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Inhibition of Multi Drug-Resistant (MDR) *Escherichia coli* and *Klebsiella pneumoniae* by bacteriocin bifidobacteria and the viability of selected bifidobacteria encapsulated with tapioca

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Abstract. *Oedjijono O, Kusharyati DF, Ryandini D, Pramono H. 2023. Inhibition of Multi Drug-Resistant (MDR)* Escherichia coli *and* Klebsiella pneumoniae *by bacteriocin bifidobacteria and the viability of selected bifidobacteria encapsulated with tapioca. Biodiversitas 24: 4175-4182.* This study was conducted to investigate the antimicrobial efficacy of eight *Bifidobacterium* spp. against multidrug-resistant (MDR) bacteria, namely *Escherichia coli* and *Klebsiella pneumoniae.* Additionally, the viability of selected encapsulated bifidobacteria was assessed at different storage durations. The non-neutralized or neutralized supernatant pH of eight bifidobacterial isolates (BCC6, BC4, BBP6, BC7, BBP1, BC13, BCC5, BCC8) inhibited the growth of MDR *E. coli* and *K. pneumoniae.* The non-neutralized supernatant *Bifidobacterium* sp. BBP6 resulted in the highest inhibition against *E. coli* or *K. pneumoniae* with a clear zone of 8 mm. In contrast, the neutralized supernatant of *Bifidobacterium* sp. BBP1 showed the highest inhibition only against *E. coli*, with a clear zone of 8 mm. The bacteriocin of BCC5 and BBP6 isolates showed the highest inhibition against MDR *E. coli*, with inhibition zones of 12.5 mm and 12 mm, respectively. Similarly, the bacteriocin of BBP6 isolate gave the highest inhibition against *K. pneumoniae*, with a zone of 7 mm. The cell numbers of the tapioca-encapsulated isolate BBP6 were quite stable in comparison to BBP1, which were 8.4 log cfu.g^{-1} and 8.1 log cfu.g^{-1} at initial storage and 6.32 log cfu.g^{-1} and 7.41 log cfu.g^{-1} after eight weeks. The viability percentage of encapsulated *Bifidobacterium* sp. BBP6 (86.91%) surpassed that of BBP1 isolate (78.41%) during the eight-week storage period at 4^{o}C. The storage time of the BBP6 at 4^{o}C was 6.05 weeks or 42.35 days.

Keywords: Bacteriocin, bifidobacteria, multidrug resistant bacteria, tapioca encapsulation

INTRODUCTION

Bifidobacteria are members of the lactic acid bacteria (LAB) family and synthesize various metabolites during their growth phase, and are generally recognized as safe (GRAS) and qualified presumption of safety (QPS) (Manoharan and Balasubramaniam 2022). These bacteria are used in different applications, such as nutraceuticals (functional food) and animal feed (silage). Furthermore, they are employed in the preservation of materials and the production of fermented beverages. Bifidobacteria, as probiotic microorganisms, typically reside within the intestines and stomach. The main characteristics of the bacteria are rod-shaped cells, non-motile, anaerobic, Gram-positive, non-spore-forming, and saccharophilic tendencies, resulting in the production of acetic acid and lactic acid from carbohydrates without concurrent CO_2 production (Turroni et al. 2019).

The resistance of pathogenic bacteria to the existing antibiotics occurs due to inappropriate use of antibiotics, such as inadequate doses of administration and irregular or discontinuous use. An alternative to replace the use of antibiotics in inhibiting MDR bacteria is the application of bacteriocin antimicrobial compounds produced by LAB (Dawwam et al. 2022). Moreover, probiotics have gained recognition for their potential to alleviate a range of ailments, including diarrhea, colitis, urinary tract infections, hypertension, allergies, and even certain forms of cancer. Probiotics have also shown efficacy in managing cholesterol levels and providing relief for individuals with lactose intolerance (Wang et al. 2022). Furthermore, they are involved in the transfer of antibiotic resistance genes to commensal or pathogenic bacteria present in the gastrointestinal tract.

Hendrati et al. (2017) isolated 17 strains from infant feces with caesar and premature delivery, and their metabolites showed inhibitory activity against the growth of *Escherichia coli* and *Salmonella typhimurium.* Efforts to develop probiotic bacteria need to pay attention to, among other things, the activity of the metabolites produced, medium composition, cultivation circumstances, and delivery methods. The factors that may have deleterious effects on probiotics during processing are low pH, high osmotic pressure, and high levels of oxygen (Ayama et al. 2014), and microencapsulation is a method to protect the inocula from their external environment and maintain the cells within an encapsulating matrix (Wang et al. 2022).

The viability of the probiotic inoculum is important to ensure that the bacteria are present in a viable form and at suitable population numbers. The probiotic products must shelf life until consumption and their high viability is maintained in the gastrointestinal tract. According to Shori (2022), several bioactive food components containing free cells reported poor survival due to the processing and

storage process as well as deleterious circumstances during transport through the gastrointestinal tract. An effort to protect probiotic bacterial cells from adverse environmental conditions was by encapsulation or immobilization techniques in the starch matrix (Hoh et al. 2021).

Encapsulation is needed to maintain high viability during development, protect bacteria from heat, oxygen, and moisture, and improve the flow properties during formulation development (Solanki et al. 2013). Probiotic products must contain at least 10^6-10^7 cfu of viable bacteria per g of the product at the time of its consumption to exert beneficial effects on human health (FAO/WHO 2002). Bifidobacteria ingested as probiotics are to improve the intestinal microflora balance, which in turn, improves the intestinal environment and contributes to the overall health of the intestine (Lim and Shin 2020).

The encapsulation materials commonly used are starch, gum arabic, carrageenan, alginate, and other ingredients (Pankasemsuk et al. 2016; Aghajani et al. 2019; Thangrongthong et al. 2020). Cassava products are widely recognized as valuable sources of resistant starch, which contributes to their favorable impact on the gastrointestinal tract (Pereira and Leonel 2014). Consequently, the potential utilization of cassava, commonly known as tapioca, as a material for encapsulating probiotics is promising. The flour presents as white granules with diameters ranging from 4 to 35 μm, with an average size of 20 μm. Tapioca starch exhibits high viscosity and boasts a significant amylopectin content, rendering the resulting gel resistant to freezing (Khumpouk et al. 2022). A specific portion possesses resistance to chemical treatment, enhancing its ability to safeguard encapsulated probiotic bacteria. Consequently, this protective effect ensures a high level of cell viability under extreme environmental conditions within the digestive tract. The encapsulation of bifidobacteria using tapioca starch has not been previously reported concerning extended storage periods.

MATERIALS AND METHODS

The isolates of bifidobacteria were from culture collections of the Microbiology Laboratory, Biology Faculty, the University of Jenderal Soedirman, Banyumas, Indonesia. Culture stocks of BC4, BC5, BC6, BC7, BC8, BC13, BBP1, and BBP6 were isolated by Hendrati et al. (2017). They were activated in 10 mL De Man Rogosa Sharpe Broth (MRSB) medium at pH 5.7, and incubated at 37ºC for 24 hours. Confirmation of the bifidobacteria isolates characteristics included Gram staining and cell morphology.

Assay of bifidobacteria cell-free supernatant against MDR *E. coli* and *K. pneumoniae*

The assay on the ability of bifidobacteria cell-free supernatant against MDR *E. coli* and *K. pneumoniae* was conducted experimentally using a completely randomized design. In addition, treatments were in the form of neutralized or non-neutralized bacterial isolate supernatant and each treatment was replicated three times. Data were analyzed using analysis of variance (ANOVA) and further analysis using honestly significance difference (HSD) was performed when significant difference among treatments was observed.

The assay referred to the Kirby's Bauer method, where the isolate (5% v/v) with a population density of 10^8 cfu. mL^{-1} was inoculated into a 10 mL MRSB medium, and incubated for 18 hours at 37ºC (Kusmarwati et al. 2014, with modification). The culture was then centrifugated in a shaker at 10,000 rpm for 10 minutes. The cell-free supernatant was divided into neutralized and non-neutralized supernatant. About 20 μL of each supernatant was pipetted onto a sterile 6 mm disc, and the disc was placed on Nutrient Agar (NA) medium inoculated with *E. coli* or *K. pneumoniae*. Subsequently, the plates were incubated at 37ºC for 24 hours. Production of the inhibition zone around the disc was measured and calculated by the formula of D (mm) = (D1 + D2)/2 (D: total diameter of inhibition zone, D1: vertical diameter of inhibition zone, D2: horizontal diameter of inhibition zone).

Production of bacteriocin by *Bifidobacterium* sp. using the ammonium sulfate method

A loopful of bacterial culture was inoculated into a 10 mL MRSB medium and incubated at 37ºC for 18 hours. About 1 mL (1%, v/v) of bacterial inoculum was transferred into a 99 mL MRSB medium and incubated in a shaker incubator with 150 rpm at 37ºC for 24 hours. The culture was then centrifugated at 10,000 rpm and 4ºC for 10 minutes. The supernatant was separated using a salting out method by adding ammonium sulfate (Martinez et al. 2013; Lei et al. 2020). Furthermore, 100 mL supernatant was added incrementally with ammonium sulfate (50%), and the solution was homogenized using a magnetic stirrer until saturated. The bacteriocin protein then precipitated with the incremental addition of ammonium sulfate. Meanwhile, the precipitated protein was separated from the solution by centrifugation at 10,000 rpm and 4ºC for 15 min. The resulting precipitate was diluted with 0.1 M phosphate buffer pH 5.3 at a volume of 2 mL, and the solution produced was bacteriocin. Each bacteriocin solution was assayed for inhibition activity against the bacteria tested.

Inhibition assay of bacteriocin against MDR bacterial tested

Bacterial cultures of MDR *E. coli* and *K. pneumoniae* were sub-cultured in a medium of Nutrient Broth (NB) and incubated at 37ºC for 8 hours. In addition, 0.1 mL of the culture was spread and inoculated onto an NA medium. A disc previously dropped with 20 μL bacteriocin was placed onto the NA medium inoculated with *E. coli* or *K. pneumoniae* and the plates were incubated at 37ºC for 24 hours. The inhibition zone produced around the disc was measured and calculated with the formula above (Oguntoye et al. 2021).

Encapsulation of bifidobacteria with tapioca and effect of storage time

Encapsulation of probiotic bacteria was performed by extrusion method (Purwandhani et al. 2007). The composition of the encapsulate was fish flour, tapioca, rice bran, probiotic *Bifidobacterium* sp., and water in a ratio of 4:2:1:2:3. The bacterial culture (10^8 cfu. mL^{-1}) and tapioca flour were mixed, then the materials were added and homogenized. Encapsulated probiotic cells were manufactured using a pellet machine (STAR 32) equipped with a mesh of 2.0 mm in size. Subsequently, the resulting pellets were dried in an oven (hot air oven) at 37°C for 5 hours. The dry pellets were then put into plastic clips, and stored in a chiller (4°C), for 8 weeks.

Viability of encapsulated bifidobacteria

A measurement of viable bacterial cells in the pellets was carried out using a total plate count method (Puspawati et al. 2010), with modification). Each 1 mL of bacterial suspension before encapsulating and 1 g of the pellet was added into 9 mL of sterile distilled water (10^{-1} dilution) to make serial dilutions until 10^{-6}. Furthermore, 1 mL of the last two dilutions (10^{-5}-10^{-6}) were inoculated into a Petri plate with 18 mL of De Man Rogosa Sharpe Agar (MRSA) medium and homogenized. The plates were incubated at 37°C for 48 hours, and the measurement of bacterial viability was expressed in percent (%) as in the following formula.

$$\text{Viability (\%)} = \frac{\text{Log total of the bacteria after treatment}}{\text{Log total of the bacteria before treatment}} \times 100\%$$

Measurement of storage time

Determination of storage time of the cells (t) is in cfu.g^{-1}. h^{-1} and cell death rate (k) until it reaches the number of encapsulated cells of 10^6 cfu.g^{-1} by using the following equation (Yulinery and Nurhidayat 2012).

$$[\text{Log } N_2 - \text{Log } N_1] = k(t)/2.303$$

Where:

Log N_1 : Logarithm of mean viability after encapsulation

Log N_2 : Logarithm of mean viability 8 weeks after encapsulation

k : The death rate per hour

t : Storage time

RESULTS AND DISCUSSION

Confirmative test of the bacteria showed that the shape of the cell was bifurcated Y, short rods, curve, non-spore-forming, non-motile, and Gram-positive (Figure 1). These properties were the characteristics of the species member of the genus *Bifidobacterium* (Martinez et al. 2013; Kusharyati et al. 2020). A wide range of bacteria belonging primarily to the genera *Bifidobacterium* and *Lactobacillus* have been characterized with different health-promoting attributes (Darbandi et al. 2022), and many strains could produce bacteriocins or antibacterial proteins highly effective against foodborne pathogens such as *Staphylococcus aureus*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, *Listeria monocytogenes*, *Escherichia coli*, and *Clostridium botulinum*.

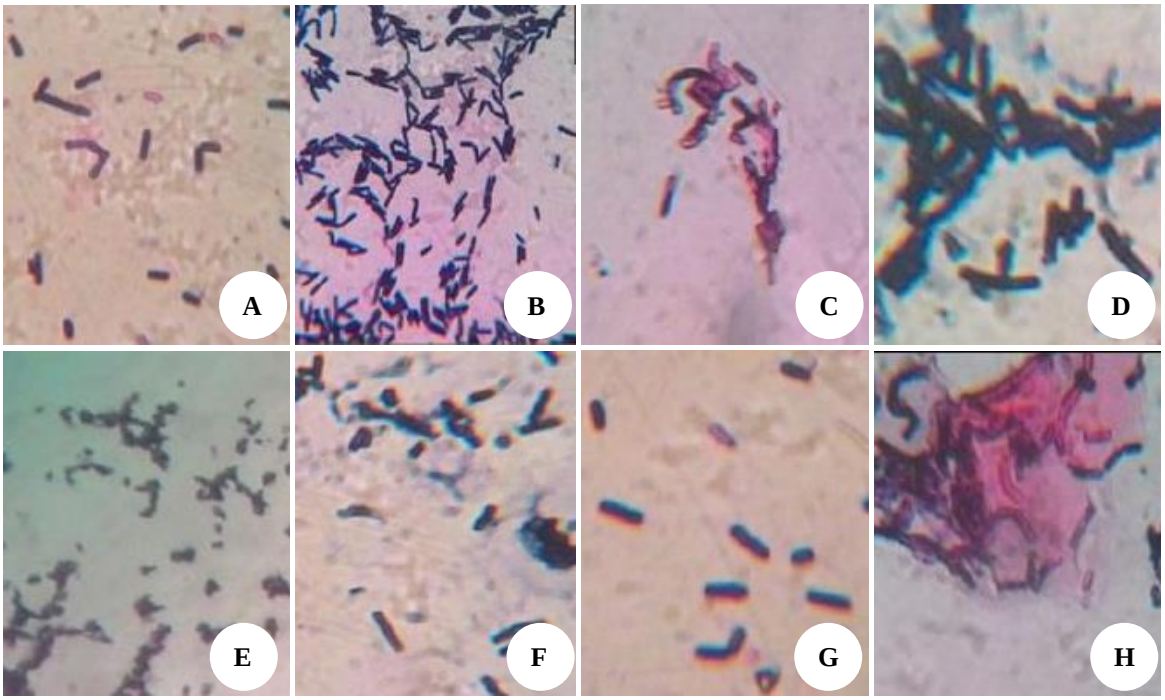

Figure 1. Cell characteristic of *Bifidobacterium* spp. A. isolate BCC6, B. isolate BC4, C. isolate BBP6, D. isolate BC7, E. isolate BBP1, F. isolate BC13, G. isolate BCC5, H. isolate BCC8. Observed under a light microscope at a magnification of 1,000 x

Inhibition of bifidobacteria cell-free supernatant against MDR *E. coli* and *K. pneumoniae*

The result of the ANOVA on the inhibition test against pathogenic bacteria showed that both supernatants from all isolates were capable of significantly inhibiting the growth of *E. coli* and *K. pneumoniae* (P>0.05). The results indicated that all isolates of bifidobacteria produced antimicrobial substances such as antibiotics, organic acid, bacteriocin, or other antimicrobial substances (Tables 1 and 2, Figure 2).

Cell-free supernatant of *Bifidobacterium* sp. BBP6 without pH neutralization resulted in the highest inhibition zone against MDR pathogens of *E. coli* and *K. pneumoniae* in comparison to the other treatments based on HSD analysis. The unneutralized supernatant isolate BBP6 resulted in the highest inhibition zones against *E. coli* or *K. pneumoniae* with a clear zone of 8 mm. Meanwhile, the BC7 isolate gave an inhibition zone of 8 mm only against *K. pneumoniae*, as shown in Table 1. The neutralized supernatant of BBP1 reported the highest inhibition against *E. coli* with a clear zone of 8 mm. The growth inhibition of *E. coli* and *K. pneumoniae* by unneutralized or neutralized supernatants of *Bifidobacterium* spp. indicated that the bacteria produced antibacterial substances such as organic acids and bacteriocin.

According to Martinez et al. (2013), bifidobacteria belong to heterofermentative LAB known to produce higher acetic acid. These organic acids also affect the growth of bacterial pathogens and can be bacteriostatic and bactericidal depending on the characteristics, types, and strains of the tested bacteria. In addition, the mechanism of inhibition of bacteria by organic acids is related to acid-base balance, proton changes, and energy production by cells (Guan and Liu 2020). The acid-base balance in microbial cells is indicated by a pH close to normal. According to Wang et al. (2021), acids can cause a decrease in pH, effectively falling below the range conducive for bacterial growth. These acids exhibit rapid diffusion into microorganism cells. Low pH renders organic acids soluble in lipids (liposoluble), which are essential constituents of the cell membrane. Consequently, the acids can readily penetrate the cell cytoplasm.

The pH-neutralized supernatant of *Bifidobacterium* spp. also inhibited the growth of *E. coli* or *K. pneumonia,* and the process was low (Table 1). In contrast, MDR *E. coli* was more susceptible to the supernatants of *Bifidobacterium* spp. compared to *K. pneumoniae*. The highest inhibition against *E. coli* was shown by the neutralized supernatant of the BBP1 isolate. Bifidobacteria were capable of producing antimicrobial compounds in the form of organic acids through heterofermentation, as well as bacteriocin peptides. These compounds exhibited inhibitory effects on the growth of other bacteria (Martinez et al. 2013).

Inhibition of bacteriocin *Bifidobacterium* spp. against MDR bacterial tested

The results showed that bacteriocins produced by *Bifidobacterium* spp. had inhibitory activity against MDR *E. coli* and *K. pneumoniae*. According to Table 2 and Figure 2, the bacteriocin produced by the isolates BCC5 and BBP6 exhibited the highest inhibition against MDR *E. coli*, with a zone of inhibition measurements of 12.5 mm and 12 mm, respectively. In contrast, the bacteriocin produced by BBP6 showed the highest inhibition against *K. pneumoniae*, with an inhibition zone of 7 mm. These findings suggested that MDR *K. pneumoniae* tended to exhibit greater resistance compared to MDR *E. coli*. According to Martinez et al. (2013) *Bifidobacterium lactis* and *Bifidobacterium longum* inhibited the growth of *Staphylococcus aureus, Bacillus cereus*, and *Salmonella typhi*. Hasan et al. (2019) reported that bacteriocin produced by *Lactobacillus casei* inhibited multidrug resistant pathogenic bacteria such as *S. aureus, K. pneumoniae*, and *Proteus vulgaris* with inhibition zones of 21, 19, and 19 mm, respectively. Meanwhile, the isolate from *B. subtilis* showed great antimicrobial activity against *Streptococcus pyogenes, Salmonella typhi,* and *Pseudomonas aeruginosa* with inhibition zone diameters of 16.94, 12.50, and 11.45 mm, respectively (Ghazaei 2022). Perez et al. (2014) stated that bacteriocin was used as an alternative to antibiotics in the suppression of clinical bacteria including multidrug-resistant bacteria. This non-antibiotic antibacterial protein was proposed as potential therapeutic agent for the control of Gram-negative species such as *Escherichia, Pseudomonas,* and *Salmonella* (Denkovskienė et al. 2019).

Table 1. The average inhibition zone diameter produced by unneutralized or neutralized supernatants of *Bifidobacterium* spp. against MDR *E. coli* and *K. pneumoniae*

Treatments	Average of inhibition zone diameter (mm)	
	Unneutralized supernatant[1]	Neutralized supernatant[2]
B1 M1	6.0 a	6.0 a
B2 M1	8.0 c	6.0 a
B3 M1	6.5 a b	7.0 b c
B4 M1	6.0 a	6.5 a b
B5 M1	6.0 a	8.0 d
B6 M1	6.5 a b	7.0 b c
B7 M1	6.5 a b	6.0 a
B8 M1	6.0 a	7.0 b c
B1 M2	6.0 a	6.0 a
B2 M2	8.0 d	6.0 a
B3 M2	7.0 b c	6.0 a
B4 M2	6.0 a	6.0 a
B5 M2	7.0 b c	6.0 a
B6 M2	7.0 b c	6.0 a
B7 M2	6.0 a	6.0 a
B8 M2	8.0 d	6.0 a

Note: [1]Numbers followed by the same letter do not differ significantly at HSD 5% of 0.948. [2]Numbers followed by the same letter do not differ significantly at HSD 5% of 0.5. B1: antimicrobial substance of *Bifidobacterium* sp. BCC8. B2: antimicrobial substance of *Bifidobacterium* sp. BBP6. B3: antimicrobial substance of *Bifidobacterium* sp. BC4. B4: antimicrobial substance of *Bifidobacterium* sp. BCC5. B5: antimicrobial substance of *Bifidobacterium* sp. BBP1. B6: antimicrobial substance of *Bifidobacterium* sp. BC13. B7: antimicrobial substance of *Bifidobacterium* sp. BCC6. B8: antimicrobial substance of *Bifidobacterium* sp. BC7. M1: MDR *Escherichia coli*. M2: MDR *Klebsiella pneumoniae*

Table 2. The ability of bacteriocins produced by *Bifidobacterium* spp. in inhibiting the growth of MDR *E. coli* and *K. pneumoniae*

Isolate code	Diameter of inhibition zone (mm)	
	E. coli	*K. pneumonia*
BCC8	9.5	6.0
BBP6	12.0	7.0
BC4	9.0	6.5
BCC5	12.5	6.0
BBP1	6.0	6.5
BC13	9.5	6.5
BCC6	6.0	6.0
BC7	9.0	6.5

The results also showed that the degree of inhibition of the bacteriocin *Bifidobacterium* spp. on the growth of *E. coli* and *K. pneumoniae* was quite low because these MDR bacteria were Gram-negative. According to Pircalabioru et al. (2021), the level of resistance of Gram-negative bacteria to bacteriocins was higher than Gram-positive counterparts. The simple composition of Gram-positive bacteria cell walls made it easier for bacteriocin activity to penetrate the cell walls. The mechanism used to inhibit the growth of bacteria was through porous formation in membranes and by inhibition of cell wall synthesis (Perez et al. 2014). This porous formation caused cell leakage resulting in disturbed membrane stability (Sari et al. 2012). Based on the results, BBP6 and BBP1 were selected for further studies on their viability in the tapioca-encapsulated matrix.

Cells number and viability of encapsulated bifidobacteria

The results showed that the number of *Bifidobacterium* sp. BBP6 in initial storage (T0) was higher than *Bifidobacterium* sp. BBP1, namely 8.4 log cfu.g^{-1} and 8.1 log cfu.g^{-1}, respectively. Meanwhile, the amounts of *Bifidobacterium* sp. BBP1 at 8 weeks of storage was lower than isolate BBP6, which was 6.32 log cfu.g^{-1} and 7.41 log cfu.g^{-1}, as shown in Figure 3. The number of *Bifidobacterium* sp. BBP6 was more stable when stored for two to eight weeks than *Bifidobacterium* sp. BBP1. The results indicated that storage time affected the viability of bifidobacteria cells.

The growth of bacteria was influenced by water, minerals, and temperature. Each bacterium had an optimum temperature for its growth, and storage temperature affected the survival of bacteria (viability) (Triana and Yulinery 2015). Furthermore, storage time caused the number of cells to change (Fatmawati et al. 2013). The longer storage time caused the bacterial population to be inhibited because the cell components became inactive or died. Cell growth was supported by the complete availability of nutrients, maximum enzyme activity, and the absence of oxygen (O_2) in the environment. However, as the storage time increased, nutrient depletion occurred, enzyme activity decreased, and the bacteria were exposed to oxygen, hindering or inhibiting their growth (Dash et al. 2022). The type of isolate also affected the ability to grow bifidobacteria. The results showed that the cell number of *Bifidobacterium* sp. BBP6 was higher compared to the BBP1 isolate (Figure 3). The temperature of bacterial storage that was not suitable for the growth temperature decreased cell growth.

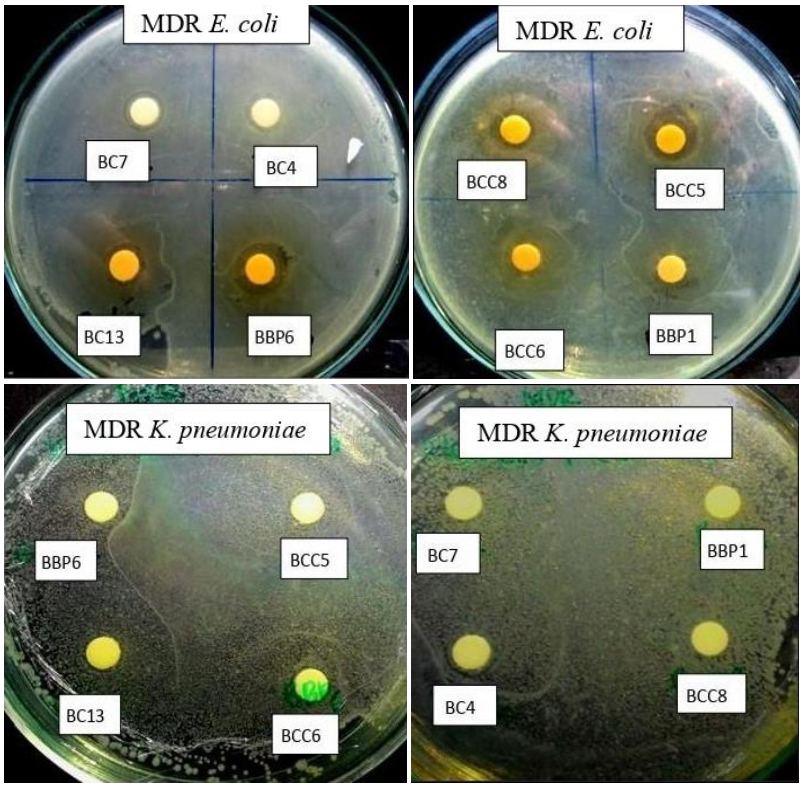

Figure 2. Inhibition of *E. coli* (*above*) and *K. pneumoniae* (*below*) by bacteriocin of *Bifidobacterium* spp.

Bacterial viability was determined by comparing the number of cells after and before storage. The results showed that the viability of encapsulated *Bifidobacterium* sp. BBP6 was higher in comparison to the encapsulated isolate BBP1 during 8 weeks of storage at 4°C. At 2 weeks of storage, the encapsulated BBP6 isolate exhibited a viability value of 86.91%, which remained stable until 8 weeks of storage at 88.67% (Figure 4). In contrast, the encapsulated BBP1 isolate showed a different pattern, with its viability value tending to decrease over time. The decrease in cell viability observed in the bacteria could be attributed to the effect of storage time, leading to reduced nutrient availability and decreased enzyme activity.

Additionally, the handling process during encapsulation and storage contributed to the decrease in viability. Bifidobacteria were known to be anaerobic, and exposure to oxygen during the encapsulation process adversely impacted their viability value. Exposure to oxygen during drying also reduced the number of cells because the element was toxic to LAB such as bifidobacteria (Talwalkar and Kailasapathy 2004). The harmful factor of oxygen for the low viability of encapsulated probiotics in yogurt was reported by Aghajani et al. (2019). According to Kawasaki et al. (2006) and Satoh et al. (2019), most of the *Bifidobacterium* species were sensitive to O_2. However, *B. boum* and *B. thermophilum* showed microaerophilic profiles.

The determination of storage time ensures the quality of products subjected to testing and distributed in the market. It is imperative to understand the impact of environmental factors, such as temperature and humidity, directly influencing the expiration date of these products. Conducting an assessment of the storage time of the product holds great significance in ascertaining the maximum duration for which the product remains beneficial to the consumer. The findings indicated that the storage time of the selected bifidobacteria varied under the rate of cell death. This rate of cell death is closely associated with the growth rate of the bacteria. The nutrients in the medium deplete quickly when bacterial growth is rapid (Yulinery and Nurhidayat 2012).

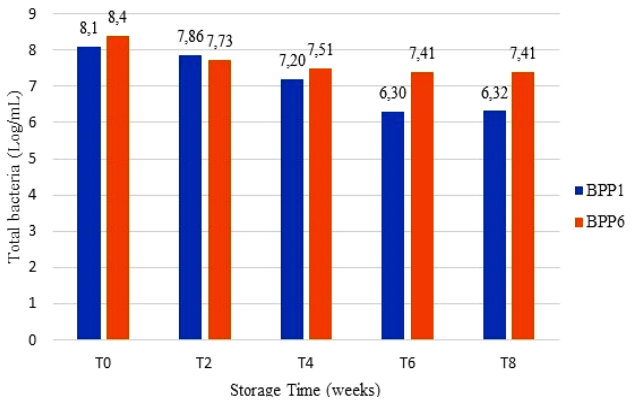

Figure 3. Cells number of encapsulated *Bifidobacterium* sp. BBP6 and *Bifidobacterium* sp. BBP1 during 8 weeks of storage at 4°C. T0: storage time 0 weeks; T1: storage time 2 weeks; T2: storage time 4 weeks; T3: storage time 6 weeks; T4: storage time 8 weeks

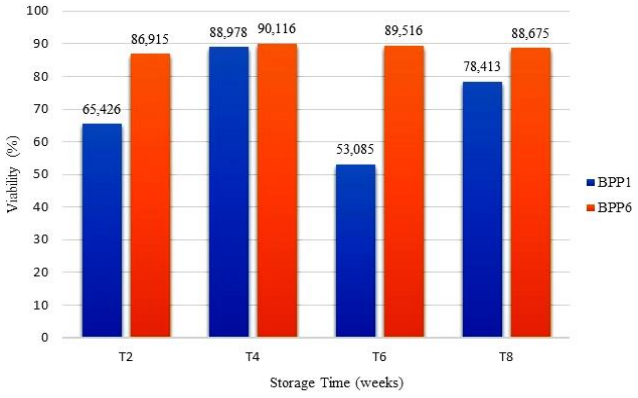

Figure 4. Cells viability of encapsulated *Bifidobacterium* sp. BBP6 and *Bifidobacterium* sp. BBP1 for 8 weeks of storage at a temperature of 4°C. T1: storage time 2 weeks; T2: storage time 4 weeks; T3: storage time 6 weeks; T4: storage time 8 weeks

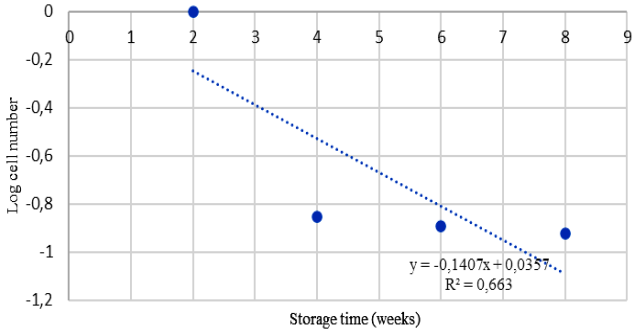

Figure 5. Relationship between the number of *Bifidobacterium* sp. BBP6 cells during 8 weeks of storage at 4°C

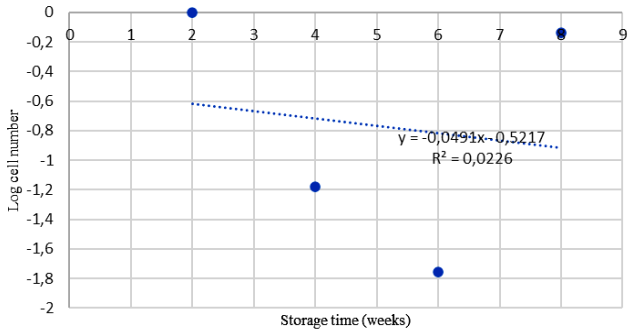

Figure 6. Relationship between the number of *Bifidobacterium* sp. BBP1 cells during 8 weeks of storage at 4°C

The death rate of *Bifidobacterium* sp. BBP6 and *Bifidobacterium* sp. BBP1 at a temperature of 4ºC with 8 weeks storage time is shown in Figures 5 and 6, respectively. The storage of BBP6 and BBP1 isolates at 4ºC resulted in the death rate of -0.1407 and -0.0491. Furthermore, the negative value showed that the cell number decreased. The BBP6 isolate exhibited the highest correlation coefficient value (R^2) of 66.3% at a temperature of 4ºC, coupled with the smallest error rate of 33.7%. This information enabled the determination and prediction of the storage time of the isolate. However, the encapsulated BBP1 isolate had a very low R^2 value of 2.29% with an error rate of 97.71%. Based on the results, the storage time of *Bifidobacterium* sp. BBP6 at 4ºC was 6.05 weeks or 42.35 days, while the BBP1 isolate was unpredicted due to the low correlation coefficient value. According to Triana and Yulinery (2015), exceeding the designated storage time limit of the test sample led to a reduction in probiotic cells in the preparation. Therefore, the consumption of probiotics must meet the minimum standard required to achieve the desired effect.

The encapsulation of probiotic cells is a process that protects the organism in various matrices. According to Orozco-Parra et al. (2020), a synbiotic film made from cassava starch and inulin showed a good result for *L. casei* preservation and delivery to the large intestine. Oguntoye et al. (2021) reported that the viability of *L. rhamnosus* GG in provitamin A cassava hydrolysates decreased rapidly within the first 30 days of storage, while encapsulated cells showed an insignificant decrease. This bacterium maintained viability above 5 log cfu. mL^{-1} and 7 log $cfu.g^{-1}$ in hydrolysates with free and encapsulated cells by the end of 60 days of storage. Furthermore, Shori (2022) reported that the increased survival of *Bifidobacterium* sp. in dairy beverages and foods during refrigerated storage, with active components such as plant phytochemicals, milk protein, inulin, and lactulose could be used to encapsulate the bacteria in processing. According to Lee et al. (2019), alginate-chitosan (Al/Chi)-encapsulated *L. acidophilus* KBL409 showed the highest survival rate after exposure to simulated gastric and intestinal fluids. The mucoadhesive abilities of alginate and Al/Chi-microspheres were higher than 94%, while the free cells *L. acidophilus* showed 88.1% mucoadhesion. Probiotic encapsulation of *L. brevis* ST-69 in alginate-nanocrystalline starch gel capsules reported a high survival rate at 94.97% of probiotic cells under simulated gastrointestinal conditions and during long-live storage at 4ºC compared to free cells (Thangrongthong et al. 2020). The addition of resistant starch and xanthan gum increased the microencapsulation efficiency for encapsulated *L. rhamnosus* GG in simulated gastric (pH 2.0, 2 hours) and intestinal juice (pH 7.5, 4 hours) (Hoh et al. 2021). The encapsulated bacterial probiotics with and without xanthan gum coating had higher survivability than free cells, indicating a positive role in promoting viability during gastrointestinal transit.

In conclusion, the highest inhibition of the antimicrobial substances against MDR-bacteria *E. coli* and *K. pneumoniae* was shown by *Bifidobacterium* sp. BBP6. The bacteriocin of the BBP6 and BBP1 isolate was potential against MDR *K. pneumoniae* and MDR *E. coli*, respectively. The different isolates and storage time affected the cell viability of the selected encapsulated bifidobacteria. Meanwhile, *Bifidobacterium* sp. BBP6 showed higher viability in comparison to *Bifidobacterium* sp. BBP1 during eight week-storage at 4ºC. Probiotic encapsulation also maintained the viability of the cells during storage.

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REFERENCES

Aghajani A, Nezhad HH, Mortazavi SA, Yazdi FT. 2019. Microencapsulation of probiotics in yogurt: A review. In: Intl Conf Eng Technol Innov.

Ayama H, Sumpavapol P, Chanthachum S. 2014. Effect of encapsulation of selected probiotic cell on survival in simulated gastrointestinal tract condition. Songklanakarin J Sci Technol 36 (3): 291-299.

Darbandi A, Asadi A, Ari MM, Ohadi E, Talebi M, Zadeh MH, Emamie AD, Roya Ghanavati R, Kakanj M. 2022. Bacteriocins: properties and potential use as nntimicrobials. J Clin Lab Anal 36: e24093. DOI: 10.1002/jcla.24093.

Dash KK, Fayaz U, Dar AH, Shams R, Manzoor S, Sundarsingh A, Deka P, Khan SA. 2022. A comprehensive review on heat treatments and related impact on the quality and microbial safety of milk and milk-based products. Food Chem Adv 1: 100041. DOI: 10.1016/j.focha.2022.100041.

Dawwam GE, Al-Shemy MT, El-Demerdash AS. 2022. Green synthesis of cellulose nanocrystal/ZnO Bio-nanocomposites exerting antibacterial activity and downregulating virulence toxigenic genes of food-poisoning bacteria. Sci Rep 12: 16848. DOI: 10.1038/s41598-022-21087-6.

Denkovskienė E, Paškevičius S, Misiūnas A, Stočkūnaitė B, Starkevič U, Vitkauskienė A, Hahn-Löbmann S. 2019. Broad and efficient control of *Klebsiella* pathogens by peptidoglycan-degrading and pore-forming bacteriocins klebicins. Sci Rep 9: 15422. DOI: 10.1038/s41598-019-51969-1.

FAO/WHO. 2002. Guidelines for the evaluation of probiotics in food. Guidelines for the Evaluation of Probiotics in Food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food. FAO Rome.

Fatmawati U, Prasetyo FI, Mega Supia TA, Utami AN. 2013. Karakteristik yogurt yang terbuat dari berbagai jenis susu dengan penambahan kultur campuran *Lactobacillus bulgaricus* dan *Streptococcus thermophillus*. Bioedukasi 6 (2): 1-9. [Indonesian]

Ghazaei C. 2022. Study of the effect of bacteriocin-producing *Bacillus subtilis* strains on beta-lactamase-producing pathogenic bacteria. J Clin Res Paramedical Sci 11 (2): e130208. DOI: 10.5812/jcrps-130208.

Guan N, Liu L. 2020. Microbial response to acid stress: mechanisms and applications. Appl Microbiol Biotechnol 104: 51-65. DOI: 10.1007/s00253-019-10226-1.

Hasan FB, Reza M, Al Masud HMA, Uddin MK, Uddin MS. 2019. Preliminary characterization and inhibitory activity of bacteriocin like substances from *Lactobacillus casei* against multi-drug resistant bacteria. Bangladesh J Microbiol 36 (1): 1-6. DOI: 10.3329/bjm.v36i1.44259.

Hendrati PM, Kusharyati DF, Ryandini D, Oedjijono O. 2017. Characterization of bifidobacteria from infant feces with different mode of birth at Purwokerto, Indonesia. Biodiversitas 18 (3): 1265-1269. DOI: 10.13057/biodiv/d180352.

Hoh PY, Lai KW, How YH, Pui LP. 2021. Microencapsulation of *Lactobacillus rhamnosus* GG with resistant starch and xanthan gum. Walailak J Sci Technol 18 (15): 9573. DOI: 10.48048/wjst.2021.9573.

Kawasaki S, Mimura T, Satoh T, Takeda K, Niimura Y. 2006. Response of the microaerophilic *Bifidobacterium* species, *B. boum* and *B. thermophilum*, to oxygen. Appl Environ Microbiol 72 (10): 6854-6858. DOI: 10.1128/AEM.01216-06.

Khumpouk P, Saichanaphan N, Khimmakthong U. 2022. The efficiency of polysaccharide microencapsulation in improving survival of probiotic bacteria. Songklanakarin J Sci Technol 44 (1): 184-190. DOI: 10.14456/sjst-psu.2022.27.

Kusharyati DF, Hendrati PM, Ryandini D, Manshur TA, Dewi MA, Khatimah K, Rovik A. 2020. Isolation of *Bifidobacterium* from infant's feces and its antimicrobial activity. Digital Press Life Sci 2: 00002. DOI: 10.29037/digitalpress.22326.

Kusmarwati A, Arief FR, Haryati S. 2014. Eksplorasi bakteriosin dari bakteri asam laktat asal Rusip Bangka dan Kalimantan. JPB Perikanan 9 (1): 29-40. DOI: 10.15578/jpbkp.v9i1.97. [Indonesian]

Lee YJ, Ji YR, Lee S, Choi M-J, Cho Y. 2019. Microencapsulation of probiotic *Lactobacillus acidophilus* KBL409 by extrusion technology to enhance survival under simulated intestinal and freeze-drying conditions. J Microbiol Biotechnol 29 (5): 721-730. DOI: 10.4014/jmb.1903.03018.

Lei S, Zhao R, Sun J, Ran J, Ruan X, Zhu Y. 2020. Partial purification and characterization of a broad-spectrum bacteriocin produced by a *Lactobacillus plantarum* Zrx03 isolated from infant's feces. Food Sci Nutr 8 (5): 2214-2222. DOI: 10.1002/fsn3.1428.

Lim HJ, Shin HS. 2020. Antimicrobial and immunomodulatory effects of bifidobacterium strains: A review. J Microbiol Biotechnol 30 (12): 1793-1800. DOI: 10.4014/jmb.2007.07046.

Manoharan M, Balasubramaniam TS. 2022. An extensive review on production, purification, and bioactive application of different classes of bacteriocin. J Trop Biodivers Biotechnol 7 (03): 72735. DOI: 10.22146/jtbb.72735.

Martinez FAB, Balciunas EM, Converti A, Cotter PD, Oliveira RPS. 2013. Bacteriocin production by *Bifidobacterium* spp.: A review. Biotechnol Adv 31 (4): 482-488. DOI: 10.1016/j.biotechadv.2013.01.010.

Oguntoye MA, Ezekiel OO, Oridupa OA. 2021. Viability of Lactobacillus rhamnosus GG in provitamin A cassava hydrolysate during fermentation, storage, in vitro and in vivo gastrointestinal conditions. Food Biosci 40: 100845. DOI: 10.1016/j.fbio.2020.100845.

Orozco-Parra J, Mejía CM, Villa CC. 2020. Development of a bioactive synbiotic edible film based on cassava starch, inulin, and *Lactobacillus casei*. Food Hydrocoll 104: 105754. DOI: 10.1016/j.foodhyd.2020.105754.

Pankasemsuk T, Apichartsrangkoon A, Worametrachanon S, Techarang J. 2016. Encapsulation of *Lactobacillus casei* 01 by alginate along with Hi-maize starch for exposure to a simulated gut model. Food Biosci 16: 32-36. DOI: 10.1016/j.fbio.2016.07.001.

Pereira BLB, Leonel M. 2014. Resistant starch in cassava products. Food Sci Technol 34: 298-302. DOI: 10.1590/fst.2014.0039.

Perez RH, Zendo T, Sonomoto K. 2014. Novel bacteriocins from lactic acid bacteria (LAB): Various structures and applications. Microb Cell Factories 13 (1): S3. DOI: 10.1186/1475-2859-13-S1-S3.

Pircalabioru GG, Popa LI, Marutescu L, Gheorghe I, Popa M, Barbu IC, Cristescu R, Chifiriuc M-C. 2021. Bacteriocins in the era of antibiotic resistance: Rising to the challenge. Pharmaccutics 13: 196. DOI: 10.3390/pharmaceutics13020196.

Purwandhani SN, Suladra M, Rahayu ES. 2007. Stabilitas thermal agensia probiotik *L. acidophilus* SNP2 terenkapsulasi metode ekstruksi dan emulsi. Prosiding Seminar Nasional Teknologi 2007, Yogyakarta, 24 November 2007. [Indonesian]

Puspawati NN, Nuraida L, Adawiyah DR. 2010. Penggunaan berbagai jenis bahan pelindung untuk mempertahankan viabilitas bakteri asam laktat yang diisolasi dari air susu ibu pada proses pengeringan beku. J Teknologi Industri Pangan 21 (1): 59-65. [Indonesian]

Sari RA, Nofiani R, Ardiningsih P. 2012. Karakterisasi bakteri asam laktat genus *Leuconostoc* dari pekasam ale-ale hasil formulasi skala laboratorium. JKK 1 (1): 14-20. [Indonesian]

Shori AB. 2022. Application of *Bifidobacterium* spp. in beverages and dairy food products: An overview of survival during refrigerated storage. Food Sci Technol 42: e41520. DOI: 10.1590/fst.41520.

Solanki HK, Pawar DD, Shah DA, Prajapati VD, Jani GK, Mulla AM, Thakar PM. 2013. Development of microencapsulation delivery system for long-term preservation of probiotics as biotherapeutics agent. BioMed Res Intl 2013: 1-21. DOI: 10.1155/2013/620719.

Talwalkar A, Kailasapathy K. 2004. A review of oxygen toxicity in probiotic yogurts: Influence on the survival of probiotic bacteria and protective techniques. Compr Rev Food Sci Saf 3 (3): 117-124. DOI: 10.1111/j.1541-4337.2004.tb00061.x.

Thangrongthong S, Puttarat N, Ladda B, Itthisoponkul T, Pinket W, Kasemwong K, Taweechotipatr M. 2020. Microencapsulation of probiotic *Lactobacillus brevis* ST-69 producing GABA using alginate supplemented with nanocrystalline starch. Food Sci Biotechnol 29 (11): 1475-1482. DOI: 10.1007/s10068-020-00812-9.

Triana E, Yulinery T. 2015. Uji stabilitas probiotik *Lactobacillus plantarum* Mar8 terenkapsulasi dalam sediaan oralit dengan analisis viabilitas. Pros Sem Nas Masy Biodiv Indon 1 (2): 278-282. DOI: 10.13057/psnmbi/m010218. [Indonesian]

Turroni F, Duranti S, Milani C, Lugli GA, van Sinderen D, Ventura M. 2019. *Bifidobacterium bifidum*: A key member of the early human gut microbiota. Microorganisms 7 (11): 544. DOI: 10.3390/microorganisms7110544.

Wang X, Gao S, Yun S, Zhang M, Peng L, Li Y, Zhou Y. 2022. Microencapsulating alginate-based polymers for probiotics delivery systems and their application. Pharmaceuticals 15 (5): 644. DOI: 10.3390/ph15050644.

Wang Y, Wu J, Lv M, Shao Z, Hungwe M, Wang J, Bai X, Xie J, Wang Y, Geng W. 2021. Metabolism characteristics of lactic acid bacteria and the expanding applications in food industry. Front Bioeng Biotechnol 9: 612285. DOI: 10.3389/fbioe.2021.612285.

Yulinery T, Nurhidayat N. 2012. Analisis viabilitas probiotik *Lactobacillus* terenkapsulasi dalam penyalut dekstrin dan jus markisa (*Passiflora edulis*). J Teknologi Lingkungan 13 (1): 109-121. DOI: 10.29122/jtl.v13i1.1411. [Indonesian]