JOTCS B
Journal of the Turkish Chemical Society
Section: B
Chemical Engineering
TURKISH CHEMICAL SOCIETY

Tetrazolium-Based Rapid Colorimetric Assay to Determine Bacteriocin Activity

Nedim ALBAYRAK

Hitit University Faculty of Engineering, Department of Food Engineering, Çorum, Turkey.

Abstract: The aim of this study is to develop a simple, rapid, and accurate method for quantitative determination of bacteriocin activity. The method involves the use of 2,3,5-triphenyltetrazolium chloride (TTC), which is a colorless water-soluble salt. Only viable cells take up the compound and reduce it intracellularly to its red-colored and water-insoluble formazan dye. Bacteriocin, nisin and lacidin-A were assayed using the sensitive indicator *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 whereas pediocin PO2 was assayed against *Lactobacillus bulgaricus* OSU 135. The major factors affecting the reduction of TTC, such as the reagent concentration, incubation period, temperature, and pH were adjusted so that optimal reduction of TTC by the indicator microorganisms could be achieved. Two-fold dilutions of the bacteriocins were mixed with a standardized indicator culture and incubated for 30 min. Then 0.2% TTC was added and the mixture (pH 6.0) was incubated at 37 °C for 20 min. After the incubation, formazan was extracted from the cells with methanol and the absorbance was measured at 485 nm. The amount of formazan formed by the survivor cells was compared with survivor counts and zone of inhibition method. The dose-response plot for the TTC-based bacteriocin assay was linear over a wide range bacteriocin concentration. A high correlation ($R^2 > 0.95$) was between viable cell count and TTC reduction for three of the bacteriocins tested. The new assay can be completed in one hour, compared to one or two days with microbiological assays. Overall, the procedure is simple and easy to carry out.

Key words: Bacteriocin; 2,3,5-triphenyltetrazolium chloride (TTC); TTC-based bacteriocin assay, Nisin, Lactacin A

Submitted: August 30, 2022. **Accepted:** September 16, 2022.

Cite this: Albayrak N. Tetrazolium-Based Rapid Colorimetric Assay For Bacteriocin Activity. JOTCSB. 2022;5(2):91–110.

***Corresponding author. E-mail:** nedimalbayrak@hitit.edu.tr.

INTRODUCTION

By definition, bacteriocins are ribosomally synthesized proteinaceous compounds showing a bactericidal mode of action against sensitive cells (1,2). Most LAB bacteriocins are active against a wide range of gram positive bacteria (3).

Although various bacteriocins have been discovered, isolated, and identified, methods for quantifying bacteriocin activity have remained with limited precision. The limitations of the available bacteriocin assay methods have been recognized in several review articles (1,4–8). Almost all methods for qualitative or quantitative determinations of bacteriocin activity are based on the microbiological assays (7). Microbiological assays depend on the inhibition of the growth of the sensitive microorganism to determine the concentration of an inhibitory substance (9,10). The methods used for determination of bacteriocin activity are usually derived from those for antibiotics. However, bacteriocins differ from antibiotics especially due to the chemical compositions and mode of actions. The

majority of antibiotics are bacteriostatic, thus they only affect the growth rate of bacteria rather than killing them. Bacteriocins, on the other hand, are protein in nature and exhibit a very rapid killing effect. Therefore, the methods used for antibiotics might not be appropriate for bacteriocins.

Most widely used methods for bacteriocin activity are modifications of agar diffusion assays (spot, well, disc diffusion) and turbidimetric assays. Since standard reference compounds for bacteriocins are not generally available, critical dilution methods are more often applied with arbitrary units to express the activity. This method is a semi-quantitative, provides only a discontinuous scale for activity and always requires a complete series of dilutions to estimate activity (11). Although this method is simple and easy to use, detection of the inhibition zone, corresponding to the highest two-fold dilution, is error-prone. There is always an uncertainty of the decisive end-point, which leads to great inaccuracy in the calculations of bacteriocin activity (4,12). In turbidimetric measurements, the main drawback is that the curve rises sharply from low to high responses within a very short dilution span, which significantly limits the useful dose range and the accuracy of the method (10,11).

Any method that is used to detect bacterial growth or cell viability can be applied to the measurement of the interaction between antimicrobial agents and bacteria. Even though cell viability is conventionally defined as the reproductive capability of the cell, several characteristics of viable cells could be measured and used to estimate the reproductive ability (13–15). For instance, the determinations of membrane integrity and/or membrane potential can be associated with viability (15). In a broad sense, the alternative approaches to viability assays include measurements of metabolic activities, redox potential, electrical conductivity, microcalorimetry, radiometry, and bioluminescence (5).

Tetrazolia have been known as vital stains and employed in histochemistry particularly for localizing oxidative enzymes in biological systems (9,16). Reduction of tetrazolium salts in living organisms is caused by enzyme activity (16–18). These salts accept electrons from oxidized substrates such as NADH and NADPH (19) by means of intracellular dehydrogenase activity of viable cells (15,20). In this system, tetrazolium salts are taken by bacterial cells and reduced into its original red-colored formazan products. Even though the tetrazolium salts can enter both viable and dead cells, only viable organisms (metabolically active) have the ability to reduce these salts into formazan (4,11,15).

The presence of cell membrane potential attracts TTC to enter the cells. TTC has one positive charge and three phenyl groups around the four-nitrogen-containing ring. Thus, the molecule possesses lipid-like and fat-like properties, which helps TTC first attracted to cells and then enter the membrane. Uptake of tetrazolium salts depends on the basic cellular structure of bacteria. Between charged and uncharged state there is potential (O/R potential) about - 0.08 V. Due to the potential difference TTC is attracted to the cells. The potential difference across the bacterial membrane is typically 0.1-0.2 V (15). In bacteria, the enzymes of energy metabolism are located on the inner surface of the cytoplasmic membrane where the reducing power (NADH) of the metabolism is converted into ATP (21,22). When H is removed from NADH (oxidation) by a particular dehydrogenase enzyme (e.g., lactate dehydrogenase), H is taken by the tetrazolium salt (reduction) leading to the formation of formazan which no longer possesses the positive charge nor the bond between the nitrogen atoms (23). It was found that most cellular reduction of tetrazolium salts was dependent on the reduced pyridine nucleotides, NADH and NADPH (17). After reduction, formazan deposits, coalesces, and becomes larger deposits located in the cytoplasm (24).

Our goal was to develop a rapid and accurate quantitative assay for bacteriocin activity. We mainly propose that the characteristic of 2,3,5,-triphenyltetrazolium chloride (TTC) can be applied to quantitate the bacteriocin activity in liquid environment. The absorbance of red-colored formazan after extraction from the cells can be determined spectrophotometrically. If the sensitive cells are exposed to the bacteriocin in a range of concentrations, a correlation should exist between the viable cell counts (survivor count) and survivors' ability to reduce TTC into formazan. Such a method should allow rapid, accurate, and more sensitive determination of the bacteriocin activity.

MATERIALS AND METHODS

Media and Indicator Microorganisms

MRS broth was prepared by dissolving 55 g of Lactobacilli MRS (Difco) in 1 L of distilled water. Portions of 50 mL of MRS broth in 160-mL milk dilution bottle (Fisher Scientific, Pittsburgh, PA) were autoclaved at 120 °C for 20 mins. MRS agar and MRS soft agar were prepared by adding 15 and 7.5 g/L of agar (Difco) in MRS broth, respectively.

Lactobacillus delbrueckii subsp. *lactis* ATCC 4797 was used as a sensitive indicator for nisin and lacidin A (25), and *Lactobacillus bulgaricus* OSU 135 was the indicator for pediocin PO2 (26). Cultures were

obtained from the culture collection of the Food Microbiology Laboratory, The Ohio State University, Columbus, OH. The cultures were maintained in MRS containing 10% glycerol and stored at -18 ^{o}C. The cultures were propagated twice in MRS and incubated at 37 ^{o}C for 18 hours before use.

MRS broth was inoculated with an overnight culture at a rate of 0.1% (10^4-10^5 CFU /mL). The inoculated medium was incubated for 12 hours in a water bath at 37 ^{o}C. After 12 h of incubation, portions of the culture were centrifuged at 10,000 rpm for 5 mins at 20 ^{o}C. The supernatant was removed and the cell pellet was resuspended to the original volume using phosphate buffer at pH 6. An amount of 700 µL of the resuspended culture was dispensed into a microcentrifuge tube for the TTC-based bacteriocin assay.

Preparation of Bacteriocins

A commercial preparation of nisin was obtained from Sigma Chemical. The preparation contains 2.5% nisin, which provides 10^6 IU nisin per g (27). Stock solution of nisin were prepared by adding 0.1 g of the powder to 1 mL of 0.05 M citrate-phosphate buffer at pH 4. The solution was filter-sterilized using a 0.22 µm pore size filter (Gelman Sciences). This solution was used to prepare various dilutions of nisin using the former buffer.

The bacteriocins Lacidin A and pediocin PO2 were produced by *L. acidophilus* OSU 133 and *Pediocactus acidolactisi* PO2, respectively. The bacteriocins were partially purified using a chloroform extraction. Stock solutions of the purified bacteriocins, or dilutions thereof, were made using phosphate buffer (pH 6). All the stock solutions and dilutions were kept frozen until use.

TTC solutions

The reagent 2,3,5,-triphenyltetrazolium chloride (TTC) was obtained from Fisher Scientific in a dehydrated state. Stock solution 1% (w/v) was prepared using 0.05 M phosphate buffer at pH 6.0. The solution was filter-sterilized using a 0.22 µm pore size filter and kept frozen in the dark. The assay solution concentration was 0.2% (w/v) and prepared from the stock solution with the same sterile buffer.

Optimizing Assay Conditions

TTC and culture viability

Experiments were done to test the susceptibility of indicator cultures to TTC. The cell suspension of strain 4797 was prepared as indicated earlier, and equal volumes (750 µL each) of 1% TTC solution and the culture were mixed. The mixtures were incubated in a water bath at 37 ^{o}C. Sample tubes were removed after 10, 20 and 90 minutes of incubation and plate count on MRS agar was performed. The experiment included two replicates of the test and blank samples which received the TTC solution and pH 6 phosphate buffer, respectively.

Culture growth

MRS broth (50 mL) was inoculated with ~10^4 CFU/mL from the culture 4797, which has been transferred twice. The inoculated MRS broth was incubated in a water bath at 37 ^{o}C. Samples were taken at 1-h intervals, and tested for cell count, turbidity, and TTC-reducing capacity by following the TTC reduction procedure.

TTC concentration

In order to determine the optimum TTC concentration, a broad range of concentrations was studied. Cell suspension of indicator microorganism was prepared and divided into six portions. The concentrations of 25, 50, 100, 500, 1000, 2000, 4000, and 5000 ppm of TTC were prepared from the stock solution using phosphate buffer (pH 6) as a diluent. Each of these concentrations was used as the TTC assay solution in the TTC reduction procedure and the results were compared.

Incubation period

The minimum time necessary for the uptake of TTC and its reduction to the formazan particulate was studied. The indicator culture 4797 was mixed with TTC assay solution and tested using the TTC reduction assay as indicated earlier. After 10, 20, 30, 40, 50 and 60 minutes of incubation, samples corresponding to the incubation period were removed from the water bath and tested according to the TTC reduction procedure. Five replicates of this experiment were performed using the same batch of the culture.

pH

In order to determine the effect of pH during the course of TTC reduction, pH values in the range of 3 to 9 were tested. Buffers with pHs of 3, 4, 5, 6, 7, 8, and 9 were prepared. For pH 3.0, 4.0, and 5.0, citrate-phosphate buffer was used (made by mixing 0.05 M solutions of citric acid monohydrate (Fisher) and dibasic phosphate (Mallinckrodt) until the desired pH is reached). For pH 6.0 and 7.0, phosphate buffer was used (made by mixing 0.05 M solutions of monobasic (Mallinckrodt) and dibasic phosphates until the desired pH is obtained). For pH 8.0 and 9.0, Tris buffer was used (made by mixing 0.05 M solutions of Tris (Fisher) and Trizma base (Sigma) until the desired pH is adjusted). A pH meter (Fisher Scientific) was used in all pH adjustments and measurements. These buffers were used for the pH adjustments of the indicator culture and the preparation of TTC solutions. The indicator microorganism was propagated as indicated and divided into seven portions. Each portion was centrifuged to remove the

medium, and the cells were resuspended in the buffer solution of desired pH. For each pH, three replicates were prepared. The stock solution of 1% TTC was also prepared in the buffer solutions of the desired pH values. Same buffers solutions were used to prepare 0.2% TTC assay solutions. Procedure for TTC reduction was done at different pH values.

Reduction Procedure of TTC
A portion (750 µL) of the indicator microorganism cell suspension was mixed with 750 µL of TTC assay solution. The mixture was incubated at 37 °C for 20 minutes in the dark. After the incubation, the cells (containing the red-colored formazan particulates) were centrifuged at 10,000 rpm for 5 min at 20 °C and supernatant was removed. Formazan in the cell pellet was extracted using 750 µL of methanol with grinding and agitation. The mixture was centrifuged at 10,000 rpm for 5 min to remove cell debris. The amount of the reduced dye in the extract was measured at OD_{485} against methanol as a reference solution.

Absorbance was measured using a spectrophotometer (Spectronic 1201). For the removal of the medium from the culture, SS-34 Rotor of Sorvall RC-5B Refrigerated Superspeed Centrifuge was used. For other centrifugations, a microcentrifuge (Biofuge A) was used.

TTC-based Bacteriocin Assay Procedure
The cell suspension of culture (700 µL) and the bacteriocin solution (100 µL) were mixed in microcentrifuge tubes and incubated at 37 °C for 30 min. After the incubation, 100 µL volume of the mixture was removed for survivor count. TTC assay solution (700 µL) was added to the remaining volume of bacteriocin-treated culture in the tubes. The mixture was incubated at 37 °C for 20 min in the dark. After the incubation, the mixture was centrifuged at 10,000 rpm for 5 mins to collect the cells. The cell pellets were received with 700 µL of methanol and were grinded with a pellet pestle (Science ware Micro Centrifuge Sample Pestle, Fisher) to extract formazan from cells. The solvent culture mixture was centrifuged at 10,000 rpm for 5 mins to remove cell debris. The absorbance of the extracted red color was measured at OD_{485} against methanol as a reference.

Survivor Count Method
For the determination of Colony Forming Unit (CFU) / mL, 100 µL of the culture was mixed with 900 µL of sterile peptone-water saline (0.85% NaCl, and 0.1% peptone-water (Difco)). Additional decimal dilutions were prepared similarly. Portions (100 µL) of the desired dilutions were spread onto MRS agar in triplicates. The CFU counts were obtained by counting the plates having colonies between 30 and 300 after the incubation for 48 hours at 37 °C.

Critical Dilution Method
Overnight culture of the sensitive microorganisms (*Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 or *Lactobacillus bulgaricus* OSU 135) were used to seed the liquefied soft agar at ca. 10^6 CFU/per mL of soft agar. The tubes were mixed well and 5 mL amounts were overlaid onto the MRS agar in petri dishes. The prepared petri dishes were refrigerated for 1 h and thereafter were spotted with the appropriate bacteriocin dilutions. Two-fold dilutions of bacteriocins were prepared as indicated earlier and 5 µL from each dilution was spotted onto the agar overlay. The plates were incubated at 37 °C for 24 hours (3). Bacteriocin activity was measured using the highest dilution showing complete inhibition zone of the indicator lawn. Bacteriocin potency was expressed as activity units, AU/mL (27,28). The results were also read as diameters of inhibition zones in mm and calculated as area of inhibition zone.

Data Analysis
Statistical analysis of the results was done using the Minitab software. The significance was determined by one way (ANOVA) and means were compared using Tukey's pairwise comparisons at 0.05 probability level. The linear regression equations and correlation coefficients obtained were used for estimation of bacteriocin concentrations.

RESULTS

Assay Development
Applying TTC as a tool to estimate the number of viable cells, rather than qualitative measure of viability, requires an understanding of the process of TTC reduction by the cultures. Thus, prior to developing TTC-based assay for bacteriocins, several parameters that may affect the reduction were studied. During the study, the factors in question were varied one at a time. After each step, the preliminary assay conditions were modified and standardized so as to serve as an ultimate procedure for the determination of bacteriocin activity. Although TTC reduction may measure intracellular dehydrogenase activity, our goal was to use this reaction as an indication of viability under standardized conditions of TTC concentration, incubation temperature, pH and culture age. The following are the factors of significance to the assay development and the obtained results.

Optimization of Assay Parameters
The growth of *L. delbrueckii* subsp. *lactis* ATCC 4797 was monitored for 14 hours of incubation by three methods, (a) plate count (CFU/mL), (b) absorbance at

$OD_{600\ nm}$, and (c) TTC reduction ($OD_{485\ nm}$). Since the growth of microorganisms follows first order kinetics, the logarithm of the responses of three methods were plotted against incubation period (Figure 1). The culture displayed regular batch growth kinetics.

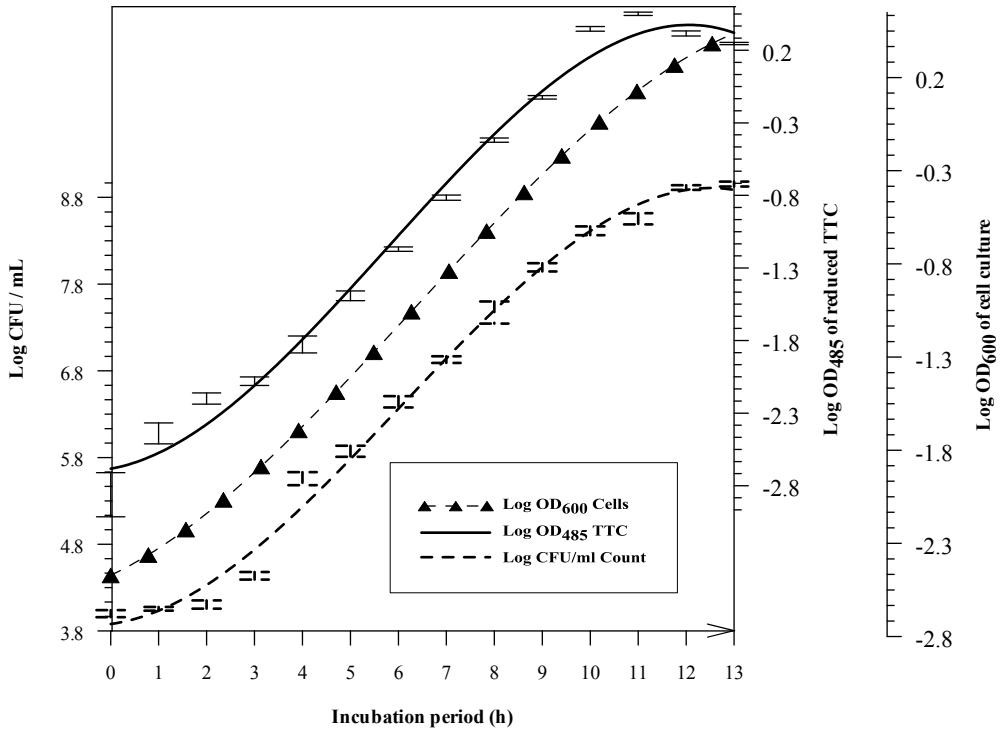

Figure 1: Growth of *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 culture monitored by plate counting (log CFU/mL), optical density (log OD at 600 nm) and TTC reduction (log OD at 485nm) after 20 min of incubation with 0.2% TTC at 37 °C. The curves indicate the best fit obtained by polynomial regression analysis of the results of three trials. The error bars show the standard deviations of the counts and TTC reduction.

The change in log CFU/mL during the first four hours was insignificant ($P > 0.05$). During that time, TTC reductions were low (absorbance between 0.001 and 0.01). The low cell count may cause this small TTC reduction and large variability in results. During the same period, $OD_{600\ nm}$ increased from 0.007 to 0.02, but this change was not significant. In fact, OD_{600} did not significantly change from the initial value until the CFU reached the 10^6 level (data not shown).

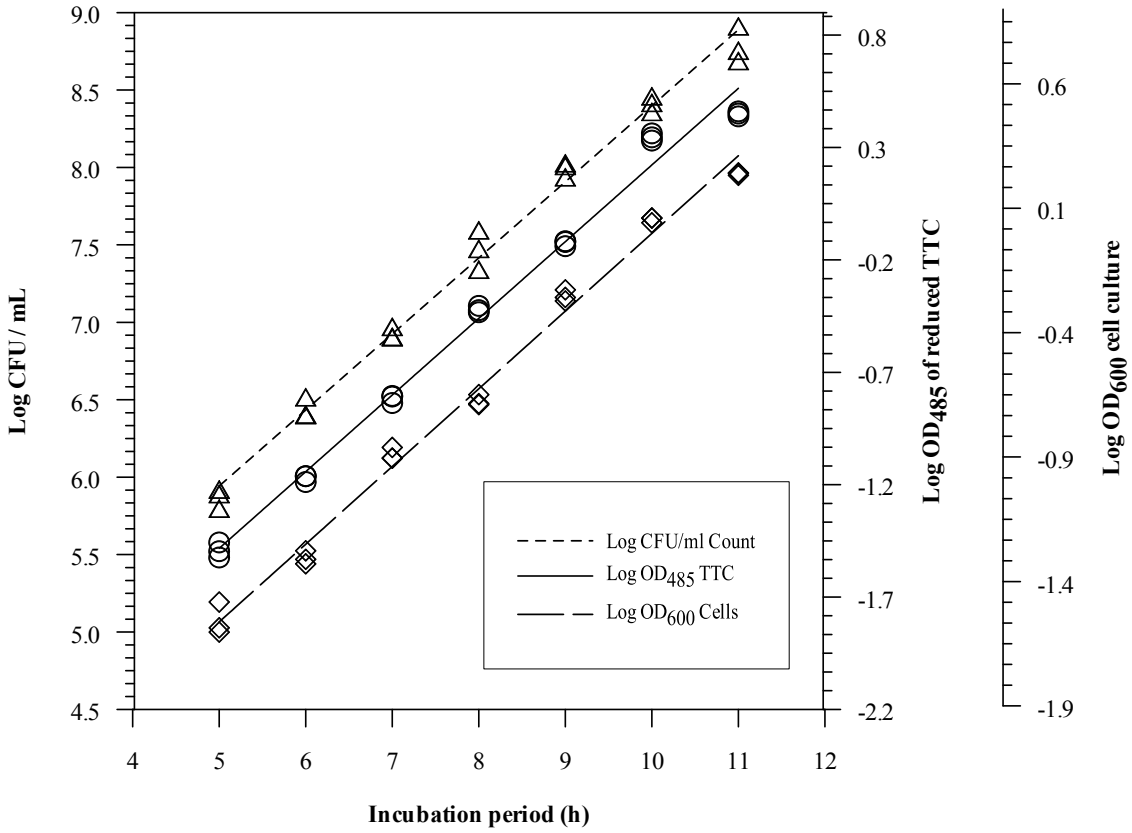

Figure 2: Growth of *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 culture between fifth and eleventh hours as measured by plate counting (CFU/mL), optical density (OD at 600 nm) and TTC reduction (OD at 485 nm after 20 min incubation with 0.2% TTC at 37 °C), (results of three independent trials).

At approximately the fifth hour of incubation, the culture began the exponential phase as indicated by the changes in log CFU/mL. This phase lasted about six hours (Figure 2). TTC reduction and OD_{600} increased with increasing plate counts until the log of CFU reached 8.5 - 8.8, which was approximately the late exponential phase of the growth. During the exponential phase, the linear growth can be clearly seen by means of the three methods (Figure 2), in which the correlation coefficients as log of response versus time were the highest ($R^2 > 0.99$). After the eleventh hour, the changes in plate counts were not significant ($P > 0.05$) and the number remained almost constant for two hours (Figure 1). However, a slight but gradual decline was observed with TTC reduction, which probably resulted from a decrease in the amounts of NADH during the stationary phase. The turbidity, on the other hand, continued to increase at a slower rate (Figure 1). When the experiment was repeated using different inoculation rates and TTC concentrations, similar results were observed (data not shown).

During the entire growth period, the correlation coefficient between log CFU and log OD_{600} was 0.96, and log CFU and log OD_{485} was 0.97 (data not shown). During the exponential growth, both R^2 results were 0.99. Overall, these results suggest that TTC reduction can be used to monitor growth instead of conventional methods. Considering the range of the linear growth (Figure 1) where metabolic activity of the culture is uniform and constant, the results were found very promising that TTC reduction could be applied to monitor viable cell counts when the number of cells is reduced by a bactericidal agent.

Based on the results of this experiment, the 12-h-old culture has the maximum TTC reduction ability. Therefore, twelve-hour-old cultures, prepared with the same inoculation rate, were used for the entire study and during all trials of bacteriocin testings. By doing so, the variations in TTC reduction associated with culture age and metabolic activity were minimized. In this state, cells possess maximum activity of reducing enzymes as well as NADH (29), which are the primary cause of the TTC reduction.

Even though tetrazolium salts have been recognized

as vital stains, they are also known to have some inhibitory effect (15). Thus, in order to rule out any inhibitory effect of TTC, the culture 4797 prepared as indicated in earlier was treated with 1% TTC and the count was monitored for up to 120 min. However, the difference in count between the treatment and control was insignificant ($P > 0.05$).

The effect of TTC concentrations was tested. As TTC concentration increased, color formation first increased rapidly then slowed down and leveled off (Fig 3). The results were best described by power 2 polynomial regression ($R^2 > 0.99$), which probably suggest that the reduction of TTC resembles the substrate effect on enzymatic reactions where the reaction follows the first order kinetics at low substrate concentrations (i.e., when substrate is limiting). At higher concentrations, however, the enzyme becomes a limiting factor and reaction follows zero order kinetics (8). Although TTC reduction may measure intracellular dehydrogenase activity, our goal is to use this reaction as an indication of viability under standardized conditions of TTC concentration, incubation temperature, pH and culture age. Therefore, 0.2% TTC concentration was chosen as an optimum concentration for measurable color formation under the standardized conditions.

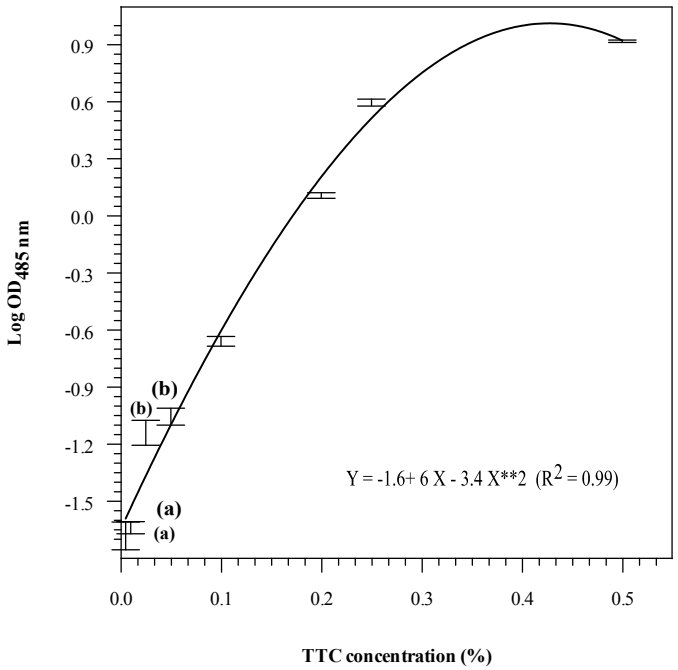

Figure 3: Color formation (measured at OD 485nm) when the cells of *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 were treated with different concentrations with of TTC. Error bars indicate standard deviations from the mean of three trials. Data points with similar superscripts were not statistically different ($P > 0.05$).

Incubation period for TTC refers to the time permitted for the uptake of the salt by the cells and its reduction to the colored dye. Experiments were performed to determine a minimum time necessary for this process. The reduction of TTC increased with increasing incubation periods (Figure 4). 20 minutes of incubation were chosen as a suitable period for adequate formation of formazan. The results were best described by linear regression ($R^2 > 0.99$) suggesting that the reaction of TTC at 0.1% concentration follows zero order kinetics.

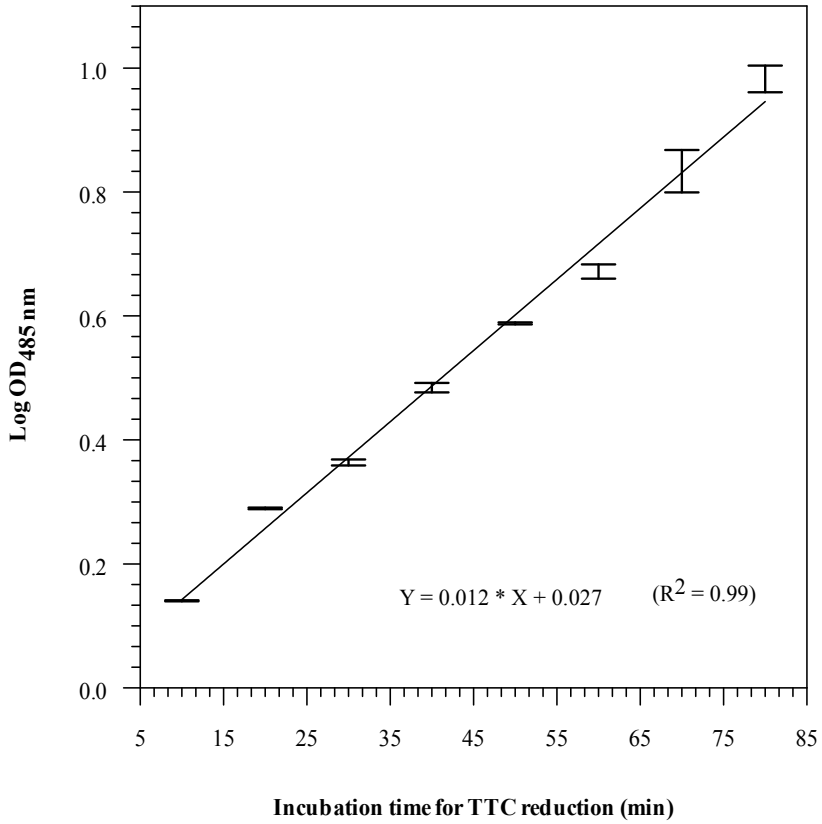

Figure 4. Reduction of 0.1% TTC, measured as color formation at OD 485nm, by *Lactobacillus delbrueckii* subsp*. lactis* ATCC 4797 after different periods of incubation at 37 °C. Error bars indicate the standard deviations from the means of two trials.

TTC reduction at pH values in the range of 3 to 9 was tested. The color formation reached the maximum at pH 6. Overall, the curve resembles to general effect of pH on enzyme activity where the activity increases as pH increases from low to optimum, and decreases as pH increases from its optimum point (data not shown).

For accurate spectrophotometric determination of the color resulting from intracellular reduction of TTC, extraction of formazan from the cells is necessary. Several solvents including methanol, ethanol, and acetone were compared in effectiveness and speed of extracting the color. Methanol was the most effective and the fastest color extractor among solvents tested (data not shown).

TTC-based Bacteriocin Assay

By taking the former factors into account, the TTC reduction procedure was standardized for the application in bacteriocin activity estimation. Bacteriocin activity was assayed by three methods (a) zone of inhibition, (b) survivor count method, and (c) TTC reduction. The responses obtained from the three methods were compared over a wide range of bacteriocins potency (two-fold dilutions). The results for nisin, lacidin A and pediocin PO_2 are summarized in Tables 1, 2 and 3, respectively. The unknown potency of bacteriocins were determined by critical dilution method. The results are expressed as AU/mL, which is determined from the reciprocal of the highest dilution possessing a clear zone (13,30). The AU/mL results as independent variable were used in dose-response curves (Figs. 5, 6 and 7).

Table 1: The survivor responses as log CFU/mL, log OD at 485 nm and diameter of inhibition zone (mm) obtained from two-fold dilution of nisin when used against *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797. Decline in responses are subtracted values of each survivor response from the last significant survivor response.

Two-fold	Survivor Response (Log)		Decline in Response (Log)(*)		Zone Diameter (mm)	
Nisin dilution	CFU/mL	OD_{485}	CFU/ml	OD_{485}	without TTC	with TTC
2^0	5.20 ± 0.07	-2.02 ± 0.07 [a]	3.84	2.62	23.33 ± 0.28	22.83 ± 0.28
2^{-1}	6.17 ± 0.05	-1.95 ± 0.16 [a]	2.86	2.55	22.16 ± 0.28	21.83 ± 0.28
2^{-2}	7.80 ± 0.05	-0.81 ± 0.08	1.23	1.41	19.66 ± 0.57	19.83 ± 0.28
2^{-3}	8.76 ± 0.03	0.32 ± 0.04	0.27	0.28	15.66 ± 1.52	17.50 ± 0.50
2^{-4}(**)	9.04 ± 0.05 [b]	0.60 ± 0.02 [c]	0.00	0.00	10.33± 1.15 [d]	12.66 ± 0.57 [e]
2^{-5}	9.08 ± 0.03	0.62 ± 0.01			0.00 ± 0.00	0.00 ± 0.00

(*) Decline in Response = { Log of Survivor Response to the last significant dose (b or c) - Log of Survivor Response to a dose}
{e.g., Decline in "Log CFU/ml" at 2^{-1} dilution =(9.04 - 5.20) = 3.84 } or {e.g., Decline in "log OD_{485}" at the same dilution =[0.60 - (-1.95)] = 2.55

(**) The highest dilution corresponding to last detectable zone of inhibition (d or e).

[a] The change in absorbance was not significant ($P > 0.05$).

[b, c] The last significant survivor response. At higher dilutions, change in responses were insignificant ($P > 0.05$).

[d, e] The last detectable zone of inhibition.

The zones of inhibition determinations were obtained from the agar plates that were used to determine AU/mL by the critical dilution method. This was done for comparison purposes and illustration of the diffusion characteristics of different bacteriocins. As zone diameters and standard deviations seen from the Table 1, 2 and 3, different bacteriocins displayed different agar diffusion kinetics. Nisin diffused very well and produced the largest and well-defined zones among the bacteriocins tested (Table 1). Nevertheless, this large zone decreased rapidly in size as the bacteriocin became more dilute. As nisin's dose was lowered by two-fold dilutions, the decline in diameter of the zone of inhibition was greater than the other bacteriocins. The fourth dilution was the last zone displaying always at least 10 mm zone diameter (Table 1). Lacidin A preparation produced zones that also decreased in size with dilution but at a smaller rate than with nisin (Tables 1 and 2). The zones of lacidin A lasted until the sixth dilution (Table 2). Pediocin PO_2 (Table 3) showed similar diffusion and zone characteristics like lacidin A. The highest concentration of nisin (2^4 x 200 = 3200 AU/mL) produced ~ 23 mm zone of inhibition and 3.8 log CFU reduction (Table 1). On the other hand, lacidin A at the highest concentration (2^6 x 200 = 12800 AU/mL) showed ~ 18 mm zone of inhibition and 5.2 log CFU reduction. Thus, based on these results, the presence of less potency of nisin or the presence of relatively high potency of lacidin A did not seem to be reflected as zones of inhibition.

Table 2: The survivor responses as log CFU/mL, log OD at 485 nm and diameter of inhibition zone (mm) obtained from two-fold dilution of **lacidin-A** when used against *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797. Decline in responses are subtracted values of each survivor response from the last significant survivor response.

Two-fold	Survivor Response (log)		Decline in Response (Log)(*)		Zone Diameter (mm)	
Lac.-A dilution	CFU/mL	OD_{485}	CFU/ml	OD_{485}	without TTC	with TTC
2^{0}	3.53 ∓ 0.12	0.04 ∓ 0.009	5.26	0.54	17.50 ∓ 0.50	18.66 ∓ 0.57
2^{-1}	4.20 ∓ 0.08	0.13 ∓ 0.009	4.59	0.45	17.33 ∓ 0.57	16.50 ∓ 0.50
2^{-2}	5.30 ∓ 0.04	0.23 ∓ 0.006	3.50	0.35	15.83 ∓ 0.76	15.66 ∓ 0.57
2^{-3}	6.13 ∓ 0.12	0.33 ∓ 0.009	2.66	0.25	14.66 ∓ 0.57	14.33 ∓ 0.57
2^{-4}	6.64 ∓ 0.12	0.49 ∓ 0.007	2.15	0.09	12.66 ∓ 0.57	13.33 ∓ 0.57
2^{-5}	8.08 ∓ 0.13	0.55 ∓ 0.011	0.71	0.03	8.66 ∓ 1.15 (c)	9.33 ∓ 0.76
2^{-6} (**)	8.80 ∓ 0.07 (a)	0.59 ∓ 0.014 (b)	0.00	0.00	0.00 ∓ 0.00	5.66 ∓ 2.08 (d)
2^{-7}	8.94 ∓ 0.05	0.60 ∓ 0.009			0.00 ∓ 0.00	0.00 ∓ 0.00

(*) Decline in Response = { Log of Survivor Response to the last significant dose (b or c) - Log of Survivor Response to a dose}
{e.g., Decline in "Log CFU/ml" at 2^{-3} dilution = (8.80 - 6.13) = 2.66 } or {e.g., Decline in "log OD_{485}" at 2^{-1} dilution = (0.59 - 0.13) = 0.45 }

(**) The highest dilution corresponding to last detectable zone of inhibition (d).

(a, b) The last significant survivor response. At higher dilutions, change in responses were insignificant ($P > 0.05$).

(c, d) The last detectable zone of inhibition.

Table 3: The survivor responses as log CFU/mL, log OD at 485 nm and diameter of inhibition zone (mm) obtained from two-fold dilution of pediocin PO_2 when used against *Lactobacillus bulgaricus* OSU 135. Decline in responses are subtracted values of each survivor response from the last significant survivor response.

Two-fold	Survivor Response (log)		Decline in Response (Log)(*)		Zone Diameter (mm)	
Ped.PO_2 dilution	CFU/mL	OD_{485}	CFU/ml	OD_{485}	without TTC	with TTC
2^{0}	6.08 ± 0.08	-0.89 ± 0.015	3.27	1.12	12.16 ± 0.3	12.63 ± 0.3
2^{-1}	6.97 ± 0.05	-0.64 ± 0.015	2.38	0.87	11.00 ± 0.0	10.83 ± 0.2
2^{-2}	7.54 ± 0.09	-040 ± 0.006	1.81	0.63	9.16 ± 0.2	9.17 ± 0.2
2^{-3}	8.07 ± 0.07	-0.22 ± 0.007	1.28	0.28	8.00 ± 0.0	8.16 ± 0.2
2^{-4}	9.11 ± 0.03	0.14 ± 0.005	0.24	0.08	6.16 ± 0.2 (c)	6.66 ± 0.1
2^{-5} (**)	9.35 ± 0.03 (a)	0.22 ± 0.003 (b)	0.00	0.00	0.00 ± 0.0	5.17 ± 0.2 (d)
2^{-6}	9.38 ± 0.02	0.23 ± 0.002			0.00 ± 0.0	0.00 ± 0.0

(*) Decline in Response = { Log of Survivor Response to the last significant dose (b or c) - Log of Survivor Response to a dose}
{e.g., Decline in "Log CFU/ml"at 2^{-2} dilution = (9.35 - 7.54) = 1.81 } or {e.g., Decline in "log OD_{485}" at 2^{-1} dilution = [0.22 - (-0.64)] = 0.87 }

(**) The highest dilution corresponding to last detectable zone of inhibition (d) with TTC.

(a, b) The last significant survivor response. At higher dilutions, change in responses were insignificant ($P > 0.05$).

(c, d) The last detectable zone of inhibition.

As indicated earlier, the TTC reduction and survivor counts were determined using the same set of dilutions of bacteriocins. Means and standard deviations of survivors' response determined by plate counts (log CFU/mL) and TTC reduction (log OD at 485 nm), are also displayed in Tables 1, 2, and 3. The significance of each response (i.e., change of response as change of dose in two fold) corresponding to the same dilution were verified by analysis of variance (one-way ANOVA).

For a meaningful comparison of the results from these

different method results, survivor responses were converted into relative values, and expressed as the decline in response. Decline in response is obtained by subtraction of the $\log_{10}$ of the response of a given dose from the $\log_{10}$ of the response of the zero dose. This relative value can also be called as 'the proportionate response' (10). In our experiments, the last detectable response was considered as the response to zero dose.

Since the area of inhibition (mm^2) is more relevant than zone diameter (mm) (31), the zone area values were used to compare with other methods. For calculation of the area of zone of inhibition, the radius of the zone, obtained from the critical dilution method with TTC, were squared and multiplied by 3.14, π-value. The area of zone of inhibition were plotted against AU/mL in Figs. 5, 6 and 7 for nisin, lacidin A and pediocin PO_2, respectively.

Comparison of Different Methods

The decline in cell count and TTC reduction were plotted against the concentrations of bacteriocin (log AU/mL obtained from the critical dilution method). The dose-response curve of nisin, lacidin A, and pediocin PO_2 were shown in Figures 3, 4, and 5, respectively. The correlation coefficients (R^2) and equations among the responses of the methods compared were summarized in Table 4. Overall, all of the responses of the methods shared the curvature characteristic of dose response curves. This was clearly observed with nisin (Figure 3). Therefore, the linear regression fit for nisin was established by only using three data points in the central range. However, in the case of lacidin-A (Figure 4) and pediocin PO_2 (Figure 5), the difference between the linear and polynomial regressions was negligibly close. Therefore, the linear fits were obtained by including all of the significant responses and used to describe the dose-response curves.

Based on the results of nisin dose-response curves, the correlation coefficients for TTC reduction, CFU count, and area of inhibition zone were 0.99, 0.98 and 0.97, respectively (Figure 5). Even though TTC reduction showed the best result in the central regions, the change in OD_{485} from zero to first dilution was not significant ($P > 0.05$) where CFU decline was evident (Figure 5A). Thus, this result probably suggests that the curve of TTC-based assay may not be useful at high doses of nisin. Overall, the correlations (Table 4) of the TTC reduction result with CFU and with the area of inhibition zone suggest that TTC-based assay provides apparent comparable results with the other methods despite the fact that higher and lower doses showed variations.

Unlike nisin, lacidin-A and pediocin PO_2 probably have a different killing kinetics, where the similar two-fold change in doses was reflected as a smaller rate of change in response. In other words, the linear dose-response curve of nisin was steeper than those of the other bacteriocins.

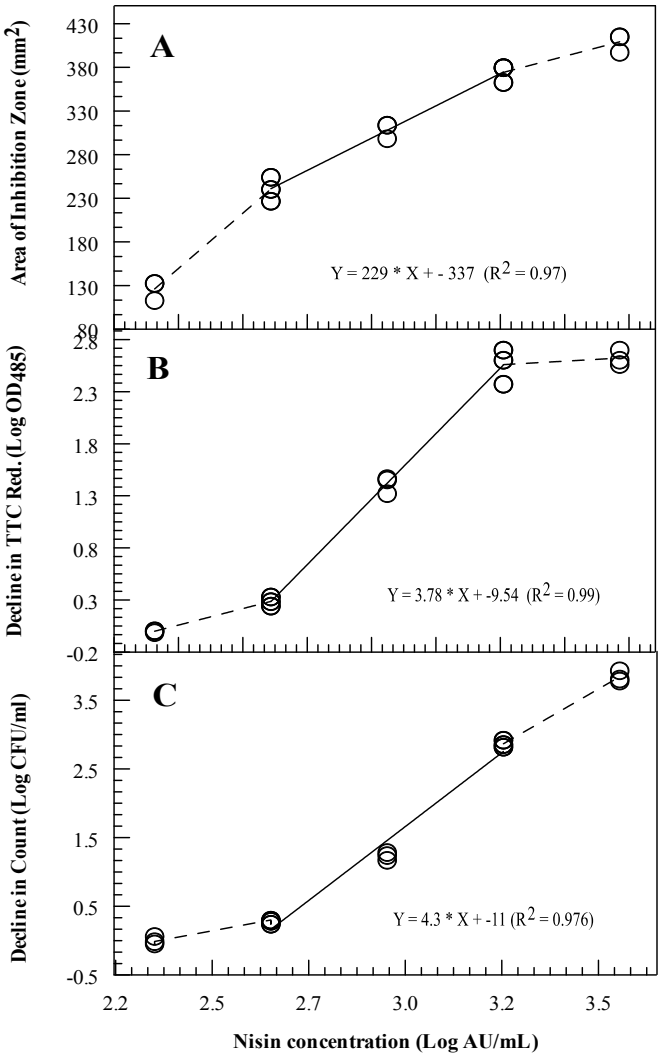

Figure 5. Nisin dose vs. area of inhibition zone (A), and decline in the responses of TTC reduction (B) and cell count (C) against indicator culture *L. delbrueckii* subsp. *lactis* ATCC 4797. Nisin activity (AU/mL) was calculated from the reciprocal of the highest dilution corresponding to the last detectable zone on agar medium with TTC. Symbols indicate the results of three independent trials, and the solid lines were obtained by linear regression fit with excluding the responses from the highest and the lowest nisin dilutions.

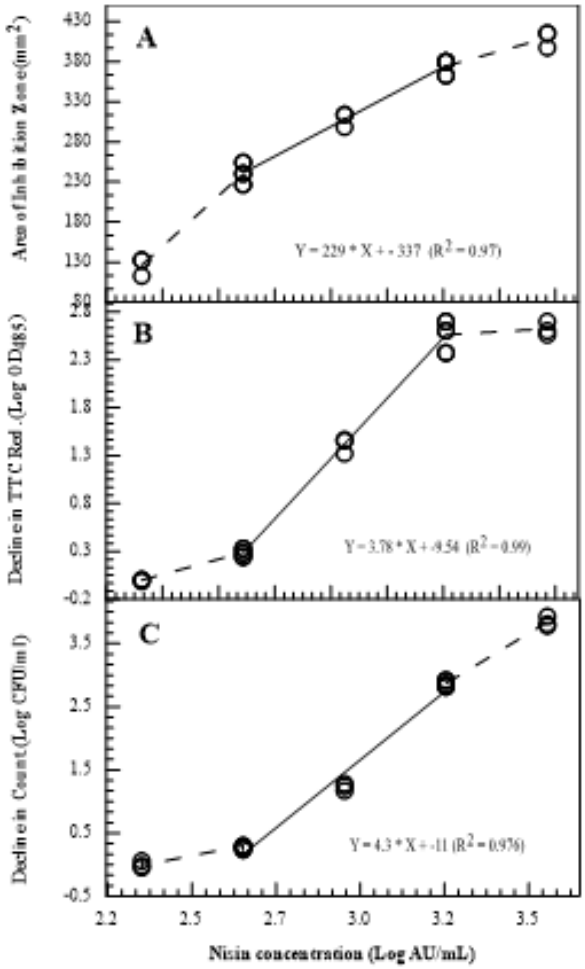

Figure 6. Lacidin-A dose vs. area of zone of inhibition (A), and the decline in responses of TTC reduction (B) and cell count (C) against indicator culture *L. delbrueckii* subsp. *lactis* ATCC 4797. Lacidin A activity (AU/mL) was calculated from the reciprocal of the highest dilution corresponding to the last detectable zone of inhibition on agar medium containing TTC. Symbols indicate the results of three independent trials.

Considering the dose-response curves for Lacidin-A (Figure 6), correlation coefficients (R^2) for CFU count, TTC reduction and the area of zone diameter were 0.99, 0.98, and 0.95. With small variations, a linear change was apparent in CFU (Figure 6A). Although the TTC reduction results (Figure 6B) were described by a linear fit, a gradual leveling off can be seen at lower dose levels. At higher dose levels, the data fit into an almost straight line. The lacidin-A was partially purified and the highest possible doses were applied yet the sigmoid shoulder at higher dose levels was not detected. This result probably suggest that TTC-based assay may provide linear responses at higher dose levels than that we used. The relationship between the relative TTC and CFU responses (Table 4) was highly correlated (R^2 = 0.96), but the correlation was lower (R^2 = 0.90) with the area of inhibition zone. Thus, TTC-based assay of lacidin-A provided comparable results with the cell count method.

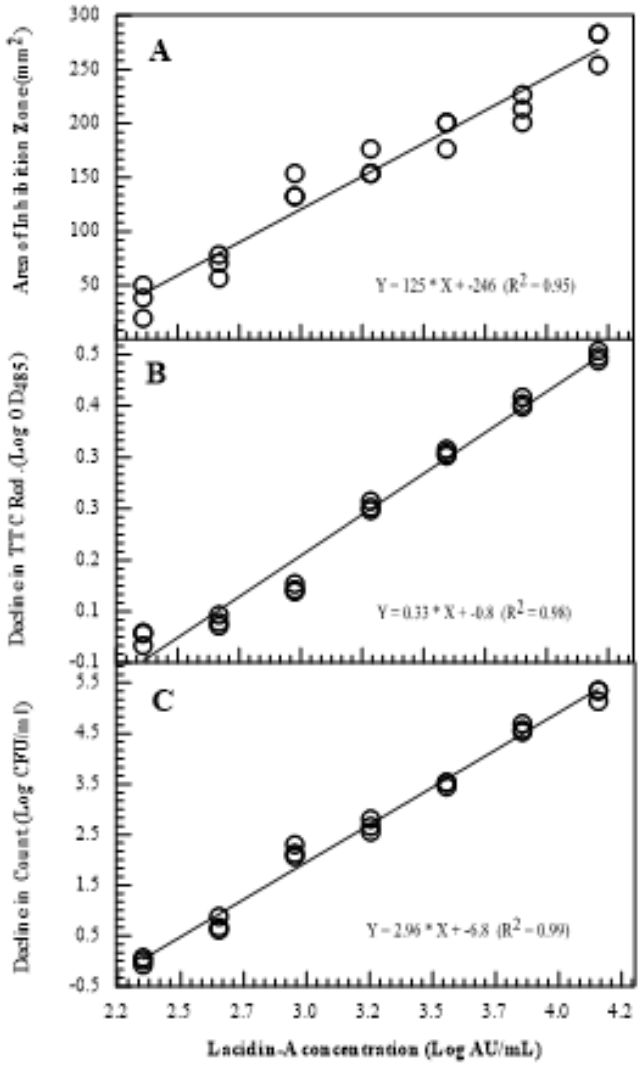

Figure 7. Pediocin PO2 dose vs. area of inhibition zone (A), and the decline in responses of TTC reduction (B) and cell count (C) against indicator culture *L. bulgaricus* OSU 135. Pediocin PO2 activity (AU/mL) was calculated from the reciprocal of the highest dilution corresponding to the last detectable zone of inhibition on agar medium containing TTC. Symbols indicate the results of three independent trials.

Overall, pediocin PO_2 dose-response curves and correlations were similar to those of lacidin-A. As shown in Figure 7, the correlation coefficients of CFU count at part A and TTC reduction at part B were high (0.98). The area of the zone of inhibition had relatively lower correlation coefficients (0.96). Like lacidin-A, a sigmoid shoulder at higher dose levels pediocin PO_2 seemed undetectable. Overall, the correlation coefficients between the TTC reduction result and log CFU/mL (Table 4) were high (R^2 = 0.97) and with the area of zone of inhibition was relatively close yet lower (R^2 = 0.95). Thus, TTC-based assay for pediocin PO_2 produced highly comparable results with the other methods.

Table 4. The correlation coefficients of the methods, TTC represents Decline in TTC reduction (log OD at 485 nm), CFU represents Decline in cell count (log CFU/mL) and MM^2 represents the area of zone of inhibition (mm^2). The results were obtained from two-fold dilution of nisin and lacidin-A against *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797; pediocin PO_2 against *Lactobacillus bulgaricus* OSU 135. The equations and correlation coefficients were generated from the corresponding data in Tables 1, 2, and 3.

	Correlations of the methods		
Bacteriocins	**X: CFU vs. Y: TTC**	**X: TTC vs.Y: MM^2**	**X: CFU vs. Y: MM^2**
Nisin	$Y = 0.86 * X + 0.17$ **(R^2=0.97)**	$Y = 58.5 * X + 225$ **(R^2 = 0.97)**	$Y = 50.3 * X + 235$ **(R^2= 0.95)**
Lacidin-A	$Y = 0.11 * X + -0.045$ **(R^2 = 0.96)**	$Y = 369 * X + 63$ **(R^2 = 0.90)**	$Y = 42.2 * X + 41$ **(R^2= 0.95)**
Pediocin PO_2	$Y = 0.35 * X + -0.031$ **(R^2= 0.97)**	$Y = 66 * X + 19$ **(R^2 = 0.96)**	$Y = 24 * X + 16$ **(R^2= 0.95)**

In conclusion, TTC-based assay for lacidin-A and pediocin PO_2, compared with the count method, can produce measurable responses at a wide range of doses of the bacteriocins. TTC-based assay for nisin provides better results at narrower range and lower dose levels. Overall, TTC reduction results were better correlated with log counts than that of with the area of inhibition zone. Under the testing conditions, it could also be concluded that different bacteriocins displayed not only different agar diffusion characteristics but probably different killing kinetics as well.

DISCUSSION

The most commonly used agar plate assay to estimate the unknown potency of bacteriocins is the critical dilution method where the end-point determination is considered (4). Although it is quite simple, this method has limitations and lacks precision. Detection of the inhibition zone, corresponding to the highest two-fold dilution, is error-prone. This leads to impreciseness in the calculations of bacteriocin activity (4). In fact, this inherent uncertainty of the decisive end-point can be considered as the biggest problem of this assay (12).

In this study, we determined bacteriocin activity (AU/mL) using the critical dilution method. The same indicator culture (4797) and MRS agar medium were used for nisin and lacidin A in the critical dilution method. Therefore, the comparison of these two bacteriocins may reveal relative degrees of susceptibility, different killing effects or different diffusion characteristics. Nisin activity was 3200 AU/mL which revealed larger zone of inhibition than that of lacidin A with activity of 12800 AU/mL. As the result, potency cannot be compared by diameters of inhibition zones. Therefore, the absolute value (mm) of zone of inhibition should not be used to compare the efficacy or potency of different bacteriocins.

TTC was used in the detection and counting of bacteria and it was reported that maximum dose of TTC allowing growth of gram positive and negative bacteria are 0.001% and 0.05%, respectively (16). Eidus et al., (32) investigated the effect of the concentrations of four different tetrazolia. The level for optimal staining occurred at about 50% of the toxic level. Tetrazolia varied in staining patterns, optimal concentration and toxicity to organisms. It was indicated that TTC is the least toxic to cells.

Tetrazolia were used for viability observations and qualitative determinations without solubilization or extraction of the colored formazans (4,24,33). In commercial cell proliferation kits that insoluble formazan of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) needs to be solubilized by dimethyl sulfoxide before the determination of color by an ELISA reader. Similarly, without solubilization, we could not have achieved sensitive absorbance measurements. Thus, we tried to solubilize the color with several solvents. It was found that methanol was the most effective.

Colorimetric procedures is based on the extraction of the biologically produced insoluble formazan by organic solvents and measurement of absorbance with spectrophotometers (18). There have been various colorimetric viability assays developed using various tetrazolium salts for different cell line or tissue

cell cultures of animal and plant origin for different purposes (34–39). For example, by employing MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), Morgan (39) was reported a colorimetric procedure to evaluate viability of tissue culture. It was indicated that water-soluble MTT is taken up by the viable cells and produced water-insoluble blue formazan by the action of mitochondrial dehydrogenases. The formazan was extracted by organic solvents. Similar to our observations, it was found that formazan production is directly proportional to cell number. Despite coinciding basic approach and taking the benefit of tetrazolium, our study differs from previous assay for direct evaluation for bacteriocin activity and comparison with currently used methods.

After examining the factors that affect the reduction of TTC by bacterial cells, a TTC-based bacteriocin assay was developed. The assay was applied for the estimation of bacteriocin activity in conjunction with survivor count and agar diffusion methods.

Microbiological assay is defined as a technique whereby the potency of an antimicrobial agent may be determined by its effect on the growth of a microorganism (10). Based on this definition, the TTC-based bacteriocin assay should not be considered as a microbiological assay due to the fact that it does not depend on the growth inhibition of the sensitive culture nor is the culture permitted to grow in liquid or agar medium. Instead, the proportion of the cells survived the treatment as a function of bacteriocin concentration is evaluated indirectly by the survivors' ability to reduce TTC. However, the procedure shares some disadvantages of microbiological assays in that survivors' viability is involved. Furthermore, this procedure could not be defined as a chemical method either, because it measures the activity of bacteriocin on the sensitive culture. Therefore, it does not share the disadvantages of chemical procedures in which reflection of activity of the compound on the culture is missing.

In recent years, new methods for quantification of nisin have been investigated. Assay methods based on the release of ATP from sensitive indicator cells, use of antibodies developed against purified nisin, and HPLC analysis have been investigated (27,29). Amount of nisin, measured by chemical means, may not correlate well with the activity of the bacteriocin. For example, nisin is quite stable to pH changes, yet the activity of nisin is greatly affected by pH (27), which may not be verified by chemical methods. This considerations have long been the case with antibiotics where bioassays have remained as the standard method for years (10). In the case of antibody-based enzyme assays which are currently developed, the minimum detectable concentration of bacteriocin is quite high, e.g, for nisin and pediocin, it was found 19,000 and 32,000 AU/mL, respectively (40).

There have been some confusions about the definition or interpretation of a quantitative assay. Some defined it as a measurable response such as mm zone inhibition of unknown potency (28,41). Others only defined it as the comparison of the responses at the same range between standard reference compound and the sample whose potency is to be determined (10). However, the standard procedures only use the term 'quantitative' when the reference standard is employed. For instance, the current procedure for the estimation of nisin in processed cheese is based on the agar diffusion and formation of zone of inhibition by the sample extract and the reference standard of nisin (12,41).

The microbiological assays, e.g., zone of inhibition measurements and turbidimetric determinations, can provide quantitative results only when a reference standard is used (4,10). Thus, any method can provide quantitative results as long as reference is used in conjunction with samples. There are several possibilities for expressing the results of the TTC-based bacteriocin assay. Bacteriocin potency may be expressed quantitatively as g/mL or ppm, but this requires the reference standard of bacteriocins. When the pure bacteriocins are available, TTC based assay can become a powerful tool to estimate the potency of bacteriocins. In the absence of a standard, one may express the bacteriocin activity using TTC-based assay to estimate AU/mL directly from the equations established in the dose-response curves. Then, measured AU can be expressed as IU (1 IU nisin = about 100 AU) for nisin and other bacteriocins (27,42).

Another arbitrary option could be a determination of some type of a median response. Median response is generally used in turbidimetric assays and defined as the dose of the inhibitory substance that permits the growth of the organism to 50% level of the zero dose under specified conditions (10). In the case of bactericidal agents, the median response is usually referred as a lethal unit, the level of 50% killing. Considering the highly significant correlation (R^2 > 0.95) between the number of cells remained viable and their ability to reduce TTC for the three bacteriocins we tested (Table 4), one can easily convert one measurement to another. Thus, the unknown potency is then quantified as the number of cells killed under the standardized test conditions.

The results of the TTC based assay can also be expressed as the amount of formazan formed by

indicator cells with or without bacteriocin when TTC reduction is tested under standardized conditions. The amount of formazan can be deduced from the standard curve depicting 2,3,5-triphenyl formazan concentration vs absorbance at 485 nm. In fact, this standard curve was obtained yet not used (data not shown). In this study, we used changes in OD_{485} to measure the activity of bacteriocins.

When compared to the current bacteriocin potency determination methods, the TTC based procedure is simple and easy to perform. Moreover, the procedure is not costly; TTC is one of the least expensive tetrazolia available. It also does not require expensive equipment nor high skills. In practice, a variety of assay volumes of tube size can be used. However, we preferred using 1.5 mL microcentrifuge tubes because of two centrifugation steps in the procedure. Thus, the centrifugations can be made directly without any transfer of assay liquid. The usage of small volumes allowed rapid heat transfer in water bath, and the use of micropipettes for all transfers. The filter sterilized stock TTC solutions can be stored frozen in small volumes and diluted with sterilized buffer to the desired assay concentration in one step.

Time saving is main advantage of TTC-based bacteriocin assay. With the other methods, using either agar or broth medium, the bioassay requires 24-48 h not including the time for preparation of the culture. In the TTC-based assay, all steps other than culture preparation, take about one hour. The ability to estimate bacteriocin potency in a short period provides great convenience for studies on bacteriocin purification, and in the determination of the optimum growth conditions (e.g., media, temperature, pH) and the productivity of bacteriocin producer strains. In these steps, investigator specifically looks forward to obtaining results in short period to be able to make necessary modifications earlier than the time is taken with current methods. There are some semi-automatic and automatic microbiological methods which are based on turbidity determinations. It was indicated that automation shortens the time up to 6 h and offers a potential for less technical errors and increase in accuracy (5,27). On the other hand, automatic methods do not assess microbial killing well. For many microorganisms, two to three log kill cannot be detected. TTC-based assay has several advantages over automatic and regular turbidimetric assays. In addition to time saving, TTC-based assay is more accurate than turbidimetric assays in that only viable cells contribute to the color formation. TTC-based method can detect up to five log decrease in count due to the action bacteriocin. Lastly, the results of TTC-based assay are less likely to be affected by bacterial clumping or aggregation.

Zone of inhibition and critical dilution tests cannot be used to differentiate whether the bacteriocins have bactericidal or bacteriostatic effect (5,7). Additionally, the diffusion of highly hydrophobic antimicrobial agents on agar becomes limited (10,12,32,43). Measuring the bactericidal effect, in general, requires the use of viable or survivor count method (4,7). Survivor count method provides measurable responses to a wider range of dose levels compared to other methods. Due to the higher detectable range of response, survivor count methods are considered more accurate than the other methods (9,32,43). The linear range is the most useful portion of dose-response curves for the assay purposes, yet generally it corresponds to only a narrow range of dose levels, which is the major problem with turbidimetric analysis (9,10,32). A clear medium is necessary for absorbance measurements otherwise the supplements of the medium may mislead the results (1). This point becomes very important in the case of opaque supplements of medium which are often required by fastidious microorganisms (5). However, in order to detect the changes in turbidity in liquid culture by spectrophotometer, at least 10^6 CFU / mL of indicator suspension is generally required (5,13,32). Formation of the clumps and chains affects the results of viable counts and turbidimetric methods (10,13,44).

Flamentation can result in an increase in turbidity where aggregation of bacterial cells can be caused by some antimicrobial agents as the number of viable cells decrease (5). Absorbance measurements can correlate with the number of cells in suspension, but it is unlikely to discriminate viable from non-viable cells (44). In this regard, optical measurements were found to be proportional to the dry weight of the culture rather than the number of viable cells (4,44).

In the TTC-based assay the metabolic activity of the culture is used as a measure of viability. It is well known that metabolic activity of the sensitive culture can change greatly with changes in the growth state. However, if the culture is harvested while the cells at the same growth phase, the metabolic response will be related directly to the number of viable cells, which is a function of the potency of the bacteriocin.

In terms of detection limits of bacteriocins, results from the TTC based assay cannot be compared with others in literature because of differences in assay conditions, sensitive strains, extraction procedure, and pH of media. For example, in the case of nisin, even though the actual nisin concentration in two-fold dilutions can be calculated from the original preparation, the effectiveness of the extraction of nisin from the commercial powder (containing 2.5% nisin) could not be determined. Thus, the results were not expressed as IU or µg of nisin /mL. For

comparison purposes, the expression AU/mL was thought to be more accurate. The equivalent of nisin was 14,300 IU/ per mL of cells at the highest concentration. The detection limit was the fourth dilution exhibiting last zone of inhibition from the critical dilution of the commercial nisin. This result agreed with the CFU count and TTC reduction in which CFU and absorbance increments were significant ($P < 0.05$) up to the fifth dilution. After that dilution, the changes were not significant suggesting that the three procedure provided similar detection limits.

Assays for antimicrobial agents usually show a sigmoidal dose-response curve when the dose has a wide range. To apply these assay methods, it is critical to choose dose levels within the linear intermediate concentrations or 'sensitive region' of the response curve where small changes in concentration give maximum change in response (10). Therefore, the linear equation for nisin dose-response curve was obtained using only the central region (Figure 3). For lacidin A and pediocin PO2, the linear fits were obtained by using all of the significant responses of TTC-based assay. Even though polynomial regressions showed higher R^2 than those of linear, the difference between the regressions were negligibly close in the central region of the plot.

In the case of TTC-based assay, the response can be obtained over a wide range of bacteriocin concentrations. The survivor cell concentration, however, should be high enough to produce a detectable color. Test sensitivity decreases when cell concentrations are lower than 10^6. Therefore, cell concentration of 10^7 and higher is preferable. It is worth to note that the parameters can easily be modified for different conditions such as low number of cells versus either increasing incubation time with TTC or higher concentration of TTC. If the number of initial indicator cells is reduced, lower bacteriocin concentrations can be detectable and the assay may become more sensitive.

REFERENCES

1. Jack RW, Tagg JR, Ray B. Bacteriocins of Gram-Positive Bacteria. Microbiol Reiveeww [Internet]. 1995;59(2):171–200.

2. Klaenhammer TR. Bacteriocins of lactic acid bacteria. Biochimie. 1988;70(3):337–49.

3. Mayr-Harting A, Hedges AJ, Berkeley RCW. Methods for Studying Bacteriocins. Methods Microbiol. 1972;7(April):315–422.

4. Mattila T. A modified Kelsey-Sykes method for testing disinfectants with 2,3,5-triphenyltetrazolium chloride reduction as an indicator of bacterial growth. J Appl Bacteriol. 1987;62(6):551–4.

5. Provonchee RB, Zinner SH. Rapid method for determining serum bactericidal activity. Appl Microbiol. 1974;27(1):185–6.

6. Tagg JR, Dajani AS, Wannamaker LW. Bacteriocins of gram positive bacteria. Bacteriol Rev. 1976;40(3):722–56.

7. Vistica DT. Tetrazolium-based assays for cellular viability: A critical examination of selected parameters affecting formazan production. In: Cancer Research. 1991. p. 2515–20.

8. Skyttä E, Mattila-Sandholm T. A quantitative method for assessing bacteriocins and other food antimicrobials by automated turbidometry. J Microbiol Methods. 1991;14(2):77–88.

9. Hewitt W, Vincent S. Theory and Application of Microbiological Assay [Internet]. San Diego, California.: Elsevier; 1989. 1–202 p.

10. Hoover DG, Harlender SK. Screening Methods for Detecting Bacteriocin Activity. In: Bacteriocins of Lactic Acid Bacteria [Internet]. Academic PressS, INC; 1993. p. 23–39.

11. Piddock LJV. Techniques used for the determination of antimicrobial resistance and sensitivity in bacteria. J Appl Bacteriol. 1990;68(4):307–18.

12. Hurst A. Nisin. In: Advances in applied microbiology [Internet]. 1981. p. 85–123.

13. Cavalieri SJ, Harbeck RJ, McCarter YS, Ortez JH, Rankin ID, Sautter RL, et al. Manual of Antimicrobial Susceptibility Testing [Internet]. Marie B. Coyle (University of Washington), editor. American Society For Microbiology. 2005. 1–241 p.

14. Cutler RR, Wilson P, Clark F V. Evaluation of a radiometric method for studying bacterial activity in the presence of antimicrobial agents. J Appl Bacteriol [Internet]. 1989;67(6):515–21.

15. Shuler M, Kargi F. Bioprocess Engineering Basic Concepts. Second Edi. Englewood Cliffs, New Jersey: Prentice Hall PTR; 2002. 61–172 p.

16. Nissen-Meyer J, Holo H, Havarstein LS, Sletten K, Nes IF. A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. J Bacteriol. 1992;174(17):5686–92.

17. Berridge MV, Tan AS, McCoy KD, Wang R. The Biochemical and Cellular Basis of Cell Proliferation Assays That Use Tetrazolium Salts. Biochemica. 1996;4:14–9.

18. Stockert JC, Horobin RW, Colombo LL, Blázquez-Castro A. Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives. Acta Histochem [Internet]. 2018;120(3):159–67.

19. Worsfold M, Marshall MJ, Ellis EB. Enzyme detection using phenazine methosulphate and tetrazolium salts:

Interference by oxygen. Anal Biochem. 1977;79(1–2):152–6.

20. Sahl HG, Kordel M, Benz R. Voltage-dependent depolarization of bacterial membranes and artificial lipid bilayers by the peptide antibiotic nisin. Arch Microbiol. 1987;149(2):120–4.

21. Nineham AW. The Chemistry of Formazans and Tetrazolium Salts. Chem Rev [Internet]. 1955 Apr 1;55(2):355–483.

22. Şenöz H. Formazanlar ve Tetrazolyum Tuzlarının Kimyası. Hacettepe J Biol Chem. 2012;40(3):293–301.

23. Debnam PM, Shearer G. Colorimetric assays for substrates of NADP+-dependent dehydrogenases based on reduction of a tetrazolium dye to its soluble formazan. Anal Biochem. 1997;250(2):253–5.

24. Zimmermann R, Iturriaga R, Becker-Birck J. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl Environ Microbiol. 1978;36(6):926–35.

25. Liao CC, Yousef AE, Chism GW, Richter ER. Inhibition of Staphylococcus aureus in buffer, culture media and foods by lacidin A, a bacteriocin produced by Lactobacillus acidophilus OSU 133. J Food Saf [Internet]. 1994 May;14(2):87–101.

26. Liao CC, Yousef AE, Richter ER, Chism GW. Pediococcus acidilactici PO2 Bacteriocin Production in Whey Permeate and Inhibition of Listeria monocyfogenes in Foods. J Food Sci. 1993;58(2):430–4.

27. Ray B. Nisin of Lactococcus Lactis ssp. Lactis as a Food Biopreservative. In: Food Biopreservatives of Microbial Origin. 1992. p. 207–64.

28. Parente E, Brienza C, Moles M, Ricciardi A. A comparison of methods for the measurement of bacteriocin activity. J Microbiol Methods. 1995;22(1):95–108.

29. Voet D, Voet JG. Biochemistry. 2nd edt. John Wiley and Sons; 1995.

30. Cabo ML, Murado MA, González MP, Pastoriza L. A method for bacteriocin quantification. J Appl Microbiol. 1999;87(6):907–14.

31. Roszak DB, Colwell RR. Survival strategies of bacteria in the natural environment. Microbiol Rev [Internet]. 1987 Sep;51(3):365–79.

32. Eidus L, Diena BB, Greenberg L. Observations on the use of tetrazolium salts in the vital staining of bacteria. Can J Microbiol. 1959;5(3):245–50.

33. Weibuli C. Observations On The Staining Of Bacillus Megaterium With Triphenyltetrazolium. J Bacteriol [Internet]. 1953 Aug;66(2):137–9.

34. Vistica DT, Skehan P, Scudiero D, Monks A, Pittman A, Boyd MR. Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. Cancer Res [Internet]. 1991 May 15;51(10):2515–20.

35. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods. 1986;89(2):271–7.

36. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods [Internet]. 1983 Dec;65(1–2):55–63.

37. Takeuchi H, Baba M, Shigeta S. An application of tetrazolium (MTT) colorimetric assay for the screening of anti-herpes simplex virus compounds. J Virol Methods. 1991;33(1–2):61–71.

38. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. Cancer Res [Internet]. 1987 Feb 15;47(4):943–6.

39. Morgan DM. Tetrazolium (MTT) assay for cellular viability and activity. Methods Mol Biol. 1998;79(4):179–83.

40. Bhunia AK. Monoclonal antibody-based enzyme immunoassay for pediocins of Pediococcus acidilactici. Appl Environ Microbiol. 1994;60(8):2692–6.

41. Benkerroum N, Ghouati Y, Sandine WE, Tantaoui-Elaraki A. Methods to demonstrate the bactericidal activity of bacteriocins. Lett Appl Microbiol. 1993;17(2):78–81.

42. Ray B. Pediocin(s) of Pediococcus acidolactici as a food biopreservatives. In: Food Biopreservatives of Microbial Origins. 1992. p. 265–319.

43. Davidson PM, Parish ME. Methods for testing the efficacy of food antimicrobials. Food Technol. 1989;43(1):148–55.

44. Daeschel MA. Procedures To Detect Antimicrobial Activities Of Microorganisms. In: Food Biopreservatives of Microbial Origin. Florida.: CRC Press; 1992. p. 58–77.