette, viscometer.

Methods

The method of this research was an experiment with a Completely Randomized Design (CRD) by using four treatments with different ratio of WPI (T_0: 2%, T_1: 2%, T_2: 4%, T_3: 6% wt) and three replications, then continued with the Duncan Multiple Range Test (DMRT) if any differences among treatments.

Emulsion Preparation

Modified glucomannan prepared based on Manab *et al.* (2016), 3% wt was dissolved in 100 ml distilled water with 4% lactic acid, then stirred for 15 minutes. After that, the solutions were heated by microwave for 10 minutes and centrifuged at 5,000 rpm for 10 min at 25 °C to separate sediments. The emulsifier and water phase was prepared by WPI (2%-6% wt) dissolved in 100 ml distilled water and then added to stabilizer glucomannan liquid at ±70 °C for 15 min. The oil phase was prepared with BPEO and corn oil (30:70). The ratio of the aqueous phase: the oil phase was set at 10:90. The colloid was homogenized at a high speed Ultra-Turrax homogenizer (IKA® T25 digital) at 20,000 rpm for 3 min. The coarse emulsion was further emulsified using an ultrasonication (Lawson®), 20 kHz

with a maximum power output of 400W for 15 min.

Viscosity Test

Viscosity measurement was investigated by a viscometer, the emulsion was transferred on pot film and measured using spindle number 4 (Tirmiara *et al.,* 2019).

Morphology Test

Morphology was tested by an optical microscope based on Rahayu *et al.* (2015). A ±5 µL drop of the emulsion was put on the slides and then covered with coverslips on the 7th day. The samples were observed under a 100×lens and the images were recorded by a camera.

Antioxidant Activity

DPPH scavenging test is a technique that demonstrated the potential of nanoemulsion to function as a donor of hydrogen atoms or electrons to convert the stable lipophilic radical DPPH (2.2′-diphenyl-1-picrylhydrazyl) into its reduced form, DPPH-H (Azizkhani *et al*., 2021). The investigation into antioxidant activity was used method by Azizkhani *et al*. (2021) with modification.

The DPPH solution (0.1 mM) was solute with methanol, and 1 mL of this solution was added to 4.0 mL of nanoemulsion at various concentrations (between 25 and 100 µg/mL), and the combination was then left at room temperature for 1 hour in the dark. Ascorbic acid is used as a control. In order to quantify the absorbance (A), a UV-vis spectrophotometer was used at 517 nm (Faithful®, Huanghua Faithful Instrument Co., Ltd, China). The half-maximal inhibitory concentration (IC_{50}), which was computed, is the concentration of a substance that can scavenge 50% of DPPH free. The following formula was used to compute the percentage of antioxidant activity:

$$\text{Antioxidant activity (Inhibition\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

Pathogenic Antibacterial Test

Antibacterial assay for pathogenic bacteria was based on Dat *et al.* (2020) with slight modification. Active cultures of *E. coli* were prepared and considered equivalent to a 0.5 McFarland standard. The EMB agar was prepared by dissolving 38.0-gram agar in 1000 ml of distilled water. The agar was sterilized using an autoclave with 1.5 atm. The 100 ηm active culture was transferred to the cultured plate and poured with ±15 ml media. The sterile filter paper disks were dipped with different concentrations of emulsion for 15 minutes and then incubated at 25°C for 72 h. The diameter of the growth-free zone containing disk diameter was measured using a calliper with 0.1 mm resolution.

Probiotic Antibacterial Test

Antibacterial assay for probiotic bacteria was based on Dat *et al.* (2020) with slight modification. Active cultures of *L. casei* were made equivalent to 0.5 McFarland standard. The 68.2-gram MRS agar was dissolved in 1000 mL of distilled water. After that, the agar was sterilized. The micropipette was used to transfer 100 ηm active culture to the cultured plate and poured with ±15 mL media. The sterile filter paper disks were dipped with different concentrations of emulsion for 15 minutes and then incubated at 25°C for 72 h. The diameter of the growth-free zone containing disk diameter was measured using a caliper with 0.1 mm resolution.

RESULTS AND DISCUSSION

Morphology

Optical microscopes have been widely used to image emulsion formations during the inversion phase (Hu *et al.,* 2017). Rahayu *et al.,* (2015) observed the morphology of a mixture of proteins and polysaccharides using a light microscope.

Figure 2 shows the surface of ultrasound-assisted emulsion from BPEO-WPI-Glucomannan. The bubble and droplets appear and spread between the water phase on T_0. The droplet's size was varied and it made a probability would merge together even though glucomannan has the ability to lock the oil movement through viscosity. The bubble might separate between oil and water phase. While at T_1, T_2, and T_3, there were no visible oil droplets in the water phase.

This result proved that the whey protein adsorbs to the oil droplet interface, producing a consistent protective film around the oil phase to help prevent droplet aggregation (Adjonu *et al*., 2014). This emulsion is made by ultrasound which is using a generated mechanical vibration and cavitation for the small-sized droplet production (Jemaa *et al.,* 2019), with WPI as a surfactant, the WPI molecules will form a layer to reach the equilibrium of interfacial tension. The first layer is called the monolayer structure, then the multilayer structure is also formed by the glucomannan molecule. It will improve the stability protection of the internal phase (Güell *et al.,* 2017).

The difference between T_1, T_2, and T_3 is the surface's smoothness along with emulsifier content. The higher the addition of emulsifier, the emulsion surface more dense and smooth. This means that the emulsifier is able to lock the bonds between the oil phase in the water phase. The compactness of emulsion because of steric repulsion, a characteristic of non-ionic and/or polymeric emulsifiers, so there is still a distance between the droplets due to the adsorption of the hydrophobic segment by the oil phase (Santamaria *et al*., 2021).

The compactness of images is because of the particle already in microemulsion or nanoemulsion so the microscope is not sufficiently capable of imaging the droplets. This result is compatible with Hu, *et al.* (2017) who explained that its means the molecule is relatively small in size if the droplets are homogeneously distributed in the image. It is difficult to distinguish the compositions of the emulsion system between the dispersed phase, whether it is made of proteins, polysaccharides, or other amphiphilic small molecules because of the small resolution.

This result is related to the viscosity where T_0 that the highest viscosity had bubble and droplet more visible in microscope image than other treatments. Satriawan *et al.* (2022) also used an optical microscope to investigate whey protein as an emulsifier in a mayonnaise emulsion and they proved that whey protein concentrate can reduce droplet size.

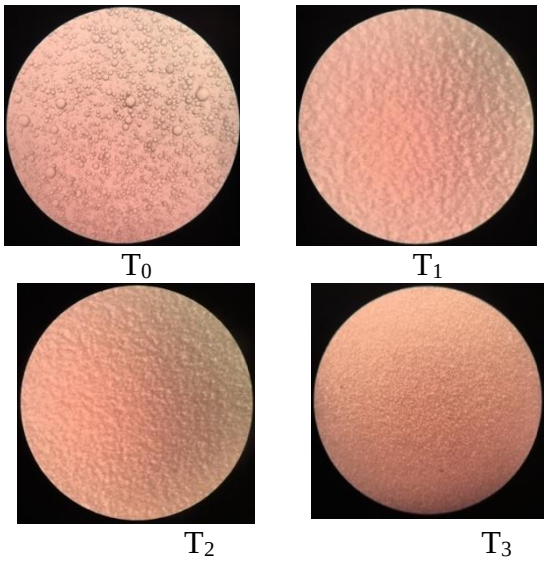
Viscosity determination

A high emulsion viscosity indicates high emulsion stability. The viscosity is influenced by the strong ionic interaction between lipophilic amino acids with the oil phase, and lipophilic amino acids with the aqueous phase (Suryanti *et al*., 2017).

Table 2 shows that different ratios gave a highly significant effects ($p<0.01$) on the viscosity. The higher viscosity at T_4 and the lowest viscosity at T_0. This proved that increasing the ratio of WPI raises the viscosity. Hammann & Schmid (2014) explained that proteins are organic macromolecules that can be arranged as α-helix, β-sheet, and stabilized by hydrogen bonds, van der Waals, electrostatic, hydrophobic, and disulfide interactions. Moreover, increasing WPI makes the emulsion contain amino acids with a large ionic charge so that it has the ability to interact ionically with larger oil and water molecules and lead to large emulsion viscosity (Suryanti *et al.,* 2017).

The viscosity of the emulsion in this research is similar to the viscosity of cumin seed oil nanoemulsion stabilized whey protein isolate and guar gum, where the viscosity is around 28-51.5 mPa.s (Farshi *et al.,* 2019). The DMRT results showed that all treatments showed significant differences. According to Mohammed *et al.* (2020), low emulsifier concentration will lower the interfacial tension between water

and oil and decrease the viscosity. Viscosity will improve the stability because the continuous phase forms 3-dimensional networks to confine the droplet movement and reduce coalescence and droplet creaming (Adjonu *et al*., 2014). Aziz *et al.*, (2019) also explicated that the increasing viscosity because of the hydrogen bonding between surfactant hydrophilic segments with water molecules that have the water molecules entrapped in the cross-linking portions of the surfactant. The more viscous the emulsion, the more denser the morphology as shown in Figure 1. This result is related to Nirmala *et al.* (2020) who made an emulsion from celery essential oil and proved that the increased viscosity is directly proportional to surfactant concentration which is attributed to the increased extent of cross-linking in the surfactant amidst which water molecules get entrapped.



Remarks:
(a) Bubble and Oil droplet was formed on T_0 after the 7th day of storage
(b) Bubble and Oil droplet was not formed on T_1 after the 7th day of storage
(c) Bubble and Oil droplet was not formed on T_2 after the 7th day of storage
(d) Bubble and Oil droplet was not formed on T_3 after the 7th day of storage

Figure 1. Optical Microscope of Emulsion BPEO-Glukomannan-WPI with 100x magnification.

Antioxidant Activities

The study on the antioxidant activities of essential oil from black pepper (*Piper nigrum* L.) proves that pepper essential oils exhibit a good antioxidant due to the major components of BPEO, such as alkaloids (piperine), flavonoids, polyphenols, and amides (Wang *et al.,* 2021).

The GC-MS from Ddistillers® essential oil showed the bioactive component of BPEO were α-Pinene, β-Pinene, Delta 3-Carene, Limonene, and Trans-Caryophyllene, with ratios of 9.79%, 11.74%, 7.98%, 11.88%, 25.73% respectively as shown in Table 1. The bioactive component content is in similar to Nikolić *et al*. (2015) who explained that the main bioactive of black pepper oil was Trans-Caryophyllene (30.33%), limonene (12.12%), α-pinene (24.42%), 3-carene (19.72%), limonene (18 0.73%) and β-pinene (10.39%).

Table 1. Formulations of BPEO Nanoemulsion

Treatment	Oil Phase		Water Phase	
	Corn Oil (mL)	BPEO (mL)	GKM (mL)	WPI (b/v%)
T_0 / Control	3.5	1.5	22.5	0
T_1	3.5	1.5	22.5	2
T_2	3.5	1.5	22.5	4
T_3	3.5	1.5	22.5	6

Table 2 shows the IC_{50} value for all treatments and every treatment shows that have strong antioxidants. The value of IC_{50} means the ability to decrease 50% of DPPH radical activity. This value is the result of the regression equation derived from the relations between % inhibition power and change in sample concentration (Nugrahani *et al.,* 2020). T_0 has the highest IC_{50} with a value 40.25 ± 7.03 µg/mL. This result is similar with Azizkhani *et al*., (2021) who study *Artemisia dracunculus* L. essential oil nanoemulsion and found that nanoemulsion exerted higher antiradical and antioxidant effect than free EO. *Artemisia dracunculus* L. The IC_{50} value for *Artemisia dracunculus* L. oil was obtained 70 µg/mL and IC_{50} for *Artemisia dracunculus* L. nanoemulsion was 52 µg/mL.

Because of their diversity of active ingredients, EOs have multiple action mechanisms for antioxidant reactions. The hydroxyl groups of antioxidants act as hydrogen donors leading to inactivating the free radicals produced by the oxidation of unsaturated fatty acids. Additionally, the antioxidants in EO have redox characteristics that enable them to donate an electron to free radicals, increasing their activity and preventing the oxidation of other substances. New radicals are created due to these reactions, and they cannot accept hydrogen atoms from unsaturated lipids.

Additionally, these secondary radicals can interact with other radicals. The ratio of whey protein give a highly significant effect on the IC_{50} ($p<0.01$). The higher the WPI concentration, the lower value of $IC_{50,}$ and it means higher antioxidant activities because it means only a small compound or concentration (9.68-40.25 µg/ml) to reduce DPPH into DPPH-H. When the free-radical molecule is reduced, the dark purple color becomes colorless or light yellow (Balliyan *et al.,* 2022).

The DMRT analysis shows that T_0 has lower antioxidant activities than other treatments. The best high antioxidant activities are T_3, followed by T_2, and T_1, respectively. This might be influenced by the whey protein antioxidant compound. Whey protein's antioxidant action is caused by lactoferrin's chelation of transition metals and sulfur-containing amino acids like cysteine and tyrosine that scavenge free radicals. Whey proteins have been found to exhibit antioxidant activity in several investigations and adding whey proteins to soybean oil emulsions enhanced their oxidative stability. Another study showed that the antioxidant characteristics of salmon oil emulsion increased after the addition of whey protein (Khan *et al.,* 2019).

Pathogen-Antibacterial Test

Soetjipto (2018) explained that some essential oils of aromatic Indonesian herbs can be antibiotic or antibacterial agents. Some of them can be applied as preservative and flavoring agents in the food industries. Various bacteria will have different sensitivities to the essential oil.

Table 2. showed that different ratios of whey protein gave no significant effects ($p>0.05$) on the inhibition diameter zone for *E. coli*. It means that encapsulation of WPI has not reduced the antibacterial properties and is successful to protect the bioactive BPEO. Sugumar *et al. (*2015) explained that encapsulation of EO represents a feasible and efficient approach to enhancing the chemical and physical stability of the bioactive constituents by protecting them from other food component interactions. One of the primary antibacterial constituents

is terpenes, which have some targets in the membrane of the bacteria and in the cytoplasm of bacteria (Allenspach *et al.,* 2020). Terpenes and ketones are capable of destroying bacterial cytomembranes and ATP (Bhavaniramnya *et al.,* 2019).

Table 2. Data of Viscosity, IC_{50} and Inhibition Zone BPEO Nanoemulsion

Treatment	Viscosity mPa.s	IC_{50} µg/mL	Phatogenic Bacteria (mm)	Probiotic Bacteria (mm)
T_0 / Control	11.93± 1.10^{a}	40.25 ± 7.03^{b}	14.19± 1.07	5.30± 0.62
T_1	23.27± 1.27^{b}	16.49 ± 3.88^{ab}	11.07± 1.62	4.45± 0.57
T_2	29.20± 4.66^{b}	12.56 ± 2.27^{ab}	10.98± 2.89	4.23± 0.47
T_3	50.87± 0.81^{b}	9.68 ± 3.50^{a}	9.87± 1.14	4.25± 0.56

Remarks: *Mean values within a column followed by the different letters are significantly different at $p < 0.05$ according to Duncan's Multiple Range Test.

Bioactive compounds can easily penetrate the cytoderm and cytomembrane, thereby maximizing the multi-path antibacterial mechanism through action on cellular intra proteins, enzyme inactivation, DNA destruction, metabolic disorders, and impaired bacterial energy production (Zhu *et al.,* 2021). Moreover, Li (2011) informed that essential oils pass through bacterial cell walls and membranes, disrupting the structure of various layers of polysaccharides, fatty acids, and phospholipids as well as bacterial permeability.

BPEO emulsion in this research included a strong antibacterial agent, based on Morales *et al.* (2003), the range of antibacterial inhibitory zone is divided into four types: weak (<5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (>20-30 mm). This inhibition zone is higher than Shahbazi (2019) who tried to make clove essential oil nanoemulsion and only has a 5.12 mm inhibition zone from the *E. coli* antibacterial test.

This result is similar to Moreira *et al.* (2005), who tested 10 types of essential oils: eucalyptus (*Eucalyptus globules*), tea tree (*Melaleuca alternifolia*), rosemary (*Rosmarinus officinalis*), mint (*Mentha piperita*), rosa moschata (*Rosa moschata*), clove (*Syzygium aromaticum*), lemon (*Citrus limonum*), oregano (*Origanum vulga*re), pine (*Pinus silvestrys*) and sweet basil (*Ocimum basilicum*) for 4 strains *E coli ATCC25158, E coli ATCC32922, E coli Cl, E coli Cll*. All of their results indicated that essential oil has an inhibition zone between 8-61 mm which is the average strong and very strong antibacterial agent. This result is similar to Mith *et al.* (2014) who tested various essential oils: cinnamon, oregano, and thyme showed strong antimicrobial *E. coli* O157:H7 S0575 an inhibition zone between 8-20 mm in various concentrations.

Specifically, Li (2011) explained his research using electron microscopy and showed that when *E. coli* strain rr98089 was given oregano essential oil it caused cell membrane damage and loss of material or cell contents. Furthermore, the essential oil is able to make the membrane more permeable so that enzymes such as ATPase located in the membrane can be disrupted. This causes leakage of ions and other cell contents.

Morsy and El-Salam (2017) explained that the antibacterial and antifungal results of black pepper essential oil showed inhibitory activity against foodborne pathogens with MIC <10 g/ml. BPEO also showed a spectrum of antibacterial activity, as well as a wider zone of inhibition against *Escherichia coli* compared to the antibiotic Gentamicin, so black pepper essential oil was suitable to be used as a bio preservative agent. This result is similar to Leimann *et al*. (2009) who explained that bioactive compounds with antimicrobial activity were not degraded during the encapsulation process. Microencapsulate lemongrass EO presented the same MIC values to *E. coli*

(22.32 mg/ml) and to *S. aureus* (2.79 mg/ml) with and without encapsulation.

It means that the encapsulation process did not cause any deterioration in the essential's antimicrobial properties. The data also show that viscosity might influence the bioactive release from essential oil to the colloid because of nano encapsulation protection.

Probiotic-Antibacterial test

Lactobacillus is one of the bacteria which is considered for use as probiotics. Even though the addition of essential oil has antagonistic action on pathogenic and spoilage microorganisms, using EO in fermentation food may influence probiotic bacteria (Moritz *et al*., 2012).

Table 3 shows that different ratios of whey protein gave no significant effects ($p>0.05$) on the inhibition diameter zone for *L. casei.* The inhibition diameter zone between 4-5 mm. It means that encapsulation of WPI has not increased the antibacterial properties of probiotic bacteria. This inhibition zone is higher than clove essential oil nanoemulsion which only has an inhibition zone of 3.76 ± 0.02 for *L. casei* (Shahbazi, 2019).

Moreover, Shahbazi (2019) found that probiotic bacteria, *L. acidophilus, L. reuteri, L. casei, L. rhamnosus,* have a lower sensitivity to the clove essential oil emulsion (probiotic microorganisms inhibition zone = 2.57-4.44) than pathogenic bacteria: *S. aureus, B. subtilis, B. cereus L. monocytogenes, S. typhimurium, E. coli.*

This result is supported by Salama *et al.*, (2021), who utilized four nanoemulsion essential oils: spearmint, lemongrass, clove, and cinnamon, and tested using three strains of lactic acid bacteria as *L. helveticus*, *L. acidophilus,* and *B. bifidum lactis*. Those experiments proved that the probiotic bacteria could survive in the presence of the different nanoemulsions with bacterial count > 10^6 cfu/mL.

Table. 3 Analysis GC-MS Primary Constituent of Black Pepper Essential Oil DDistillers®

Constituent	Result
α-Pinene	9.79%
β-Pinene	11.74%
Delta 3-Carene	7.98%
Limonene	11.88%
Trans-Caryophyllene	25.73%

Data Source: PT. Syailendra Bumi Investama

These phenomena may be influenced by WPI character which is flexible to encapsulate any type of hydrophilic, hydrophobic, or viable probiotic cell (Abd El-Salam, & El-Shibiny, 2015). There is a possibility that milk protein gel formation gives double protection between essential oil bioactive and probiotic bacteria, this theory is supported Abd El-Salam, & El-Shibiny (2015) who explain that whey proteins (WP) are considered as a biomaterial for encapsulation of probiotics. Whey protein increases the resistance of probiotic *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* from acid and bile salts. Moreover, milk proteins are rich sources of bioactive peptides.

Those bioactive give a synergistic effect on the probiotic bacteria. Furthermore, the BPEO nanoemulsion could be an additive to meat and milk products. The effect of essential oil on meat products was studied by Badia *et al.* (2020). The interesting finding is both oregano essential oil and rosemary essential oil slowed the growth of the lactic acid bacteria but they did not change the maximum bacterial population on vacuum-packed Tuscan sausage. Salama *et al.* (2021) used essential oils nanoemulsion for the flavoring agent on functional stirred yogurt.

CONCLUSION

Based on the results of this research, we concluded that the increase ratio of WPI influences morphology, viscosity, antioxidant, and antibacterial properties of BPEO ultrasound-assisted emulsion. WPI can protect the BPEO's bioactive compound so the emulsion still has good antioxidant and antibacterial properties during storage and interactions with food components. It is also possible to use essential oil for fermented food products because this study indicated black pepper essential oil has lower sensitivity for *L. casei* than for *E. coli*.

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