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Supplementation of Sucrose and n-source in Culture Medium Towards Bacteriocin Production of Lactic Acid Bacteria Isolated from Ampel Bamboo Shoot (*Bambusa vulgaris*) Pickle

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Study on the use of sucrose in media for LAB from bamboo shoot is limited. The aim of this study was to determine the effect of sucrose in the culture medium to produce bacteriocins of LAB isolated from Ampel bamboo shoot pickle under different fermentation conditions. Fermentation of bamboo shoot was done in 2.5% of salt concentration at 15ºC for 5 days (condition A) and in 5.0% of salt concentration at 30ºC for 4 days (condition B). Isolation of LAB from the brine water of bamboo shoot fermentation yielded 35 isolates consisting of 16 isolates from condition A and 19 isolates from condition B. Antimicrobial activity analysis showed that all the isolates had antimicrobial activity against pathogenic bacteria (*E. coli* FNCC 0091, *L. monocytogenes* FNCC 0156 and *S. aureus* FNCC 0047). All isolates were grown in MRS-B media supplemented with sucrose as a carbon source and yeast extract and peptone as nitrogen source. The inhibitory activity of bacteriocin was between 13-3274 mm^2 ml^{-1}. The highest inhibition was found from isolate B16 grown in MRS-B supplemented with 2% sucrose against S. aureus FNCC 0047 (3274.99 mm^2 ml^{-1}). Thirteen showed bacteriocin production after inoculated in MRS-B with a combination of carbon and nitrogen source. From the 32 isolates in the present study, only 4 isolates A14, A17, A18 and B9 showed bacteriocin inhibitory activity against all the indicator pathogens *E. coli* FNCC 0091, *L. monocytogenes* FNCC 0156 and *S. aureus* FNCC 0047.

Keywords: bacteriocin, inhibitory activity, bamboo shoot, media supplementation

Penelitian tentang penggunaan sukrosa dalam media BAL dari rebung masih terbatas. Tujuan dari penelitian ini adalah untuk mengetahui pengaruh sukrosa dalam media kultur terhadap produksi bakteriosin BAL yang diisolasi dari acar rebung Ampel pada kondisi fermentasi yang berbeda. Fermentasi rebung dilakukan pada konsentrasi garam 2,5% pada suhu 15ºC selama 5 hari (kondisi A) dan pada konsentrasi garam 5,0% pada suhu 30ºC selama 4 hari (kondisi B). Isolasi BAL dari air garam fermentasi rebung menghasilkan 35 isolat yang terdiri atas 16 isolat kondisi A dan 19 isolat kondisi B. Analisis aktivitas antimikroba menunjukkan bahwa semua isolat memiliki aktivitas antimikroba terhadap bakteri patogen (*E. coli* FNCC 0091, *L. monocytogenes* FNCC 0156 dan *S. aureus* FNCC 0047). Semua isolat ditumbuhkan dalam media MRS-B yang dilengkapi dengan sukrosa sebagai sumber karbon dan ekstrak yeast serta pepton sebagai sumber nitrogen. Aktivitas penghambatan bakteriosin antara 13-3274 mm^2 ml^{-1}. Daya hambat tertinggi ditemukan pada isolat B16 yang ditumbuhkan pada MRS-B yang disuplementasi sukrosa 2% terhadap *S. aureus* FNCC 0047 (3274,99 mm^2 ml^{-1}). Tiga belas isolat menunjukkan produksi bakteriosin setelah diinokulasi pada MRS-B dengan kombinasi sumber karbon dan nitrogen. Berdasarkan 32 isolat dalam penelitian ini, hanya 4 isolat A14, A17, A18 dan B9 yang menunjukkan aktivitas penghambatan bakteriosin terhadap semua indikator patogen *E. coli* FNCC 0091, *L. monocytogenes* FNCC 0156 dan *S. aureus* FNCC 0047.

Kata kunci: bakteriosin, aktivitas penghambatan, rebung, suplementasi media

Bamboo shoots are one of the abundant food items in Indonesia. There are about 1,300 bamboo species in the world, and 145 of them are indigenous to Indonesia (Putra *et al.* 2013). Some bamboo species have high economic value and could be processed as an edible product. One of them is "Ampel" bamboo (*Bambusa vulgaris*) shoot. Fresh bamboo shoots are seasonal, perishable in nature and short-lived. Fermentation is one of the ways to preserve bamboo shoots, while the process also brings out typical taste as vegetable pickles. Bamboo shoots pickles are part of Indonesian diet, but aside from nourishment,

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the fermentation process opens more potential benefit in microbial technology. Fermentation of bamboo shoot can occur spontaneously by natural lactic bacterial surface microflora since such vegetable could provide a natural medium for lactic acid fermentation (Swain *et al.* 2014). Food fermentations by lactic acid bacteria (LAB) have a great economic value and contribute to improving human health, due to their potential uses as bio-preservatives and probiotics because of their abilities to produce several functional metabolites, like bacteriocins (Mokoena 2017).

Singhal *et al.* (2021) mentioned that different preparation style of fermented bamboo shoots has been reviewed and compiled by various researchers in the past. In this research, used previously method by Lindayani *et al.* (2018). Young bamboo shoots (*Bambusa* sp.) can be processed into salted fermented bamboo shoots by trimming, slicing, and mixing bamboo shoots with (2-3%) brine, placed into a sealed container and incubated for several weeks. Salt concentration, pH, nutrient, and temperature are the important factors of the succession of LAB fermentation and metabolism. LAB isolated from Ampel Bamboo Shoot Pickles, fermented in 2.5% of salt concentration at 15ºC for 5 days and in 5.0% of salt concentration at 30ºC for 4 days, showed antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* (Armando 2016; Mariana 2016). The antimicrobial activity of LAB played a role on its preservatives effect in the fermentation process, which was attributed to the production of bacteriocins and other antimicrobial compounds such as lactic acid, acetic acid and hydrogen peroxide (Hu *et al.* 2019). The study by Armando (2016) and Mariana (2016) revealed the succession of LAB species in fermented Ampel bamboo shoot but did not investigate the presence of bacteriocin-producing LAB in the process.

Bacteriocins produced by LAB have attracted special interest as potential alternative safe commercial food preservatives. LAB have been used as food and feed preservatives for centuries, and bacteriocin-producing LAB could replace chemical preservatives for the prevention of bacterial spoilage and the outgrowth of pathogenic bacteria in food products (Castellano *et al.* 2017). In order to reach optimum bacteriocin production, LAB need appropriate biochemical and biophysical environment to grow and express normal metabolic activities. The biochemical environment conditions are provided by the nutrients in the culture media. For LAB cultivation, supplementary nutrients like carbon source and the widely used complex nitrogen sources such as yeast extract, beef extract, and peptone are important necessities for growth (Hayek and Ibrahim 2013). In addition, Desmukh and Thorat (2013) found that supplementation with 1% of peptone and 0.8% of yeast extract yielded maximum bacteriocin production of *Lactobacillus rennanquilfy* WHL 3.

This study reports the effect of sucrose and nitrogen (peptone and yeast extract) supplementation in the culture medium, and different initial fermentation conditions (salt concentration and temperature) towards bacteriocin production of LAB isolated from Ampel bamboo shoot pickle.

MATERIAL AND METHODS

Materials. Thirty-two isolates of LAB obtained from Ampel Bamboo Shoots Pickle were used in this study. Bacterial growth media used were MRS (de Man Rogosa and Sharpe) broth, MRS agar, Nutrient agar, and Nutrient broth. Supplementations used were glucose, tryptone, and yeast extract.

Methods. The study was done in 2 steps, i.e., production of crude bacteriocins, and bacteriocin inhibitory activity test.

- **Production of Crude Bacteriocins.** The isolates which had been selected as potential antimicrobial producers were grown in MRS-B at 37ºC for 48 hours to reactivate the isolates. The isolates were adjusted to McFarland 5 and 1 ml of isolates were added to 9 ml of the following media: (i) MSRB containing 2% sucrose; (ii) MSRB containing 2% sucrose and 1% peptone; (iii) MRSB containing 2% sucrose and 0.8% yeast extract; (iv) MRSB containing 2% sucrose, 1% peptone and 0.8% yeast extract. The sterilization of the media was done by vacuum filter using Whatman membrane filter 0.45 µm. After that, the media were adjusted to pH 6.0 with 1 M of NaOH and 0.5 M of HCl (Desmukh and Thorat 2013).
- **Bacteriocin Inhibitory Activity Test.** Bacteriocin activity was performed by using the well diffusion method (Ivanova *et al.* 2000). Ten µL of pathogen inocula equivalent to McFarland 3 was inoculated with 10 ml of nutrient agar (NA, Merck, Germany) as a pour plate and was allowed to solidify. Cell-free supernatant was neutralized by adjusting pH to 6.0 with 1 M NaOH in order to prevent the inhibitory effect of lactic acid (Venigalla *et al.* 2017). Twenty µL of neutralized cell-free supernatant was inoculated in 5.5 mm diameter wells and incubated for 3 hours at 4°C to let it absorbed. Sterile MRSB was used as a negative control. Bacteriocin activity was performed against pathogenic bacteria *Staphylococcus aureus* FNCC 0047 and *Escherichia coli* FNCC 0091. Clear zone around the wells was measured after 24 hours incubation at 37°C.

Bacteriocin activity was expressed as activity units (AU) per ml. One AU was defined as the unit area of clear zone per unit volume of sample tested bacteriocins (mm^2/ml). Bacteriocin activity was calculated using the following formula (Thirumurugan *et al.* 2015; Venigalla *et al.* 2017):

Bacteriocin activity (mm^2/ml) = (Lz - Ls) / V

Lz : clear zone area (mm^2)
Ls : well area (mm^2)
V : sample volume (ml)

RESULT

In the present study 23 isolates showed bacteriocin inhibitory activity against pathogenic bacteria (Table 1).

The inhibitory activity ranged between 13.10-3274.99 mm^2 ml^{-1}. Each isolate had different response to each media treatment and indicator pathogens. Isolate A18 and B2 were able to produce bacteriocin after cultured in MRS-B without supplementation, indicating that this isolate gained enough nutrient contained in MRS-B media. DeMan Rogosa Sharpe Broth (MRS-B) is a common and

Table 1. Bacteriocin inhibitory activity (mm^2 ml^{-1}) on supplemented MRS-B medium against pathogenic bacteria

Isolate code	Pathogenic bacteria	Activity unit of bacteriocin (mm^2 ml^{-1}) on supplemented MRS-B medium				
		MRS-B (control)	MRS-B supplemented with sucrose	MRS-B supplemented with sucrose and peptone	MRS-B supplemented with sucrose and yeast extract	MRS-B supplemented with sucrose, peptone, and yeast extract
A7	1	-	-	-	22.13±38.33	-
	2	-	-	-	-	-
	3	-	-	-	102.67±177.82	-
A8	1	-	-	-	-	-
	2	-	-	57.75±100.03	-	-
	3	-	-	-	-	-
A9	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
A10	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
A11	1	-	13.10±22.68	-	23.87±41.34	-
	2	-	-	-	-	-
	3	-	-	-	-	-
A12	1	-	-	69.27±119.99	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
A13	1	-	327.38±567.04	-	-	-
	2	-	487.80±491.10	-	-	-
	3	-	-	-	-	-
A14	1	-	99.03±171.53	297.29±183.86	-	-
	2	-	-	558.97±562.26	-	-
	3	-	37.85±65.55	301.71±522.58	-	-
A15	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
A16	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
A17	1	-	-	-	99.03±171.53	-
	2	-	838.10±1451.62	471.43±816.54	22.13±38.33	-
	3	-	-	-	913.52±1390.91	-
A18	1	117.86±204.13	-	198.65±175.07	-	13.10±22.68
	2	-	-	-	57.75±100.03	52.38±90.73
	3	-	-	-	22.13±38.33	72.32±125.26

Table 1. Continued

Isolate code	Pathogenic bacteria	Activity unit of bacteriocin ($mm^2 ml^{-1}$) on supplemented MRS-B medium				
		MRS-B (control)	MRS-B supplemented with sucrose	MRS-B supplemented with sucrose and peptone	MRS-B supplemented with sucrose and yeast extract	MRS-B supplemented with sucrose, peptone, and yeast extract
A19	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
A20	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
A22	1	-	-	-	-	-
	2	-	104.27±120.25	553.27±958.30	471.43±816.54	253.52±439.12
	3	-	57.75±100.03	142.61±247.00	63.38±109.78	-
A24	1	-	-	-	-	-
	2	-	1484.64±1924.08	-	-	471.43±816.54
	3	-	187.56±163.19	-	-	88.52±120.91
B1	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
B2	1	35.65±61.75	-	-	-	-
	2	-	-	-	-	-
	3	33.52±58.06	-	-	-	-
B3	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	29.46±51.03
B4	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	117.86±204.13
B5	1	-	-	-	-	-
	2	-	-	-	-	134.39±232.01
	3	-	-	-	-	-
B6	1	-	-	-	-	-
	2	-	-	-	-	52.38±90.73
	3	-	-	-	-	-
B8	1	-	-	57.75±100.03	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
B9	1	-	-	-	1600.93±1289.46	-
	2	-	-	-	849.39±765.48	-
	3	-	-	-	356.85±511.81	-
B10	1	-	-	-	47.27±81.88	-
	2	-	-	-	-	-
	3	-	-	-	-	-
B11	1	-	-	327.38±567.04	-	242.13±419.38
	2	-	-	-	-	-
	3	-	-	-	-	-
B12	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
B13	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-

Table 1. Continued

Isolate code	Pathogenic bacteria	Activity unit of bacteriocin ($mm^2 ml^{-1}$) on supplemented MRS-B medium				
		MRS-B (control)	MRS-B supplemented with sucrose	MRS-B supplemented with sucrose and peptone	MRS-B supplemented with sucrose and yeast extract	MRS-B supplemented with sucrose, peptone, and yeast extract
B14	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	455.85±789.55	209.52±362.91	-
B15	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	209.52±362.91	-	-
B16	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	3274.99±2932.35	-	-	-
B19	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	204.32±353.89	-	-

1: *E. coli* (FNCC 0091), 2: *L. monocytogenes* (FNCC 0156), 3: *S. aureus* (FNCC 0047), -: no bacteriocin inhibitory activity

effective medium for growth of *Lactobacillus* species. Thirteen isolates (A7, A8, A12, B4, B5, B6, B8, B9, B10, B11, B14, B15 and B19) showed bacteriocin production after inoculated in MRS-B with a combination of carbon and nitrogen source (Table 1).

Various species of bacteria existed in both fermentation conditions (Table 2). This may be caused by the natural fermentation method, which enables both desirable and associated microorganisms to grow during the process. Surprisingly, the same LAB species from different fermentation had different bacteriocin inhibitory activity after cultivation in supplemented media. *Lb. fermentum* I (A14, A17 and A18) generally could be induced by most of the carbon and nitrogen supplementation, but Lb. fermentum I (B3) could be induced only when supplemented with a combination of carbon and all nitrogen sources.

Table 2. Result of API software identification (24 hours incubation)

Isolate code	Species identification	Significant (%)	Remarks
A8	*Lactobacillus pentosus*	97.3	Good identification
A13	-	-	-
A14	*Lactobacillus fermentum* 1	99.8	Very good identification
A17	*Lactobacillus fermentum* 1	99.8	Very good identification
A18	*Lactobacillus fermentum* 1	99.9	Very good identification
A22	*Lactococcus raffinolactis*	99.7	Doubtful profile
A24	*Lactobacillus brevis* 1	60.3	Doubtful profile
B3	*Lactobacillus fermentum* 1	99.8	Very good identification
B9	*Leuconostoc mesenteroides* ssp. mesenteroides/dextrancium 2	-	Unacceptable profile

-: not grow/not identified

DISCUSSION

Isolates A14, A17, A22, A24 and B16 produced bacteriocin when grown in MRS-B supplemented with sucrose as sole carbon source (Table 1). Sucrose is the traditional and most commonly used sweeteners in the dairy industry. It is a nutrient and energy source for living things. Commonly, glucose is considered as a carbon source by all microorganisms due to its size, rapid uptake, utilization and cellular energy conversion. However, some bacteria have a complete enzymatic machine that allows them to use complex carbohydrates; for example, *E. faecium* (Ramsey *et al.* 2014). Hayek and Ibrahim (2013) observed that LAB species had different preferences to sugar, which could influence growth and functional properties of LAB.

The highest bacteriocin inhibitory activity (3274.99 ± 2932.35 mm^2 ml^{-1}) was found in isolate B16 with supplementation of 2% sucrose. Bacteriocin from *L. lactis* cultured in CM medium supplemented with sucrose yielded higher activity than supplemented with other sugar (Indriati *et al.* 2014). Sucrose as a carbohydrate source is able to result in high biomass and bacteriocin production as it is consumed faster than lactose or glucose when present as a sole carbohydrate source (Indriati *et al.* 2014). These findings revealed that sucrose could be the best alternative sugar to glucose to yield higher bacteriocin activity.

Some of them yielded better result by the presence of peptone or yeast extract and the combination of both. Different nitrogen source yielded different result for each isolate as well. Peptones are water-soluble protein

hydrolysates, containing peptides, amino acids, and inorganic salts as well as other compounds, such as lipids, vitamins, and sugars, which is a good source of nitrogen, carbon and other nutrition for microorganism culture (Davami *et al.* 2015). On the other hand, yeast extract contains peptides, amino acids, nucleotides and other soluble components of yeast cells (Zarei *et al.* 2016). pH, temperature inoculums size, and other environment factors, besides nutrient source, strongly affected LAB bacteriocin production (Mokoena 2017). Thus, the environmental factor, such as temperature and pH should be optimized for maximum bacteriocin production in future study.

More isolates from condition B (13 isolates) had inhibitory activity than the isolates from condition A (10 isolates), which were differentiated by initial fermentation conditions. This result showed that initial fermentation condition (salt concentration and temperature) affects the ability to produce bacteriocin on LAB isolates from Ampel bamboo shoot fermentation. Fermentation condition B, performed in higher temperature (30°C) and higher salt concentration (5.0%) facilitated better the growth of bacteriocin-producing LAB during the process. Similarly, Ln. mesentroides isolated from fermentation at higher temperature had better bacteriocin inhibitory activity than those isolated from colder temperature (Danial *et al.* 2016).

From this result (Table 2), *Lb. fermentum* I from fermentation condition A had better bacteriocin activity than from fermentation condition B. It has been reported that higher temperature could alter fermentation process to homofermentative (Danial *et al.* 2016). Since *Lb. fermentum* is categorized as hetero-fermentative LAB (Adebayo *et al.* 2014), lower temperature (fermentation A) could be a better condition for the growth of *Lb. fermentum* 1, which may affect its potential to produce bacteriocin. Ray (2014) explained that the species and the strains of a species growing under similar conditions differ greatly in the amount of bacteriocin production. A nutritionally rich medium is always better, and for many bacteriocins, growing the strains in a fermentor under a controlled terminal pH can produce more bacteriocin.

The antibacterial spectrum of bacteriocin frequently includes spoilage organisms and food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* (Khandare and Patil 2015). From all 32 isolates in the present study, only 4 isolates – A14 (*Lactobacillus fermentum* 1), A17 (*Lactobacillus fermentum* 1), A18 (*Lactobacillus fermentum* 1) and B9-showed bacteriocin inhibitory activity against all the indicator pathogens *E. coli* FNCC 0091, *L. monocytogenes* FNCC 0156 and *S. aureus* FNCC 0047 (Figure 1). Optimum bacteriocin inhibitory activity was found in bacteriocin B9 which was able to inhibit all pathogens when cultured in MRS-B supplemented with 2% sucrose and 0.8% yeast extract, while the highest inhibitory activity was by B16 against *S. aureus* FNCC 0047 (Gram-positive pathogen) but it had no bacteriocin activity against *E. coli* FNCC 0091 and *L. monocytogenes* FNCC 0156 (Figure 1).

Bacteriocins are frequently not active against Gram-negative bacteria (Stevens *et al.* 1991). However, some isolates (A11, A12, A18, B8, B9, B10 and B11) showed broader inhibitory activity against *E. coli* FNCC 0091 (Figure 2). This may be caused by different bacteriocin production characteristics of each genus and species (Danial *et al.* 2016). Bacteriocin inhibitory activity generally act through depolarization of the target cell membrane or through inhibition of cell wall synthesis and range in specificity from a narrow spectrum of activity (lactococcins which only inhibit lactococci) to those which have a broad range of activity such as the lantibiotic nisin. In general, the bacteriocin molecules initially adsorbed on the membrane surface and form transient pores, leading to loss of protein motive force as well as the pH gradient across the membrane (Ray 2014). This alters the permeability of the membrane, causing leakage of small nutrient molecules, as well as affecting the transport of nutrients and synthesis of ATP. These changes finally cause the cell to lose viability. In addition, some bacteriocins can cause lysis of sensitive cells.

In conclusion, fermentation condition B (30°C and 5% salt concentration) has better result on the growth of bateriocin-producer LAB than fermentation condition A (15°C and 2.5% salt concentration). Optimal bacteriocin inhibitory activity against pathogenic bacteria was found in isolate B9, cultured in MRS-B supplemented with 2% of sucrose and 0.8% of yeast extract.

Conflict of Interest. There is no conflict of interest.

Data Availability Statement No Data. The data used to support the findings of this study are included within the article.

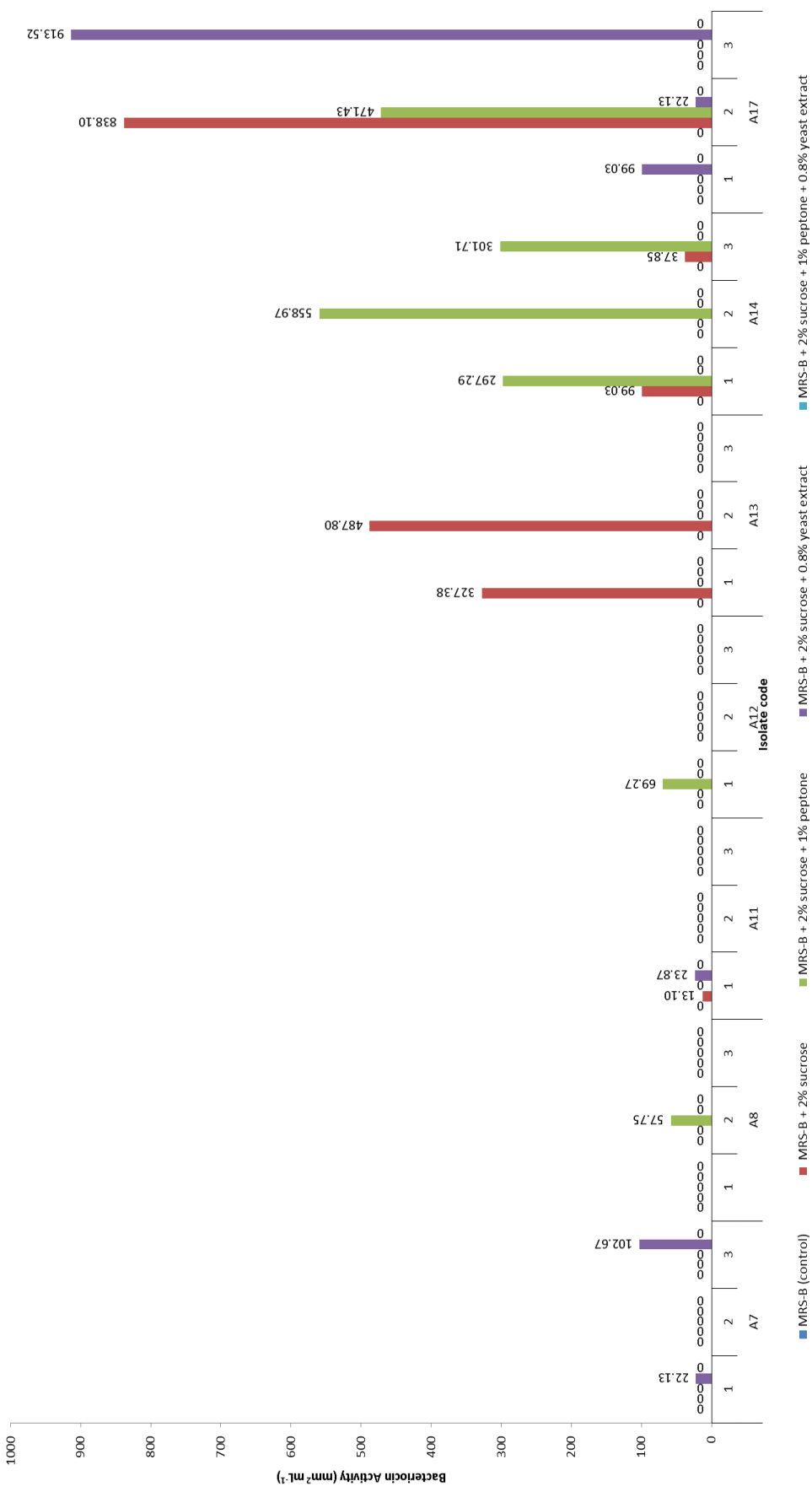


Figure 1. Effect of medium compositions on bacteriocin inhibitory activity ($mm^2 ml^{-1}$) of LAB isolates against *E. coli* FNCC 0091, *L. monocytogenes* FNCC 0156 and *S. aureus* FNCC 0047. 1: *E. coli* FNCC 0091, 2: *L. monocytogenes* FNCC 0156, 3: *S. aureus* FNCC 0047

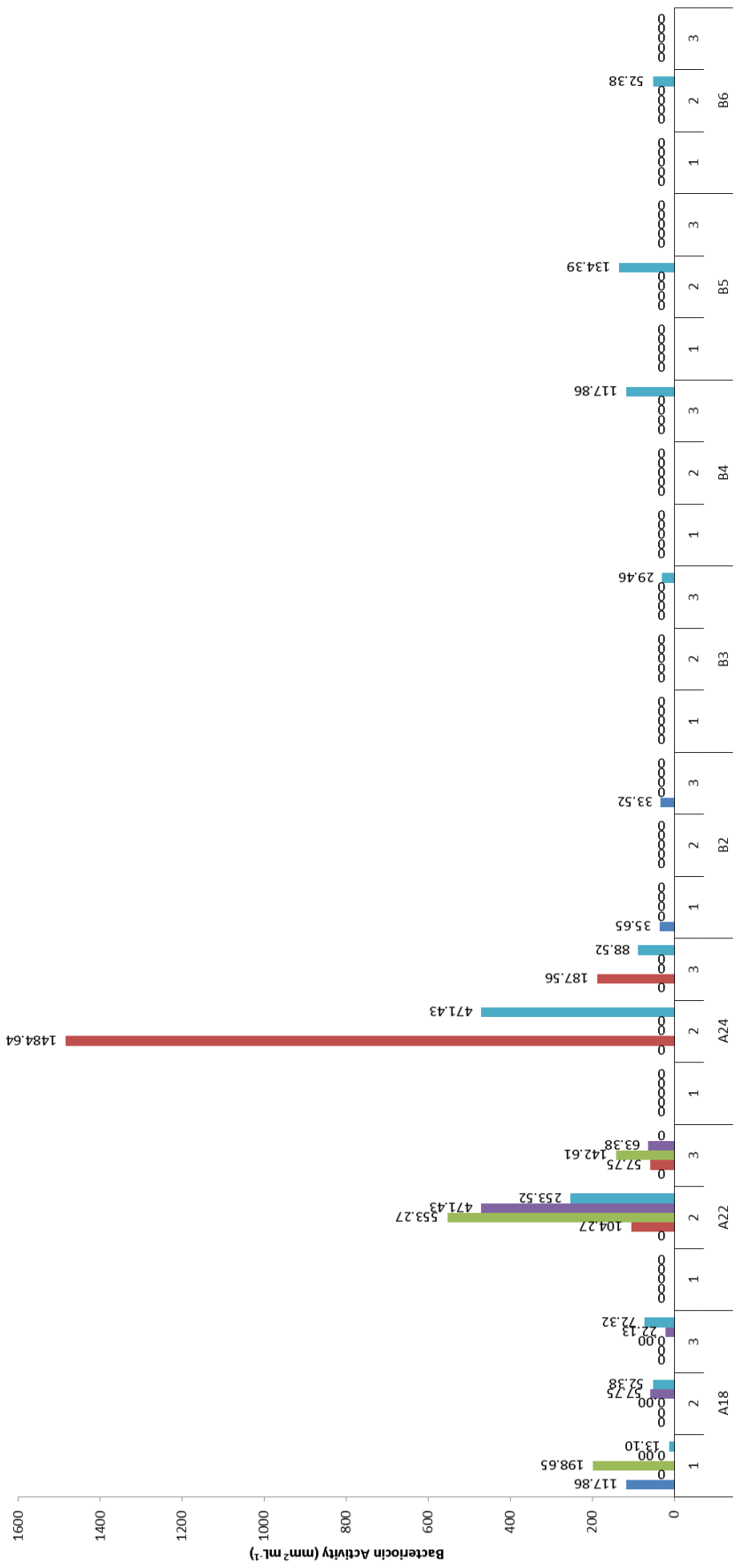
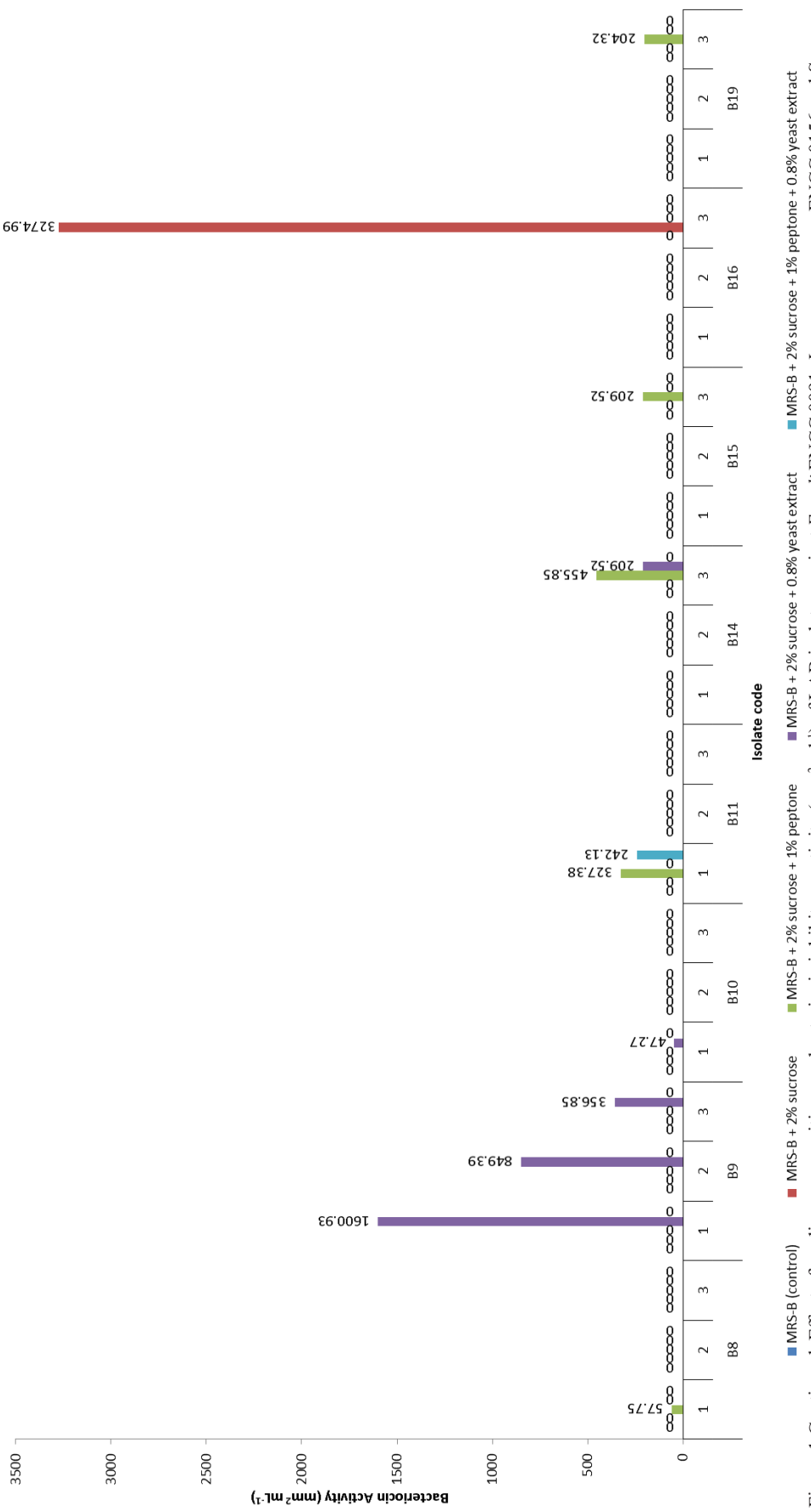


Figure 1 Continued. Effect of medium compositions on bacteriocin inhibitory activity (mm^2 ml^{-1}) of LAB isolates against *E. coli* FNCC 0091, *L. monocytogenes* FNCC 0156 and *S. aureus* FNCC 0047. 1: *E. coli* FNCC 0091, 2: *L. monocytogenes* FNCC 0156, 3: *S. aureus* FNCC 0047



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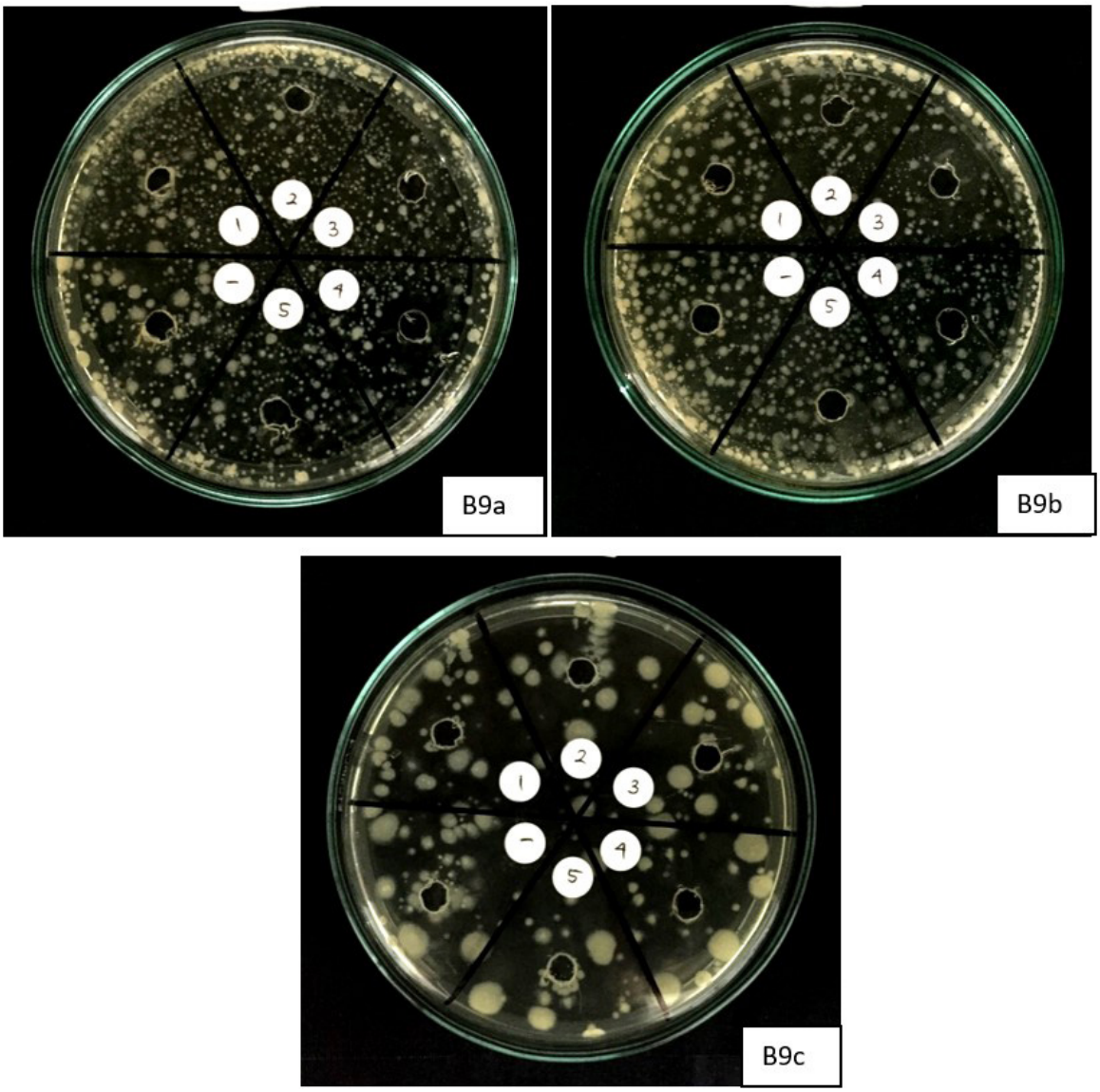

Figure 2. Effect of medium compositions on bacteriocin B9 inhibitory activity test against *E. coli* FNCC 0091 (a), *L. monocytogenes* FNCC 0156 (b), and *S. aureus* FNCC 0047 (c), negative control (-), MRS-B media (1), MRS-B supplemented with 2% of sucrose (2), MRS-B supplemented with 2% of sucrose and 1% of peptone (3), MRS-B supplemented with 2% of sucrose and 0.8% of yeast extract (4), MRS-B supplemented with 2% of sucrose, 1% of peptone, and 0.8% of yeast extract (5)

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