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FULL PAPER

Prebiotic properties of fermented ceri Terengganu (*Lepisanthes fruticosa*) beverage by survival of lactic acid bacteria and its antibacterial activity

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Abstract

The present study was undertaken to investigate the survival of lactic acid bacteria (LAB) in the fermented ceri Terengganu beverage and its antibacterial activity. The preliminary study of the survival of five selected strains of LAB involved was *Bifidobacterium bifidum* UABb-10™, *Lactobacillus acidophilus* DDS®-1, *Lactobacillus paracasei* UALpc-04™, *Lactobacillus plantarum* UALp-05™, and *Streptococcus thermophilus* UASt-09™. The viability of each strain was tested in fermented ceri Terengganu beverage (pH 3.25) where all strains showed a survival rate of at least 92.5%. A total of five types of foodborne pathogens namely *Escherichia coli* O517:H7 UPMEC32, *Listeria monocytogenes* ATCC®51772™, *Salmonella enterica* serovar Enteritidis MDC15, *Salmonella enterica* serovar Typhimurium ATCC®53648™ and *Streptococcus gallolyticus* ATCC®9809™ were selected to determine the antibacterial activity and minimum bactericidal concentration ($MBC_{>99}$) of fermented ceri Terengganu beverage. Antibacterial activity using the agar well diffusion method inhibited five tested food-borne pathogens at varying extents of inhibition zone ranging between 12.92 to 18.50 mm in diameter. Another antibacterial activity assay using the broth microdilution method also confirmed the 100% inhibition effect of fermented ceri Terengganu beverage against these selected pathogenic microorganisms even though the beverage has been diluted to 50%. The synergetic effect of a significant amount of multiple organic acids present in fermented ceri Terengganu beverages was the main factor contributing to its potent antibacterial properties. This finding indicated the potential of fermented ceri Terengganu beverage as a prebiotic beverage and might be able to reduce the risk of food poisoning incidence as it has shown a good antibacterial effect against selected foodborne pathogens.

1. Introduction

Malaysia is a country rich with a variety of sources of fruits possessing diverse genetics. Currently, with more than 370 species of fruits being planted and growing wild, most fruits that are planted and grown are common fruits that have a commercial value (Rukayah, 1999; Osman, 2011). There are sixteen types of fruits that are classified as common fruits and grown extensively whereas fruits that are not grown on a commercial scale are classified as rare fruits (Rukayah, 1999; Osman, 2011). Underutilised or rare cultivated fruits refer to fruit species which are not grown on a commercial scale. One of the examples is ceri Terengganu which has been identified to be potential fruit of commercial importance.

Ceri Terengganu or its scientific name, *Lepisanthes fruticosa* (Roxb.) Leenh is a non-seasonal fruit (Salahuddin *et al*., 2017). *L. fruticosa* belongs to the Sapindaceae family and it has a widespread distribution throughout Peninsular Malaysia, Sarawak and Sabah in Malaysia, Kalimantan and Java in Indonesia, Thailand, Philippines, Indo-China and Myanmar (Lim, 2013; Salahuddin *et al*., 2017). In Malaysia, ceri Terengganu is widely distributed in the East Coast of Peninsular Malaysia in Terengganu, Johor and Pahang (Lim, 2013); growing on a medium-sized shady tree called the Johor tree or also known as the Perupok tree which can be

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found growing naturally in the forests. This species is cultivated occasionally and it is often used as a landscaping ornament and shade tree because of its aesthetic values due to its attractive shape, light purple young leaves and long violet inflorescence. *Lepisanthes fruticosa* fruits form clusters on long stalks giving a bright red, smooth and shiny skin (Figure 1). The size of the fruit is around 2 to 3 cm and it has a mix of tart, and sweet tastes its flowering season can happen throughout any time of the year.

Figure 1. Ceri Terengganu

Ceri Terengganu fruits are found in the open areas and are usually not large trees but shrubs the fruits are usually harvested and enjoyed by children (play with these fruits), and insects, being a source of food for birds and other animals. Based on ethnobotanical studies, ceri Terengganu is usually consumed as a food source and is also used in traditional medicine by rural folks (Salahuddin *et al*., 2017). Consequently, ceri Terengganu is lacking market demand and is sold at very low prices. Ceri Terengganu can be found at outdated stalls along village roads or local markets (*pasar tani*) since the fruit is not on sale in supermarkets. It was commonly overlooked by the developed population and considered to be low in prestige and nutritionally inferior to other comparable fruits. Currently, there is no industry specialising in ceri Terengganu processing in Malaysia and the fruit remains under-utilised by the general population. Therefore, a strategy has been made to use modern microbial precision fermentation technology to produce a new value-added fermented beverage from ceri Terengganu juice using a consortium of kombucha strains (Abdul Manan *et al*., 2020). Fermentation is an age-old art that is now teaming up with ground-breaking science. Through microbial precision fermentation, more active metabolites will be produced to enhance the nutritional value of the original substrates (Marco *et al*., 2021). Consequently, it is desirable to process fresh ceri Terengganu fruits into fermented beverages in order to avoid waste and increase their marketability.

Ceri Terengganu juice could serve as suitable media for cultivating a consortium of kombucha strains for fermented beverage production. The ceri Terengganu juice supplemented with sugar was allowed to ferment under aerobic conditions. The consortium of kombucha strains involves yeasts and acetic acid-producing bacteria that live together symbiotically to convert the sugared ceri Terengganu juice to form new fermented products through fermentation. The fermentation affected the physiochemical and sensory properties of the juices (Abdul Manan *et al*., 2020). With a focus on promising new value-added healthy products, the fermentation enhanced the antioxidant and antibacterial activities of the resulting product, also were the bioactive compounds, with the desirable overall properties of the fermented beverage. As the fermentation progresses, the taste of fermented ceri Terengganu juice changes from a pleasurably fruity, sour and tart taste, thus increasing the consumer acceptability of the flavour and other sensory aspects of the beverage. Ultimately, a pleasantly sweet and sour, slightly pungent, apple cider-like beverage is produced. The low pH of the beverage is attributed to the production of various organic acids during fermentation. *In vivo* studies showed that both fermented products had a non-toxicity effect (Abdul Manan *et al*., 2020).

Fermented beverages are being embraced by consumers who believe good digestive health influences both body and mind. Prebiotic is a term that means a substrate that is selectively utilized by microorganisms that benefit or confer a health benefit to humans and animals (Makwana and Hati, 2019). Whilst, this favourable fermentability property of the ceri Terengganu juice can be explored for prebiotic functional properties and their potential application as a functional healthy ingredient. Building on the non-dairy fermented beverages, fermented ceri Terengganu is catching on as a ready-to-drink beverage product. To the best of our knowledge, this is the first fermented beverage in Malaysia that has been successfully developed by MARDI using ceri Terengganu. The current study aimed to study the survival of selected lactic acid bacteria in the presence of fermented ceri Terengganu beverage to improve the number and variety of bacteria which supports better overall gut health. On top of that, this study also identified the antibacterial activity of fermented ceri Terengganu beverage towards selected foodborne pathogens.

2. Materials and methods

2.1 Fermented ceri Terengganu beverage

The fermented ceri Terengganu beverage produced at the Malaysian Agricultural Research and Development Institute (MARDI) under optimised conditions was used as the sample in this study. The fermentation of ceri

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Terengganu starts with a dilution of ceri Terengganu juice until a final 2.1-2.2% Brix value. This is followed by adding 15% of sugar to the juice, and pasteurisation at 90°C for 10 mins. The medium is then inoculated with 10% of the culture containing the consortium of kombucha strains. The juice at the initial pH of 5.25 was then incubated at 28°C for 21 days, under static and aerobic conditions, until achieved 0.9-1.0% total acidity and final pH of 3.25. Finally, the fermented ceri Terengganu juice (Brix 14%) was filtered using an ultrafiltration filter (0.22 um) to obtain a final product free of microorganisms. The final filtered product was kept in a cold room (4°C) prior to use in further analysis.

2.2 Determination of organic acids content

Analyses of the organic acids profile of fermented ceri Terengganu beverage phytonutrients were carried out with high-performance liquid chromatography (HPLC), Alliance Separation Module (Waters, 2695), equipped with a diode array detector (Waters, 2996). A 10 µL aliquot of sample solution was separated using Synergi 4 µm, Hydro-RP80A (250 × 4.6 mm) with the temperature controlled at 30°C. The mobile phase consists of mobile phase A (20 mM KH_2PO_4 with adjusted pH 2.9) and mobile phase B (water) with a flow rate of 0.6 mL/min. Gradient elution was performed as follows: from 0 to 30 min, 100% A; from 30 to 45 min, linear gradients from 100 to 0% A; from 45 to 55 min, linear gradient from 0 to 100% A. Peak identification was made by comparing retention times and UV spectra at 190, 210 and 254 nm with authentic organic acids compounds. Quantification was made using calibration curves obtained by injecting known amounts of pure organic acids as an external standard. All analyses were performed in triplicate.

2.3 Lactic acid bacteria and culture condition

A total of five strains of lactic acid bacteria (LAB), *Bifidobacterium bifidum* UABb-10™, *Lactobacillus acidophilus* DDS®-1, *Lactobacillus paracasei* UALpc-04™, *Lactobacillus plantarum* UALp-05™, and *Streptococcus thermophilus* UASt-09™ were obtained from UAS Laboratories, Edina, USA. Strains were sub-cultured twice on de Man-Rogosa-Sharpe (MRS) agar (Becton, Dickinson, Company, France) individually and incubated overnight at 37°C, 48 hrs. A loopful of LAB strain from each MRS agar was inoculated into MRS broth (Becton, Dickinson and Company, France) and incubated at 37°C for 18 hrs in the growth incubator (Labcon, USA).

2.4 Determination of lactic acid bacteria tolerance in fermented ceri Terengganu

A volume of 1 mL LAB cultures (OD 600 nm ~ 1.0) was harvested by centrifugation at 10 000 rpm for 5 min and washed with PBS pH 7.2 twice, before being re-suspended in fermented ceri Terengganu broth. The cultures were then incubated at 37°C for 0, 1, 2, 3 and 4 hrs, reflecting the time spent by food in the small intestine. After every interval sampling, the cells were immediately washed and re-suspended with 1.0 mL saline phosphate buffer (PBS) (Sigma-Aldrich, USA) pH 7.2 twice and seeded on MRS agar. Plates were incubated at 37°C for 48 to 72 hrs. The results were expressed as colony-forming unit per millilitre (CFU/mL) relative to the control sample (t = 0). The survival rate (%) was calculated using Equation 1 as the percentage of LAB colonies grown on MRS agar compared to the initial bacterial concentration (Mulaw *et al*., 2019):

$$\text{Survival rate (\%)} = \frac{\text{Final log CFU/mL}}{\text{Initial log CFU/mL}} \times 100 \quad (1)$$

where final log CFU/mL is the viable count of LAB after incubation and initial log CFU/mL is the initial viable count (t = 0).

2.5 Antibacterial activity of fermented ceri Terengganu beverage against foodborne pathogens

2.5.1 Foodborne pathogenic strain and culture condition

In this study, five types of foodborne pathogens: *Escherichia coli* O517:H7 UPMEC32, *Listeria monocytogenes* ATCC®51772™, *Salmonella enterica* serovar Enteritidis MDC15, *Salmonella enterica* serovar Typhimurium ATCC®53648™ and *Streptococcus gallolyticus* ATCC®9809™ were selected to determine the antibacterial activity using agar well diffusion method and minimum bactericidal concentration ($MBC_{>99}$) of fermented ceri Terengganu beverage. All bacterial cultures were revived from glycerol stock and streaked onto selective agar media as prepared according to the manufacturer's protocol and incubated at 37°C for 24 hrs.

2.5.2 Measurement of the inhibition zone of fermented ceri Terengganu beverage against foodborne pathogens

The antibacterial activity of fermented ceri Terengganu beverage against five selected food-borne pathogens was determined using the agar well diffusion method. Each pathogen was swabbed evenly over the surface of the Mueller-Hinton agar (Becton, Dickinson, Company, France) plate with a sterile cotton swab to give an inoculum level of 10^8 CFU/mL. A sterile pipette tip was used to make wells with a diameter of 6 mm. Approximately 15 µL of Mueller-Hinton broth was added to each well in order to seal it to avoid leakage. Then, 50 µL of the sample was added into the wells and

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allowed to diffuse onto agar for the first 60 mins in the chiller with a temperature of 20°C, followed by another addition of 50 µL of sample and allowed it to diffuse onto agar for another 60 mins in the chiller before incubation at 37°C for 24 hrs. The antibacterial effect was recorded by calculating the diameter of the inhibition clear zone that appeared. The chemical inhibitors, 1% of acetic acid (Acros Organics, Netherlands), tartaric acid, lactic acid, malic acid and antibiotic penicillin-streptomycin (Nacalai Tesque, Inc., Kyoto, Japan) were used as a comparison control.

2.5.3 Determination of minimum inhibitory concentration ($MBC_{>99}$) of fermented ceri Terengganu beverage against foodborne pathogens

Escherichia coli O517:H7 UPMEC32, *L. monocytogenes* ATCC®51772™, *S. enterica* ser. Enteritidis MDC15, *S. enterica* ser. Typhimurium ATCC®53648™ and *S. gallolyticus* ATCC®9809™ were cultured onto tryptone soy agar (Merck, Germany) individually and incubated at 37°C, 24 hrs. A loopful of food-borne pathogen culture from each tryptone soy agar was later inoculated into tryptone soy broth (Becton Dickinson, France) and incubated at 37°C for 16 hrs at the agitation rate of 160 rpm using a shaking incubator (Labcon, USA) to obtain the suspension culture with the initial colony count of 10^9 CFU/mL. $MBC_{>99}$ for each pathogenic strain were determined using the broth microdilution technique. Fermented ceri Terengganu beverage at different concentrations (12, 25, 50 and 100%) was used as an initial working concentration to determine its $MBC_{>99}$. Two wells served as control, consisting of pathogen culture without treatment and sterilized growth media. Each well contained 120 µL of the test material in serially descending concentration. An amount of 20 µL of inoculum containing about 1×10^9 CFU/mL foodborne pathogen suspension was added onto each well and was rotated using a microtiter plate shaker before being incubated at the temperature of 37°C for 24 hrs. After incubation, the serial dilutions were performed in the microcentrifuge tube using a micropipette and the plate count for each dilution was analysed in triplicate.

2.6 Statistical analysis

All experiments were repeated three times independently. The data were reported as mean±standard deviation (SD).

3. Results and discussion

3.1 Characterization of fermented ceri Terengganu beverage

During the fermentation process, sugared ceri Terengganu juice that first gets fermented into alcohol by yeast and then taken a step further and converted to acetic acid and other organic acids, which gives fermented ceri Terengganu beverage its tangy taste. It is not as easy as it sounds, as there are many microorganisms competing to ferment sugar substrates. The final fermented beverage must have the right amount of sweetness (sugar content) if carbohydrate is a label claim. Moreover, this fermented ceri Terengganu beverage is more tasteful after going through the microbial fermentation process with a consortium of kombucha strains. Table 1 shows the profile of organic acids produced in the final fermented ceri Terengganu beverage. The highest organic acid obtained from fermented ceri Terengganu beverage was L-malic acid (15,833.34±83.21 ug/mL), acetic acid (12,007.67±131.85 ug/mL) and tartaric acid (1,318.05±10.54 ug/mL). The remarkable increment of L -malic, acetic and tartaric acid in fermented ceri Terengganu beverage was produced by acetic acid bacteria indicating that this consortium of kombucha strains can adapt and grow well in ceri Terengganu juice media. Other organic acids identified were oxalic acid (403.19±3.30 ug/mL), succinic acid (350.03±9.69 ug/mL), lactic acid (188.58±40.22 ug/mL), glucuronic acid (100.06±0.52 ug/mL), galacturonic acid (100.01±2.44 ug/mL), ascorbic (11.44±0.05 ug/mL) and kojic acid (2.09±0.08 ug/mL) also exhibited an increment trend with fermentation days as shown in Table 1, except for citric acid (24.77±1.24 ug/mL). There is a decrease in citric acid content after the fermentation process.

The occurrence of organic acids, which lower the pH of the beverages, may also confer several health benefits. Health benefits associated with fermented beverages such as vinegar and fermented tea (kombucha) include antibacterial activity, antioxidant activity, modulation of the glycaemic response, positive effects on cardiovascular health, such as cholesterol-lowering and antihypertensive action, positive effects in weight loss, improvement of appetite, reduction of fatigue, and anticancer activity (Chen *et al*., 2016). Organic acids, primarily acetic acid, and polyphenols have been attributed as the main functional compounds in vinegar and fermented tea and are present in all varieties at varying levels (Chen *et al*., 2016). According to Gogineni *et al*., 2013, the composition of the organic acid is most correlated with the multiple health benefits of fermented tea beverage, rather than just a microbial-gut interaction.

L-malic, acetic and tartaric acids are important organic acids found in fermented ceri Terengganu beverages after the fermentation process. L-malic acid is mainly used in food and applications including candy and beverages. This acid has antibacterial activity and

Table 1. Profile of organic acids content in fermented ceri Terengganu beverage

Organic acids	Unfermented ceri Terengganu juice (µg/mL)	Fermented ceri Terengganu beverage (µg/mL)
Glucuronic acid	ND	100.06±0.52
Galacturonic acid	ND	100.01±2.44
Oxalic acid	18.91±0.2	403.19±3.30
Tartaric acid	Not Detected	1,318.05±10.54
L-malic acid	Not Detected	15,833.34±83.21
Lactic acid	Not Detected	188.58±40.22
Acetic acid	192.93±0.08	12,007.67±131.85
Citric acid	7,585.3±15.09	24.77±1.24
Succinic acid	ND	350.03±9.69
Kojic acid	1.95±0.09	2.09±0.08
Ascorbic acid	Not Detected	11.44±0.05

Values are presented as mean ± standard deviation from triplicate analyses. ND: Not detected

confers special blending and flavour-fixing properties (Glodberg and Rokem, 2009). According to Tang *et al.* (2013), a combination of L-malic acid and citric acid was reported to have an important role in the therapy of ischemic heart disease. Acetic acid is known to improve many conditions. It is able to lower cholesterol and triglycerides (Fushimi *et al*., 2006), has anti-hypertensive effects (Kondo *et al*., 2001), controls blood sugar (Mitrou *et al*., 2015), antibacterial properties (Wali and Abed, 2019), able to treat pseudomonal wound infection (Nagoba *et al*., 2013), helps prevent constipation (Wang *et al*., 2020), prevent and treat ulcers (Wang *et al*., 2017). Tartaric acid is lauded with antioxidant and anti-inflammatory properties that keep the immune system healthy and can act as a boosts immunity, excellent digestive aid and improve glucose intolerance (Gurtler and Mai, 2014).

The presence of glucuronic acid, one of the major primary metabolites in fermented tea beverages, is believed to improve detoxification aiding excretion through the kidneys by binding toxin molecules (Wang *et al*., 2014). Oxalic acid is a low molecular-weight organic acid produced by microorganisms that it can bind to minerals in the gut and prevent the body from absorbing them (Palmieri *et al*., 2019). Lactic acid improved digestibility, stimulation of peristalsis in the intestines, improved blood circulation, normalises acidity of the stomach acids, maintenance and balances the body's pH, reduction of the bad bacteria while maintaining the balance of the good, and increased nutrient absorption (Dufresne and Farnworth, 2000; Hati *et al*., 2019). Citric acid and ascorbic acid possess strong antioxidants and protect the body from damaging free radicals (Rostamzad *et al*., 2011), whilst, kojic acids are generally used as an antioxidant in food to act as a preservative (Burdock *et al*., 2001)

3.2 Survivability of lactic acid bacteria in fermented ceri Terengganu beverage

The lack of understanding of how consuming fermented foods is capable of affecting gut microbial communities. Fermentation is known to alter nutritional availability and biogenic compounds, including bioactive peptides, which might be expected to give an impact on the gut microbiota (Dimidi *et al*., 2019). The survival of five selected LAB with in vitro probiotic potential was studied at low pH (3.25) medium condition of fermented ceri Terengganu beverage which is vital for gastrointestinal tract condition. The survival rate of LAB in fermented ceri Terengganu beverages is shown in Figure 2. All of the five selected LAB were able to survive above 90% in the presence of fermented ceri Terengganu beverage. *L. plantarum* UALp-05™ was the most tolerant with 110.63% survival rate followed by *L. acidophilus* DDS®-1, *L. paracasei* UALpc-04™ and *B. bifidum* UABb-10™ with survival rates of 102.22%, 100% and 96.55%, respectively. Meanwhile, *S. thermophilus* UASt-09™ was the least tolerant with a 92.5% survival rate.

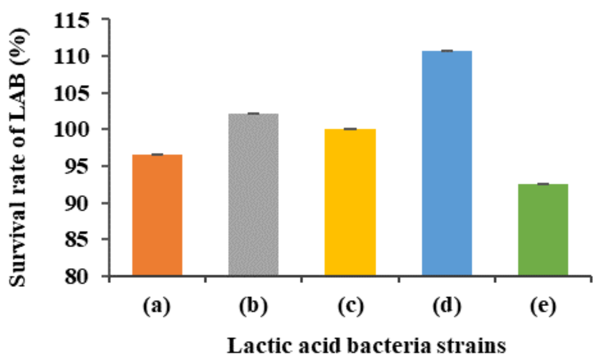

Figure 2. Survival rate of LAB in fermented ceri Terengganu beverage. Results are expressed in percentage (%). (a): *B. bifidum* UABb-10™, (b): *L. acidophilus* DDS®-1, (c): *L. paracasei* UALpc-04™, (d): *L. plantarum* UALp-05™ and (e): *S. thermophilus* UASt-09™. Values are presented as mean±standard deviation from triplicate analyses.

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Prebiotic food including fermented food encourages the growth of probiotic bacteria whereas the combination of prebiotics and probiotics generates a higher potential for a synergistic effect (Florou-Paneri *et al*., 2013). The result has shown that fermented ceri Terengganu beverage was able to give a positive effect by encouraging the growth of all LAB. More than 90% survival rate was shown by all tested LAB in fermented ceri Terengganu beverages despite the fermented beverage having a low pH of 3.25. More interestingly, there were increments in the microbial count of *L. acidophilus* DDS®-1 and *L. plantarum* UALp-05™ after 4 hrs incubation period. This is due to the presence of various nutrients or biogenic compounds such as sugars content in fermented ceri Terengganu beverage, which are sucrose, glucose and fructose that act as a carbon source and minerals such as magnesium, potassium and calcium to support their survival and growth of LAB (unpublished data). Compared to unfermented juice, fermented ceri Terengganu has high nutritional characteristics due to the activity of enzymes and microorganisms during the fermentation process.

This study showed that fermented ceri Terengganu beverages have the prebiotic potential that supports the growth of LAB. Rezac *et al.* (2018) have reported that the most commonly consumed fermented products including fermented sausage, fermented vegetables and fermented cereal products contained 10^5 – 10^7 CFU/mL of LAB per millilitre or gram. Rettedal *et al.* (2019) reported that the consumption of fermented milk products containing active beneficial bacteria has shown an increased survival of the bacteria within the gut microbiota. These findings have substantiated the ability of fermented ceri Terengganu beverage to be a potential prebiotic and support the growth of beneficial bacteria.

Many prebiotic products currently belong to dairy product categories, but lactose intolerance and the cholesterol content of these products might discourage consumers. Therefore, interest is increasing in the possibility of using fruits and vegetables for the preparation of prebiotic products, with the industry concentrating on the fermentation of cucumbers, cabbages and olives using LAB thus far (Blana *et al*., 2014; Blana *et al*., 2016). In order to grow, different LAB require different amino acids and vitamins, and some probiotic strains are able to grow in fruit matrices. Increasing awareness of the link between diet and health results in a change in food choices among consumers. The major functional food market comprises food fortified with prebiotics, probiotics and synbiotics. There are likely to be additional health benefits resulting from the addition of probiotic bacteria to fruits and vegetables that have already contained large amounts of bioactive substances (Blana *et al*., 2014; Blana *et al*., 2016).

3.3 Antibacterial activity of fermented ceri Terengganu on foodborne pathogens by measurement of inhibition zone

A total of five foodborne pathogenic microorganisms were selected to examine the inhibitory activities of fermented ceri Terengganu beverage, which were *E. coli* O517:H7 UPMEC32, *S. enterica* ser. Typhimurium ATCC®53648™, *S. enterica* ser. Enteritidis MDC15, *L. monocytogenes* ATCC®51772™ and *S. gallolyticus* ATCC®9809™. Fermented ceri Terengganu beverage showed an inhibitory effect against all five foodborne pathogens by using an agar diffusion assay. After incubation, antibacterial activity was measured by the zone of inhibition. The diameter of inhibition zones showed that fermented ceri Terengganu beverage had an antibacterial effect against each tested food-borne pathogen (Figure 3). The average zones of inhibition by which the fermented ceri Terengganu beverage inhibited the growth of the tested foodborne pathogens ranged between 12.92 to 18.50 mm (Table 2).

Fermented ceri Terengganu beverage displayed the highest antibacterial activity against *S. enterica* ser. Typhimurium ATCC®53648™ with an inhibition zone of 18.50 mm in diameter. Meanwhile, fermented ceri Terengganu beverage showed a minimum inhibition zone with a diameter of 12.92 mm against *S. gallolyticus* ATCC®9809™ as shown in Table 2. Fermented ceri Terengganu beverage exhibited varying degrees of antagonism against *E. coli* O517:H7 (13.33±1.03 mm), *L. monocytogenes* ATCC®51772™ (14.75±1.75 mm), *S.*

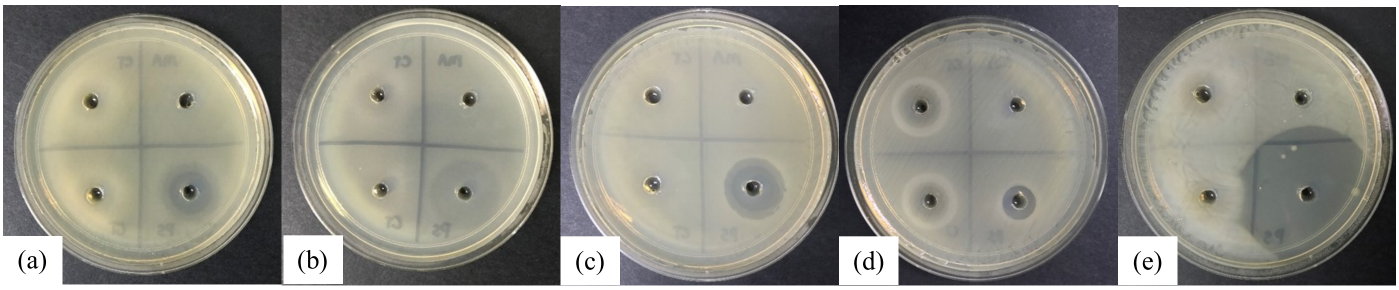

Figure 3. Inhibition zone exhibited by fermented ceri Terengganu beverage against five tested foodborne pathogens; (A) *E. coli* O517:H7 UPMEC32, (B) *L. monocytogenes* ATCC®51772™, (C) *S. enterica* ser. Enteritidis MDC15, (D) *S. enterica* ser. Typhimurium ATCC®53648™ and (E) *S. gallolyticus* ATCC®9809™.

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Table 2. Inhibition zone of fermented CT against selected foodborne pathogens by well diffusion assay

Pathogens	Diameter of inhibition zone (mm)±Standard Deviation						
	Fermented ceri Terengganu beverage	Penicillin Streptomycin (1%)	Mixture of acids (1%)	Acetic acid (1%)	Tartaric acid (1%)	Lactic acid (1%)	Malic acid (1%)
E. coli O517:H7	13.33±1.03	20.83±0.76	7.33±1.44	7.75±0.35	7.50±0.00	6.75±0.35	7.05±0.64
L. monocytogenes ATCC®51772™	14.75±1.75	11.17±0.29	8.83±2.75	ND	8.00±0.00	7.25±0.35	6.50±0.71
S. enterica ser. Enteritidis MDC15	15.92±1.86	20.00±0.00	6.67±0.76	4.00±0.00	8.00±0.00	7.50±0.71	7.00±0.00
S. gallolyticus ATCC®9809™	12.92±3.09	47.17±1.04	6.67±0.29	ND	11.50±2.12	14.00±0.00	13.00±1.41
S. enterica ser. Typhimurium ATCC®53648™	18.50±1.22	26.17±1.26	9.00±1.73	ND	8.50±0.71	7.00±1.41	7.00±1.41

Values are presented as mean±standard deviation from triplicate analyses. ND: Not detected

enterica ser. Enteritidis MDC15 (15.92±1.86 mm), *S. gallolyticus* ATCC®9809™ (12.92±3.09 mm) and *S. enterica* ser. Typhimurium ATCC®53648™ (18.50±1.22mm). According to Mulaw *et al*. (2019), a clearance zone of equal to or less than 9 mm indicates poor antibacterial activity while strong antibacterial activity is demonstrated by equal to or more than 12 mm diameter against the tested pathogens. Accordingly, fermented ceri Terengganu beverage exhibited strong antibacterial activity against all selected food-borne pathogens, where the highest antibacterial activity was shown by fermented ceri Terengganu beverage against *S. enterica* ser. Typhimurium ATCC®53648™ with a diameter of 18.50±1.22 mm inhibition zone. On the contrary, commercial chemical inhibitors (1% acetic acid) showed no inhibitory activities at all towards *L. monocytogenes* ATCC®51772™, *S. gallolyticus* ATCC®9809™ and *S. enterica* ser. Typhimurium ATCC®53648™ as confirmed in agar diffusion assay.

In general, the antibacterial activity of fermented ceri Terengganu beverage might be caused by the presence of antibacterial compounds such as organic acids, short-chain fatty acids, biophenolic, bioflavonoids and other biogenic compounds produced by a consortium of kombucha strains during fermentation of ceri Terengganu juice. According to Gurtler and Mai, (2014), tartaric acid is one of the organic acids known with the least antibacterial effect to inactivate fewer microorganisms and inhibit less microbial growth in comparison with other organic acids (including acetic, ascorbic, benzoic, citric, formic, fumaric, lactic, levulinic, malic, and propionic acids). The acetic acid present in fermented tea beverages has been reported to be involved in the antibacterial activity of the broth against pathogenic bacteria, thus providing protection against contamination of the tea fungus (Dufresne and Farnworth, 2000). According to Lynch *et al*. (2019), the antibacterial mechanism of fermented beverages produced by using acetic acid bacteria is primarily due to their acetic acid content. When the bactericidal effects of a number of organic acids, including lactic acid, acetic acid, citric acid, and malic acid on *E. coli* O157:H7 were investigated, acetic acid was found to be most effective, followed by lactic acid, citric acid, and malic acid. The antibacterial activity of organic acids is influenced by the target bacterial strain(s), temperature, pH, acid concentration, and ionic strength (Budak *et al*., 2014).

3.4 Minimum bactericidal concentration ($MBC_{>99}$) of fermented ceri Terengganu beverage against foodborne pathogens

To further investigate the capability of the antibacterial activity of fermented ceri Terengganu beverage, another antibacterial assay using the broth microdilution method was conducted to determine its $MBC_{>99}$. Different concentrations of fermented ceri Terengganu beverage were studied to determine the $MBC_{>99}$ against the five selected foodborne pathogens. $MBC_{>99}$ is identified by determining the lowest concentration of substrate that reduces the viability of the initial pathogen inoculum by 99.9%. The results showed that fermented ceri Terengganu beverage exhibited a different degree of growth inhibition rates against specific types of pathogenic microorganisms (Figure 4).

Overall, fermented ceri Terengganu beverage at 100% and 50% diluted concentration, revealed a 100% inhibition effect against these selected pathogenic microorganisms. Whilst at 25% diluted concentration of fermented ceri Terengganu beverage was capable to cause a 100% killing rate against *E. coli* O517:H7 and *S. enterica* ser. Typhimurium ATCC®53648™ (Figure 4a and Figure 4e). More interestingly, in fermented ceri Terengganu beverage at 12% diluted concentration, the antibacterial efficacy retained above 50% inhibition effect against all selected pathogenic microorganisms. These findings demonstrated that fermented ceri

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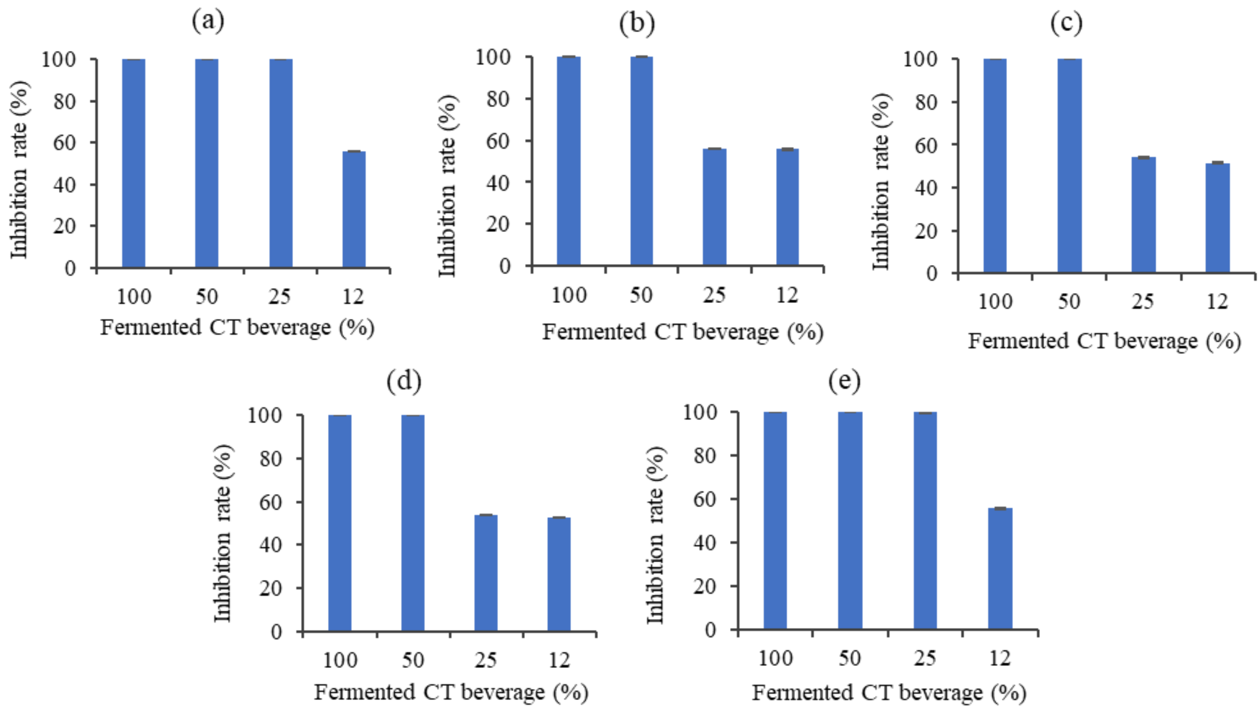

Figure 4. MBC$_{>99}$ of fermented ceri Terengganu (CT) beverage against five selected foodborne pathogens; (a): *E. coli* O517:H7; (b): *L. monocytogenes* ATCC®51772^{TM}; (c): *S. gallolyticus* ATCC®9809^{TM}; (d): *S. enterica* ser. Enteritidis MDC15; (e): *S. enterica* ser. Typhimurium ATCC®53648^{TM} at different concentrations. Each value in the graph represents the mean±standard deviation from triplicate analyses.

Terengganu beverage exhibited an inhibitory effect adequately against all selected foodborne pathogens. As a comparison, no antibacterial inhibitory activity was observed in unfermented ceri Terengganu juice. One of the factors is the presence of a group of organic acids in fermented ceri Terengganu beverage after the fermentation process with a consortium of kombucha strains was probably the main contributing factor for its potent antibacterial inhibitory activity.

This occurrence indicated the presence of soluble solid compounds such as soluble organic acids and phenolic acids in the fermented beverage may contribute to its potent inhibition against food-borne pathogens (Corbo *et al*., 2014). A previous study reported that fermented papaya and mango leaves beverages showed a significant reduction of soluble solid content when it is diluted (Koh *et al*., 2017; Koh *et al*., 2019). Yeast and acetic acid bacteria that are involved in the fermentation process are presumed to break down the complex phytochemical structure of the fruit into functional bioactive metabolites that contribute to the antibacterial activity against selected foodborne pathogens (Liburdi *et al*., 2020). Previous research on fermented tea beverages has demonstrated their antibacterial efficacy against pathogenic microorganisms of both Gram-positive and Gram-negative origin. The antibacterial activity of fermented tea beverages is largely attributable to the presence of organic acids, particularly acetic acid, large proteins, and catechins (Sreeramulu *et al*., 2000; Jayabalan *et al*., 2014). According to Sreeramulu *et al*., (2001), acetic acid and catechins are known to inhibit a number of Gram-positive and Gram-negative microorganisms. The industry can expect more plant-based fermented beverages as the movement grows. To assist, fermented ceri Terengganu beverage introduced a portfolio of new cultures specially formulated for such products.

4. Conclusion

This research is important in increasing the use and value of ceri Terengganu through the fermentation process to produce a product that has both probiotics and antibacterial properties. The fermented ceri Terengganu beverage exhibited prebiotic potential as all selected lactic acid bacteria showed a survival rate of more than 90% in the presence of fermented ceri Terengganu. This result offers insights into the effects that fermented ceri Terengganu has on the prebiotic properties. The fermented ceri Terengganu has also been found to exhibit antibacterial activity against all tested food-borne pathogens where it displayed the largest inhibition zone on *S.* Typhimurium ATCC®53648^{TM} with 18.50 mm of diameter. Additionally, fermented ceri Terengganu beverage was capable to cause 100% killing rate against *E. coli* O517:H7 and *S.* Typhimurium ATCC®53648^{TM} with MBC$_{>99}$ value of 25%. Future studies on the nutrient composition of the fermented ceri Terengganu beverage that can impose health benefits and the influence that product has towards as functional beverage, benefits for gut microbiota and human health,

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in general, is much needed. This study has revealed that the fermented ceri Terengganu beverage is a good source of bioactive compounds which make it a functional beverage.

Conflicts of interest

The authors declare no conflict of interest.

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