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Original Research Article

Characteristics and *In Vitro* Antibacterial Activity of *Lactobacillus acidophilus* FNCC-0051 Probiotic Microspheres against *Propionibacterium acnes* ATCC 11827

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ABSTRACT

Lactobacillus acidophilus FNCC-0051 is a probiotic which contains bacteriocin metabolites, lactic acid, and organic acid which are effective as antibacterial for acne treatment. This study aims to encapsulate probiotic, characterise and determine the antibacterial activity of *L. acidophilus* FNCC-0051 at pH 4.5 and 6.0. The probiotic microspheres were made using ionic gelation aerosolization technique with 2% sodium alginate and 1.0 M $CaCl_2$. The characterization included surface morphology, size, moisture content, swelling index, and viability. Antibacterial activity was carried out by diffusion method using *Propionibacterium acnes* ATCC 11827. Activity was determined by inhibition zone diameter every 1 hour for 6 hours. Results showed that the microsphere was spherical with smooth surface with a size of 1.7841 ± 0.0253 µm. Moisture content was 3.38 ± 0.18% and the swelling index at pH 4.5 and pH 6.0 were 384.29% and 507.37%, respectively within 6 hours. The results of inhibition zone diameter measurement against *Propionibacterium acnes* at pH 4.5 for 6 hours were 10.11 ± 1.07; 12.16 ± 1.25; 15.73 ± 2.36; 18.81 ± 3.20; 22.99 ± 2.38; and 30.74 ± 5.24 mm. while the inhibition zone diameter at pH 6.0 were 20.98 ± 2.41; 25.58 ± 2.40; 31.04 ± 3.57; 36.09 ± 2.62; 40.47 ± 2.12; and 48.38 ± 4.73 mm. Swelling capacity at pH 6.0 was higher than at pH 4.5. Therefore, the released probiotics were higher at pH 6 and caused a higher antibacterial activity at pH 6.0 than at pH 4.5.

Keywords: Probiotics, Microspheres, Antibacterial activity, pH.

Introduction

Acne is a dermatological disorder caused by many factors, including *Propionibacterium acnes* bacterial proliferation.[1] Probiotics are living microorganisms that can provide health benefits if consumed in sufficient quantities (up to 10^6-10^8 cfu/mL.[2] *Lactobacillus acidophilus* probiotics are lactic acid bacteria that produce bacteriocins which are known to have *in vitro* antibacterial activity towards *Propionibacterium acnes*, which are bacteria that cause acne.[3,4] Probiotics can provide health benefits when used around 6-7 Log cfu g^{-1} or mL^{-1} per day, but probiotics are very sensitive to environmental conditions (pH, temperature, etc.) during the manufacturing process and during storage, because these factors can reduce their viability.[5] Therefore, a delivery system that is suitable to maintain the viability of these probiotics is was required , one of which is the microsphere delivery system.[6] Besides being able to protect the viability of probiotics, the microsphere delivery system can also produce a gradual release, so that the therapeutic effect lasts longer.[7,8] In this research, the microspheres are made by ionic gelation method aerosolization technique using 2% sodium alginate polymer as a matrix and $CaCl_2$ (1.0 M) crosslinking solution by freeze drying method and using 5% maltodextrin as a lyoprotectant.[9]
The characteristics of the microsphere can affect the release of active ingredients and their activity, especially the viability of probiotics and microsphere swelling index. Probiotic viability affects the ability of probiotics to achieve Minimum Inhibitory Concentration (MIC) in order to work effectively as an antibacterial. Viability is influenced, among others, by pH, where the addition of acid can cause a decrease in bacterial viability.[10,11] The release of active ingredients is influenced by swelling ability of microspheres which depends on swelling character of the matrix building polymer.[12] The polymer matrix of sodium alginate is stable at pH 4-10 and expands optimally at neutral pH.[13]
This study aims to determine the characteristics and antibacterial activity of *L. acidophilus* FNCC-0051 probiotic microspheres towards *Propionibacterium acnes* ATCC 11827 at pH 4.5 and 6.0 and compare to clindamycin phosphate. [14]

Materials and Methods

Organisms and Reagents
L. acidophilus FNCC-0051 from UGM Center for Food and Nutrition Studies, *Propionibacterium acnes* ATCC 11827, Sodium alginate *low viscosity* (Pharmaceutical Grade), $CaCl_2.2H_2O$ (Pharmaceutical Grade), sterile aquadest, maltodextrin (Pharmaceutical Grade), H_2SO_4, *de Man Ragosa Shorpe* (MRS) sterile broth media, MRS agar medium, Sodium chloride (Pro Analysis), Nutrient Agar media, sodium citrate (Pro Analysis), citric acid (Pro Analysis), disodium hydrogen phosphate (Pro Analysis) sodium dihydrogen phosphate (Pro Analysis), Phosphate Buffer Saline (PBS), clindamycin phosphate.

Equipment
Some instruments used included Incubator (Memmert INB 500), Vortex (Labinco L46), Analytical Balance (Chyo balance corporation Kyoto Japan), Autoclave (Huxley HL340), Scanning Electronic Microscope (SEM), FTIR, Spectrophotometer, pH meter (SCHOTT glass mainz type CG 842), Buchner Funnel, Colony Counter (Suntex 570), Optical Microscope, Hot Plate, Aerosolized Atomizer (nozzle size of 45 µm), Ohaus MB45 Moisture Analyzer, Freeze Dryer.

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Qualitative Analysis of Materials
Qualitative examination was carried out on *L. acidophilus* FNCC-0051 with gram stain and cell shape test. Calcium chloride, sodium alginate, and maltodextrin were tested organoleptically and examined using FTIR by KBr pellet technique.

Optimization of L. acidophilus Growth Time (probiotic starter)
Growth optimization was carried out by entering 1 loop of *L. acidophilus* pure culture *on* MRS broth media and incubated at 37°C for 48 hours. Sampling was carried out at 0, 6, 12, 18, 24, and 48 hours for *L. acidophilus* viability testing with the Total Plate Count (TPC) method.[15]

Microspheres Preparation
As shown in Table 1, 2 g of sodium alginate was spread into 100 mL of aquadest and sterilized at 121°C for 20 minutes. About 10 mL of probiotic suspension was added to the alginate solution while stirring until homogeneous. Meanwhile, 200 mL of 1.0 M $CaCl_2$ solution was prepared. The mixture of alginate solution and probiotics were sprayed using nozzle (aerosolization technique) into a solution of $CaCl_2$ with a spraying distance of 8 cm from the surface of the solution and a pressure of 40 Psi, then stirred using a magnetic stirrer at a speed of 1000 rpm for 1 hour. The microspheres formed were separated from the $CaCl_2$ solution by centrifuging at 2500 rpm for 6 minutes and washed with sterile aquadest until free of $CaCl_2$. Microspheres that were free of $CaCl_2$ were drained to a minimum amount of water and constant weight. Furthermore, the microspheres obtained were dispersed into 5% maltodextrin solution 5 times the weight of wet microspheres, then dried using freeze dryer for 24 hours at -80°C.[16]

Table 1: *Lactobacillus acidophillus* Probiotic Microsphere Formula

Materials	Functions	Formula
Lactobacillus	Active	10 mL (6-7 Log
acidophilus	ingredients	$CFUmL^{-1}$)
Sodium Alginate	Polymers	2.0% (2 g)
Sterile Aquadest	Solvent	ad 100 mL
$CaCl_2$	Cross linker	1.0 M 200 mL
Maltodextrin	Lyoprotectant	5.0%

Characteristics of Probiotic Microspheres
a. *Surface Shape and Morphology*
The evaluation of the shape and surface of the probiotic microspheres was carried out using optical microscope and Scanning Electron Microscope (SEM).

b, *Determination of Particle Size*
Particle size measurement was carried out using an optical microscope with a sample of 300 microsphere particles and a magnification of 400 x.

c. *Measurement of Moisture Content*
Measurement of the moisture content of probiotic microspheres was done using Ohaus MB45 Moisture Analyzer by weighing 1-1.5 g of sample and adjusting the temperature and time. The percentage (%) of moisture content was displayed on the light emitting diode (LED) of the instrument.

d. *Determination of Swelling Index*
Determination of swelling index was carried out based on changes in mass at a certain time interval. After the sample was put into the media, the microspheres were weighed at approximately 50 mg for 6 samples, then each 5 mL of buffer pH 4.5 was added and left at 32 ± 0.5°C. Changes in weight were observed at 1, 2, 3, 4, 5, and 6 hours. The same steps were taken at a pH of 6.0.[17]

e. *Measurement of Viability*
Measurement of entrapment efficiency/viability was carried out using the aseptic ALT test. The probiotic microspheres were weighed as much as 1 g, then dissolved in sterile 1% sodium citrate (w/v) at pH 6.0 and shaken at room temperature for 1 hour. Furthermore, 10 times dilution was done on MRS broth media and incubated at 37°C in an aerobic atmosphere for 48 hours. The calculation of the number of probiotic bacteria was done using log cfu/mL units. Percentage of viability of *L. acidophilus* probiotic content in the microsphere was calculated using the following formula:[18]

$$viability(\%) = \frac{log_{10}N}{log_{10}N_0} x\ 100$$

Evaluation of the Antibacterial Activity of Probiotic Microspheres at pH 4.5 and pH 6.0
The probiotic microspheres (550 mg) were weighed (equivalent to 6 log cfu/mL) and suspended in 10 mL of buffer media at pH 4.5±0.05 and pH 6.0±0.05, respectively. Activity testing was carried out on 3 samples of probiotic microsphere suspension at pH 4.5 and on 3 samples of probiotic microsphere suspension at pH 6.0. Measurement of the diameter of the inhibition zone in each sample was carried out at 1, 2, 3, 4, 5, and 6 hours.[14]
The relationship curve between time as the x-axis and the diameter of the inhibition zone as the y-axis was created in order to obtain the probiotic microspheres antibacterial activity profile.

Equivalence Test against Clindamycin Phosphate
The preparation was done by making 1000 ppm clindamycin phosphate (standard stock solution) in aquadest. From the standard stock, a working standard solution of clindamycin phosphate was made at 10, 20, 30, 40, 50, and 60 ppm, then planted in the media and incubated. Furthermore, the diameter of the inhibition zone was measured for each concentration, and a regression equation was made between the diameter of the inhibition zone and the concentration (ppm).
Furthermore, the antibacterial activity of probiotic microspheres was compared against the antibacterial activity of clindamycin phosphate by interpolating the inhibition zone diameter data into the antibacterial activity regression curve from positive control or clindamycin phosphate comparison standards.[19]

Statistical Analysis
Statistical analysis was performed using SPSS 20 software (SPSS, Chicago, IL, USA). Data were analyzed using a one-way analysis of variance followed by post hoc Tukey's multiple comparisons test in SPSS 20 software. Values of $P< 0.05$ were considered to be statistically significant.

Results and Discussion

Optimization of L. acidophilus Growth Time
Log TPC value for *L. acidophilus* FNCC-0051 growth increased with increasing time and reached optimal growth time at 24 hours. After 24 hours, the log TPC value decreased (as seen in Figure 1) This is because there is a decrease in the availability of nutrients needed by bacteria to keep growing so that the bacteria begin to die.[15] Optimization of growth time is used to determine the best time to harvest probiotics before the optimal growth time.

FT-IR Spectra analysis
Result of the infrared spectroscopic analysis of *L. acidophilus* probiotic microspheres with the sodium alginate matrix showed that there was a shift in the sodium alginate carbonyl group wave number (C=O) from 1618.31 cm^{-1} to 1631.70 cm^{-1} and the loss of guluronic cluster fingerprints at a wave number of 892.7 cm^{-1} (Figure 2). This showed that there was a cross-linking process between sodium alginate and $CaCl_2$ solution.

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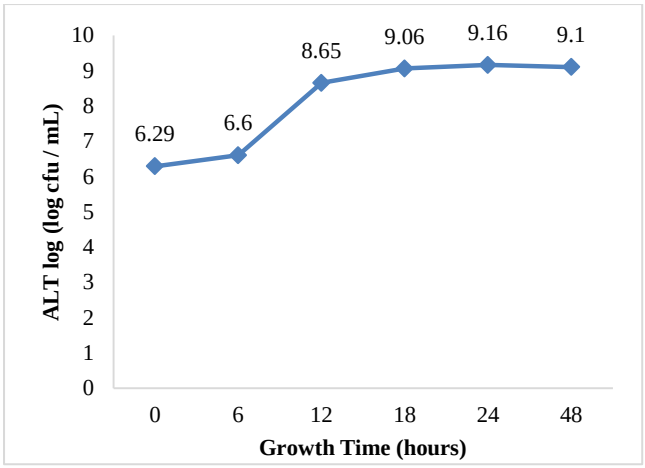

Figure 1: Result of Optimization of *Lactobacillus acidophilus* Probiotic Growth Time

Morphology of L. acidophilus Probiotic Microspheres
The surface morphology test of the probiotic microsphere particles by SEM showed that the probiotic microspheres were spherical with a flat and smooth surface. This result can be seen in Figure 3.

Determination of Particle Size
The determination of the particle size was conducted using an optical microscope with 400x magnification and the average particle size was between 1-50 µm, the result shown in Table 2. This met the criteria for microsphere particle size for topical use.[20] The small and uniform particles will provide good occlusivity to the surface where it is applied, thereby increasing the penetration and effectiveness of the drug.

Measurement of Moisture Content
The moisture content of the probiotic microspheres as shown in Table 3, was in the range of 3-5%. Too high moisture content will cause the microspheres to be easily damaged by the growth of other microorganisms. However, microspheres that are too dry will also complicate the swelling process from the microsphere matrix so that the release of active ingredients is less than optimal.

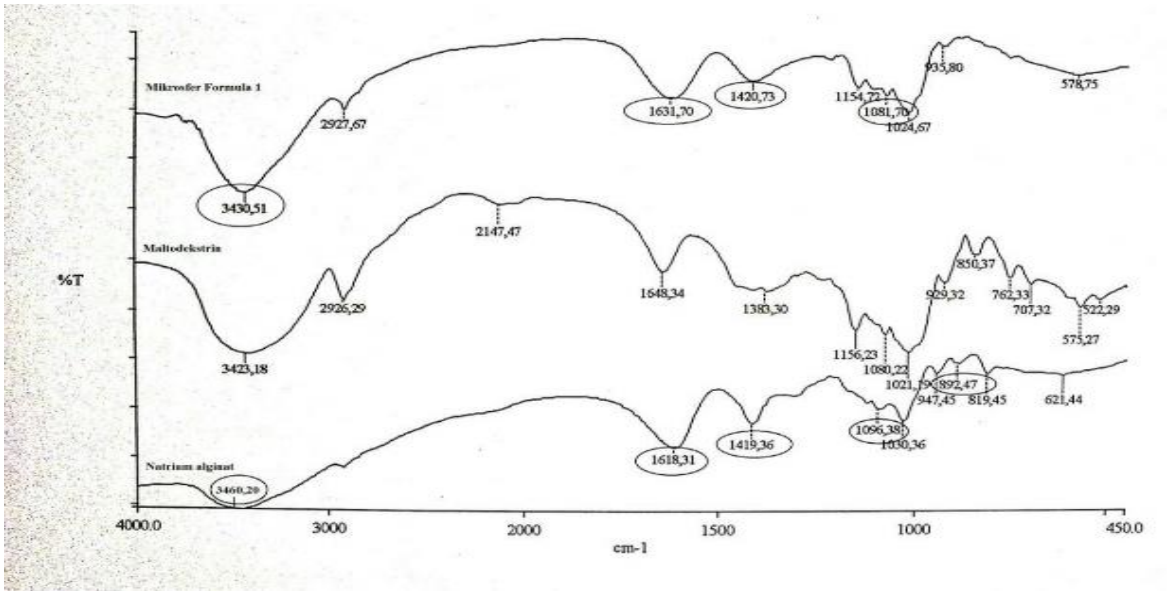

Figure 2: FTIR Spectra of *Lactobacillus acidophilus* Probiotic Microsphere

Determination of Swelling Index
Result of the swelling index determination of probiotic microsphere at pH 4.5 and pH 6.0 can be seen in Table 4, Table 5, and Figure 4. Examination of swelling index was conducted to determine the ability of the microsphere matrix to expand so that the active ingredients could be separated from the matrix and exert it effect. Swelling index of probiotic microspheres with 2% sodium alginate polymer at pH 4.5 and pH 6.0 increased from the 1st hour to the 6th hour. From the test results of swelling index, it was noted that the swelling index of the probiotic microspheres at pH 6.0 was greater than the swelling index of the probiotic microspheres at pH 4.5. This is because the sodium alginate polymer matrix is stable at pH 4-10 and expands well at neutral pH.[12,13]

Measurement of Trapping Efficiency/Viability
The result of the bacterial viability test using the TPC method. on dry microspheres (after freeze drying) was higher than wet microspheres (before freeze drying), as shown in Table 6. This might be possible because maltodextrin, which is an oligosaccharide used in the microsphere drying process as a lyoprotectant, can be a nutrient for

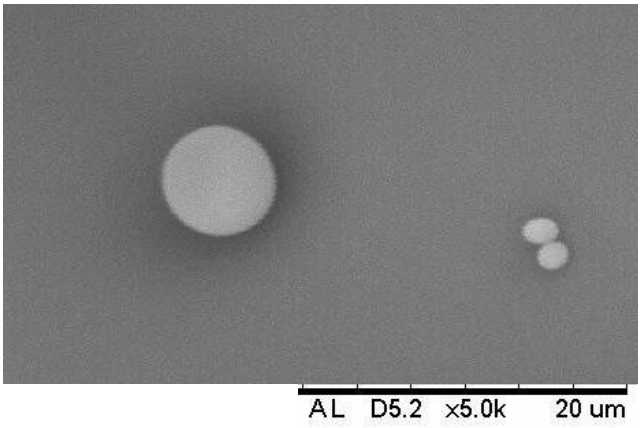

Figure 3: Result of *Lactobacillus Acidophilus* probiotic microspheres morphological examination with SEM

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probiotic bacteria. So that after going through the drying process the bacteria can regenerate.[15] Viability or high number of living bacteria will also provide high activity. Probiotic viability is influenced by pH and the addition of acid can cause a decrease in bacterial viability because high acidity levels can damage bacterial cell membranes.[10]

Antibacterial Activity Test Results at pH 4.5 and pH 6.0

Probiotic microsphere antibacterial activity test at pH 4.5 and 6.0 were carried out for 6 hours with observations at the 1st, 2nd, 3rd, 4th, 5th, and 6th hour. As shown in Table 7 and Figure 5, the observed inhibition zone diameter at pH 4.5 and at pH 6.0 increased until the 6th hour. Statistical test results using one way ANOVA with a confidence interval of 95% ($\alpha = 0.05$) on the diameter of the inhibition zone between times resulted in significant value greater than 0.05, meaning that there was no significant difference in the resulting inhibition zone diameter between times at pH 4.5 and pH 6.0. *L. acidophilus* antibacterial activity in the microsphere at pH 4.5 and at pH 6.0 had continued for 6 hours.

The result of the antibacterial activity test at pH 6.0 was greater than the antibacterial activity at pH 4.5. This was evidenced by the statistical test result obtained by the significant value that was smaller than 0.05, meaning that there was a significant difference between probiotic microsphere activity at pH 4.5 and probiotic microsphere activity at pH 6.0. The result of activity test at pH 6.0 that was greater than at pH 4.5 was due to the release of the drug from the alginate matrix which was crosslinked with the Ca^{2+} ion dependings on the pH of the media and the solubility of the drug.[8] Under acidic condition, sodium alginate cannot expand optimally because alginic acid is an anionic polymer and the carboxyl groups do not ionize at a pH below the pKa of the polymer (3.35). Ionization will occur when the pH is above the pKa of the polymer. The increase in pH in the polymer solution causes an increase in the number of carboxylate groups in the alginate which ionizes to become COO^-. These ions repel each other so that there is relaxation of bonds/bonds becoming looser and resulting in an increase in swelling degree. Under neutral condition, the anionic polymer ionizes and expands so that the release of the drug at a pH of 6.0 is greater.[13]

Probiotic Microsphere Activity Equivalence Test towards Clindamycin Phosphate

The equivalence test was carried out to compare the equivalence of the inhibitory concentration of probiotic microspheres with clindamycin phosphate towards *Propionibacterium acnes* ATCC 11827. The clindamycin phosphate activity test was carried out at concentrations of 10, 20, 30, 40, 50, and 60 ppm. This concentration range was chosen because it is above the clindamycin sulphate MIC (0.047 ppm) towards *Propionibacterium acnes*.[19]

The results of the clindamycin phosphate activity test at 10, 20, 30, 40, 50, and 60 ppm were 12.75, 14,98, 16.48, 16.90, 17.18, and 18.10 mm, respectively. Furthermore, a linear regression equation was made with the diameter of the inhibition zone (mm) as y and the log of clindamycin phosphate (ppm) as x and the regression equation $y = 6.6000x + 6.3191$ was obtained with a correlation coefficient (r) = 0.9913 (Figure 6)

Based on Figure 7, the zone of inhibition generated by probiotic microspheres from the 1st to the 6th hour at pH 4.5 and pH 6.0 was interpreted in the linear regression equation. The result of intrapolation is an illustration of the equivalence between hourly probiotic microspheres and clindamycin phosphate. The results of the equivalence curve were made with the diameter of the inhibition zone of the probiotic microspheres per time (hour) as x and the equivalent concentration of probiotic microspheres with clindamycin phosphate (ppm) as y. The equivalent results compared to the MIC of Clindamycin phosphate towards *Propionibacterium acnes* ATCC 11827 for topical use was 0.047 ppm.[19] This shows that in the first hour, at pH 4.5 and pH 6.0, probiotic microspheres have shown activity because in the first hour the equivalent concentration is above MIC and it can be seen in Figure 6 that the antibacterial activity of the probiotic microspheres produced is equivalent to the activity of clindamycin phosphate.

Table 2: Probiotic microsphere particle size determination

Observation Replication	Average Size (µm)	Mean ± Standard Deviation
1	1.7461	1.78±0.02
2	1.8182	
3	1.7880	

Table 3: Probiotic microsphere MC examination

Replication	Moisture Content (%)	Mean±Standard Deviation
1	3.32	3.38±0.18
2	3.18	
3	3.65	

Table 4: Swelling index examination of probiotic microspheres at pH 4.5

Hour	% swelling index
1	84.16
2	100.4
3	132.34
4	245.28
5	293.5
6	384.29

Table 5: Swelling index examination of probiotic microspheres at pH 6.0

Hour	% swelling index
1	191.04
2	233.46
3	282.18
4	365.94
5	440.28
6	507.37

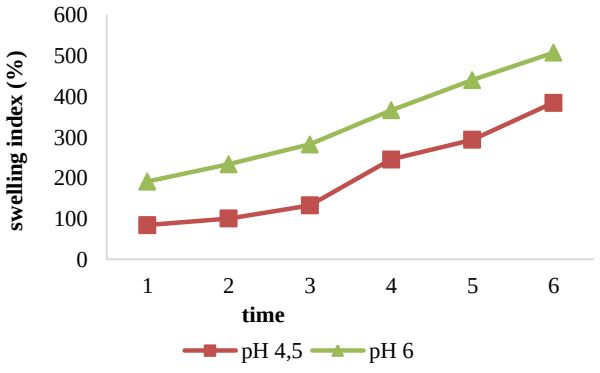

Figure 4: Swelling index curve of Lactobacillus acidophilus probiotic microspheres at pH 4.5 and pH 6.0

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Table 6: Log TPC value of probiotic microspheres before and after the freeze drying process

Group	log TPC (log cfu/mL)		
	Probiotic starter	Before freeze drying	After freeze drying
Replication 1	8.73	8.09	9.30
Replication 2	8.76	5.78	10.49
Replication 3	8.80	8.03	10.95

Table 7: The diameter of *Lactobacillus acidophilus* probiotic microspheres inhibition zone at pH 4.5 and 6.0

Time (hour)	Inhibition Zone Diameter at pH 4.5 ± SD (mm)	Inhibition Zone Diameter at pH 6.0 ± SD (mm)
1	12.94 ± 0.33	15.03 ± 0.31
2	13.47 ± 0.30	15.61 ± 0.28
3	14.18 ± 0.44	16.13 ± 0.34
4	14.69 ± 0.51	16.60 ± 0.20
5	15.28 ± 0.30	16.90 ± 0.15
6	16.10 ± 0.51	17.42 ± 0.28

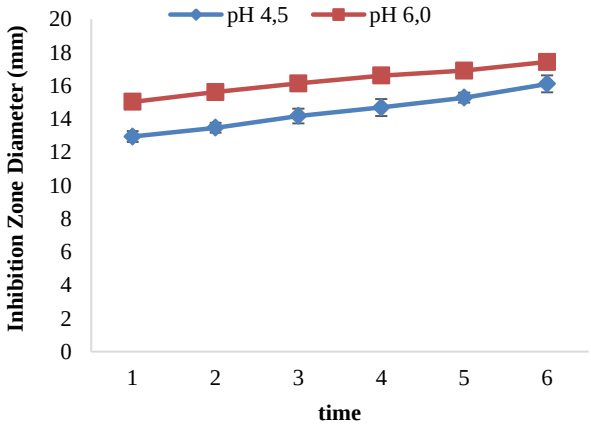

Figure 5: Inhibition zone diameter curve of probiotic microspheres

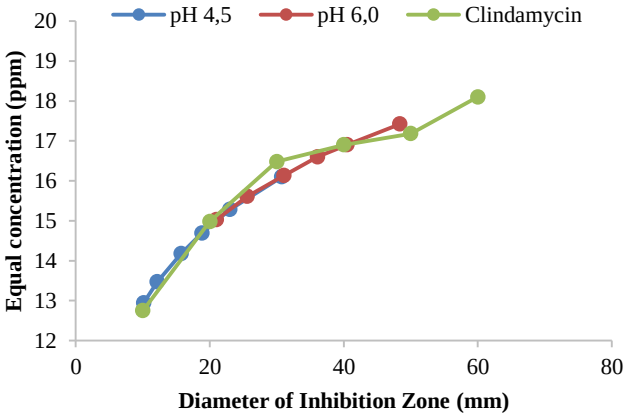

Figure 7: Curve of *Lactobacillus acidophilus* probiotic microspheres antibacterial activity equivalence towards clindamycin sulphate at pH 4.5 and 6.0.

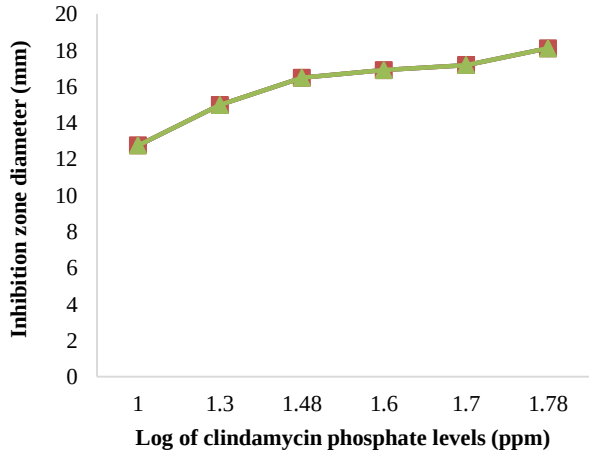

Figure 6: Relation curve of inhibition zone diameter (mm) with log clindamycin sulphate content (ppm)

Conclusion

The swelling capacity of probiotic microspheres at pH 6.0 for six hours observation is greater than its swelling capacity at pH 4.5. Therefore, the released probiotics is higher and causes higher antibacterial activity at pH 6.0 than its activity at pH 4.5. Antibacterial activity of *Lactobacillus acidophilus* FNCC-0051 probiotic microspheres with sodium alginate matrix at pH 6.0 is greater than the activity at pH 4.5.

Conflict of interest

The author declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is their original work, and that they will be held liable for any claims relating to the content of this article.

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