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Assessment of *Lactobacillus spp.* Populations from Tuba Inoculated in different Beverages

Rey G. Tantiado[1]*, Jacinth L. Legario[1] and Connie C. Durana[1]

[1]*College of Arts and Sciences, West Visayas State University, La Paz, Iloilo City, Philippines*
*reygtantiado@yahoo.com(*corresponding author)*
jacinthlegario@yahoo.com, conniedurana@gmail.com

Abstract

This study isolated Lactobacillus spp. from tuba and inoculated in four selected canned beverages, beer, coffee, pineapple juice, and soft drink stored. Serial dilution and spread-plate methods were used to assess the population count of Lactobacillus isolates on Rogosa agar incubated in anaerobic condition for 48 hours. Lactobacillus counts were monitored in a 28-day sampling period together with the pH condition of the beverage used. Results showed that Lactobacillus spp. from tuba survived in four canned beverages with an acceptable viable count that ranged from log 5-6 CFU/ml and with a pH ranged from 3 to 4 at the end of the sampling period. Bacterial population count showed no significant difference in the different types of beverages, p=0.053>0.05. A medium positive correlation between the count of Lactobacillus spp. and pH in beverages was observed, rho (0.450). Thus, the Lactobacillus from tuba was able to survive in the different beverages during storage at room temperature, therefore implying that these beverages could be good vehicles for probiotics.

Keywords*:* beverage, *Lactobacillus spp., population count, pH, Tuba*

1. Introduction

1.1. Background of the Study

Tubâ or coconut wine is a sweet exudate from tapped unopened spathe of coconut [3] and dirty brown in color, containing 10-12 % sugar, mainly sucrose [14]. Upon storage tubâ turns to a whitish, effervescent, acidic alcoholic beverage.

Tubâ in the Philippines is commonly referred to as mnazi [15]. The coconut sap starts fermenting immediately after assortment, due to natural yeasts in the air (typically spurred by residual yeast left in the gathering container) [38]. Within two days, fermentation yields a fragrant wine of up to 4 % liquor con tent, mildly intoxicating and sweet. The coconut wine, tubâ may be enabled to ferment longer, up to a day, to yield a stronger, more sour and acidic taste, which some folks favor [39].

Mnazi, or tubâ, like any other sugary plant sap, can be processed into an alcoholic beverage through fermentation of the sugars present in the sap, yielding alcohol and carbon dioxide [19]. It is sweet, dirty brown in color, containing 10-12% sugar, mainly sucrose [26]. The lactic acid bacteria isolated from mnazi or tubâ were found to be Gram positive and catalase negative [27]. A total of 86 isolates were obtained for preliminary identification. After further screening based on morphological and growth at different conditions, they were identified as *Lactobacillus paracasei ssp. paracasei*. Specifically, 47% were identified as *Lactobacillus paracasei ssp. paracasei* 2, 27% *Lactobacillus paracasei ssp. paracasei* 1 while 7% were identified as *Lactobacillus paracasei ssp. paracasei* 3. Other species included *Lactobacillus plantarum* (13%) and *Lactococus lactis ssp. lactis* 1 (6%) [28, 30, 32, 33].

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Probiotics, live cells with different beneficiary characteristics, have been extensively studied and explored commercially in many different products in the world. Their benefits to human and animal health have been proven in hundreds of scientific research. *Lactobacillus* and *Bifidobacterium* are the main probiotic groups; however, there are reports on the probiotic potential of *Pediococcus, Lactococcus, Bacillus,* and yeasts [13, 28, 29]. Some of the identified probiotic strains exhibit powerful anti-inflammatory, antiallergic and other important properties. Apart from that, the consumption of dairy and non-dairy products stimulates the immunity in different ways. Various food matrices have been used with probiotics, which are briefly documented [13, 32, 33].

Although probiotics have traditionally been added to yogurt and other fermented dairy products [18,20,29], nowadays, there has been an increasing demand for non-dairy probiotic products, and these organisms have been incorporated into drinks, as well as marketed as supplements in the form of tables, capsules, and freeze-dried preparation [4,35].

When probiotics are added into a new probiotic food or drink, many important variables must be considered in order to guarantee viability, which is considered essential for their health benefits [28]. The physiologic state of the probiotics added to food is of considerable importance, and it depends on the time of harvesting of the culture (whether during the logarithmic or stationary phase of growth), on the condition leading to transition to the stationary phase, on the treatment of the probiotics during and after harvesting, and, finally, on the composition of the growth medium in relation to the composition of the food to which they will be added [30, 32]. Thus, probiotic foods or preparations should have an extended shelf life so that they can contain a large number of viable cells at the time of consumption (typically at least 10^6 CFU /g of product) [29].

As mentioned above, current industrial probiotic foods are basically dairy products, which may represent inconveniences due to their lactose and cholesterol content [16, 24]. Recently, new formulates such as fruit juices, cereals, chocolate, ice cream, and desserts appear to be good vehicles for delivering probiotics to humans [11, 12, 19]. In this scenario, the combinations of probiotic strains with different beverages, already positioned as a healthy food product, could be very successful, and the consumption of probiotics could be extended to certain segments of the population such as vegetarians, children, and those who are allergic to dairy products [22]. Researchers have reported that the cell viability in beverages depends on the strains used, the characteristic of the substrate, the oxygen content, and the final acidity of the product [33]. This study seeks to provide a wider variety of consumer products that will provide accessible reach to probiotic products such as drinks or beverages available commercially. This was done by assessing the tolerance of *Lactobacillus spp*. from tubâ on selected commercial beverages through their population counts.

1.2. Objectives of the Study

This study was conducted to isolate *Lactobacillus spp*. from tubâ and to assess their populations incorporated in several types of beverages. It also seeks to determine the population counts of *Lactobacillus spp*. from tuba incorporated in different types of beverages, the pH value of different types of beverages inoculated with Lactobacillus spp. from tuba, the possible significant differences in the population count of *Lactobacillus spp.* from tubâ incorporated in different types of beverages and the possible significant correlation between the population count of the *Lactobacillus spp.* and pH in different types of beverages.

2. Methodology

2.1. Collection of FreshTuba Samples

Tubâ samples were taken from coconut trees in Brgy. Simsiman, Calinog, Iloilo. These samples were placed directly in five sterile plastic bottles, having 100 ml of tubâ in each bottle, as soon as the harvester of tubâ reaches the ground. It was taken from the coconut trees in the afternoon and was immediately brought to the laboratory for *Lactobacillus* isolation, purification, inoculation, and population counting in four beverages.

2.2. Isolation of *Lactobacillus spp*. from Fresh Tuba Samples

About 500 ml of fresh tubâ sample was collected right after harvesting from the coconut tree in the afternoon. The pH of the sample was determined at the sampling site. The samples were kept at 4°C and transported in cool boxes packed with dry ice to the Central Science Laboratory. The 500 ml of tubâ was transferred in sterile glass container and serially diluted and plated in Rogosa agar plates.

For each collected 100 ml tubâ samples, 1ml of tubâ sample was suspended in 9 ml of normal saline solution up to the 10-fold dilution. The mixtures were allowed to settle, and serial dilutions up to 10^{-10} were prepared using sterile normal saline solution and were agitated normally. An aliquot of 0.1 ml of each dilution from 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10} were taken with the use of pipette and were spread evenly over the surface of Rogosa agar medium. The inoculated plates were placed in an anaerobiosis jar with an atmosphere enriched with 5-10% CO_2 [31].

The plates were then placed in heat-resistant cellophane. Moist tissue paper was added for the maintenance of the humidity to the plastic container with burning candle inside it. The candle was then allowed to completely burn off which indicates the full consumption of oxygen present and replaced with carbon dioxide (about 5% CO_2) for the growth of the *Lactobacillus spp.* Plates were incubated at 37 ^{0}C, and monitored after 72 hours.

Representative colonies of *Lactobacillus spp.* bacteria based on colony characteristics as described were picked from incubated plates and purified further by repeated streaking on prepared Rogosa agar plates. The pure cultures of the isolates were maintained on Rogosa agar slants kept at refrigeration temperature (4 ± 1°C).

2.3. Acquisition of Different Beverages

Three samples of each commercial yogurt containing live *Lactobacillus spp.* and 4 types of beverages (limited to one brand only and commonly purchased by consumers) were bought from a local grocery store. After gathering the drinks, the samples were immediately processed in the laboratory. The initial pH of each beverage used was determined with the used of pH paper. Two replicates of each representative beverage were done in three trials.

2.4. Purification of *Lactobacillus spp*

For the purification of tubâ *Lactobacillus spp*., isolates from the Rogosa agar plates were subcultured in a new Rogosa agar plate with the use of sterile inoculating loop. This subculturing process was repeated until pure tubâ *Lactobacillus spp*. isolates were obtained. The purified colonies were described based on the colony characteristics on agar plate as seen with the naked eye. Bacteria colonies were described according to form, elevation, margin, pigmentation or color, appearance, optical characteristics and texture. The isolated pure bacterial strains were preserved at 4 ^{0}C overlaid with sterile mineral oil for further use and maintained for longer period by serial subculture.

2.5. Characterization and Identification of *Lactobacillus spp*

The actual isolation and identification of suspected *Lactobacillus spp.* was carried out in Rogosa agar. Duplicate spread plates and serial dilution were prepared for each sample. The plates were then incubated at 25 ^{0}C for the tubâ and 37°C for the plates for a period of 3 days. Isolates were picked from plates with less than 30 colonies. Pure colonies were obtained by transferring three times from the Rogosa agar plates formerly plated to the nutrient broth. Thereafter, one loopful of inoculum from each tube, which showed positive growth, was streaked onto plates with Rogosa agar for further isolation and purification of cultures. After incubation for 3 days, the same procedure was repeated until three transfers were made. The isolates were Gram-stained and tested for catalase production.

Presumptive *Lactobacillus spp.* was phenotypically characterized by Gram staining, determination of morphology by light microscopy under oil immersion objective, and catalase activity. Only Gram-positive, catalase negative, nonmotile rod and cocci isolates strains were selected [23]. The presence of catalase activity was assessed by the formation of gas bubbles after the suspension of bacterial cells in a droplet of 3% hydrogen peroxide.

2.6. Broth Culture for *Lactobacillus spp*

Successful isolated colonies of tubâ bacteria characterized as bacilli, Gram-positive, and catalase negative were inoculated in 50 ml nutrient broth. It was then incubated at 37 ^{0}C for 72 hours under anaerobic condition to ensure the growth of bacteria. The bacterial growth in nutrient broth was compared to the 0.5 McFarland Standard prior to inoculation in different beverages.

2.7. Inoculation of *Lactobacillus spp*. on Different Beverages as Substrates

About 100 ml of each beverage was inoculated with 50ml broth cultures [10]. The cell suspension obtained from nutrient broth was compared with the 0.5 McFarland Standard containing 1.5 X 10^8 CFU/ml prior to inoculation. About 50 mL of this suspension was inoculated in 100 mL of each beverage [25]. The treated beverages were vortexed to ensure thorough mixing and then incubated at 37ºC for 72 h under anaerobic condition.

Treatment A consisted of 150 ml probiotic drink (*Lactobacillus spp.*) only was used as a positive control group. Treatment B consisted of 50 ml probiotic drink and 100 ml distilled water as the negative control. Treatments C, D, E, and F were used as the experimental groups. Each treatment is composed of one separate beverage (beer, coffee, juice, and softdrink). About 100ml of the beverage was mixed with 50ml culture of the *Lactobacillus spp.* from nutrient broth. The ratio of the mixture is 2:1 of beverage to *Lactobacillus* culture. Treatments were done in 3 trials and 2 replicates each.

2.8. Determination of the Viability of *Lactobacillus spp*. in the Beverage Samples

After 72 hours of incubation at 37°C, the beverage samples (150 ml) were stored at room temperature for four weeks [2]. Samples were taken at the end of every week for 4 weeks. The viability of *Lactobacillus* cultures in probiotic-enriched beverages were determined and expressed as colony forming unit (CFU) per milliliter. Serial dilutions of each of the sample stored at room temperature were prepared and from each beverage. About 0.1 ml of the aliquot was plated out on Rogosa agar medium from all even diluents. The plates were then incubated at 37°C for 72 hours and for four weeks. After incubation, plates with colonies between 30 and 300 were counted using a colony

counter. The experiment was done in duplicate and three trials to determine the population counts of *Lactobacillus spp.*

2.9. Determination of pH

The pH of each beverage sample was determined from day 0 up to the 28-day sampling period. A one milliliter aliquot in duplicate and three trials was submerged in an electrode with temperature probe in the sample of solution and when the reading stabilized, the result was recorded. A pH paper was also dipped in a sample solution wherein after soaking for a few seconds, any color change was compared to the standard color chart of the pH for the value of the pH of each beverage.

2.10. Data Analysis

2.10.1: Descriptive Data Analysis: The population of *Lactobacillus spp.* was assessed through colony counting after 28 day sampling period and 72-hour incubation. The assessment of populations of *Lactobacillus spp.* were recorded in a table and computed for their colony-forming units (CFU) per milliliter of sample using the formula:

$$\text{CFU per ml} = \frac{\text{Total number of colonies}}{\frac{\text{N}}{10} + \frac{\text{N}}{100} + \frac{\text{N}}{1000}} \text{ X Dilution Factor}$$

Where: N = number of replicates

Dilution Factor = first countable dilution

The computed CFU/ml was converted to log CFU to minimize disparities among the values that were used for data analysis. Mean and standard deviation were used to evaluate the population count of *Lactobacillus spp.* and pH in the different treatments. Furthermore, these were used to determine the highest and lowest bacterial count and pH in the different beverages.

2.10.2 Inferential Data Analysis: One-Way Repeated Measures of Analysis of Variance (rANOVA) was used to assess the mean log CFU/ml of *Lactobacillus spp*. for any significant differences in the population count in different beverages. Spearmann rho correlation was used to determine any significant correlation between the population count of *Lactobacillus spp*. and the pH in a 28-day sampling period. The level of significance was set at 5% α level.

3. Results

3.1. Growth and Morphology of Lactobacillus spp. on Rogosa Agar

The isolates were round shape, off-white to creamy color, shiny colonies (Figure 2.B) those were quite similar to the reference *Lactobacillus* spp. grown on MRS agar medium (Figure 1.A). Isolates when Gram stained were found to be rod-shaped, short-medium chain and Gram positive (Figure 1.B) while Figure 2.A shows the typical characteristics of *Lactobacillus spp.*

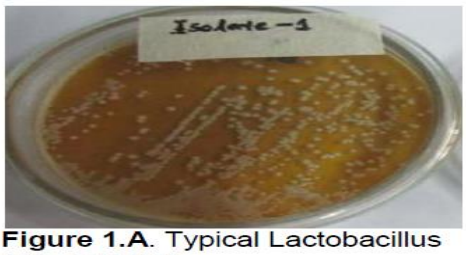

Figure 1.A. Typical Lactobacillus spp. colonies.

Figure 1.B. Lactobacillus spp. colonies from tubâ samples

Figure 2.A. Typical Lactobacillus spp. cells (600X total magnification).

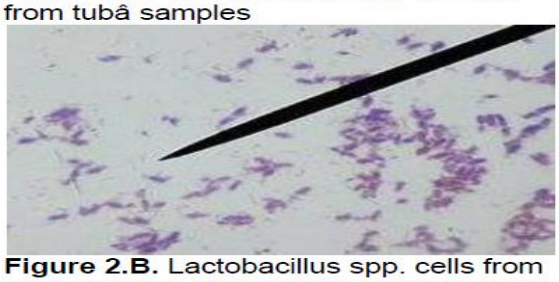

Figure 2.B. Lactobacillus spp. cells from tubâ samples (1000x total magnification).

3.2. Growth of *Lactobacillus spp*. in Different Beverages

All tested *Lactobacillus spp*. were capable of growing well on different beverages without nutrient supplementation over a 28-day sampling period. Figure 4 shows the growth pattern of *Lactobacillus spp.* from tuba in different beverages in a 28-day sampling period. After day 3 of sampling period, probiotic drink has the highest colony count (M = 9.8 log CFU/ ml; SD = 3.27) while after the 28-day sampling period, juice has the highest colony count (M = 6.68 log CFU/ml; SD = 1.32). The beer has lowest colony count (M = 5.16 log CFU/ml; SD = 0.05) after the day 3 sampling period while the softdrink has the lowest colony count (M = 5.08 log CFU/ ml; SD = 0.53) after the 28-day sampling period.

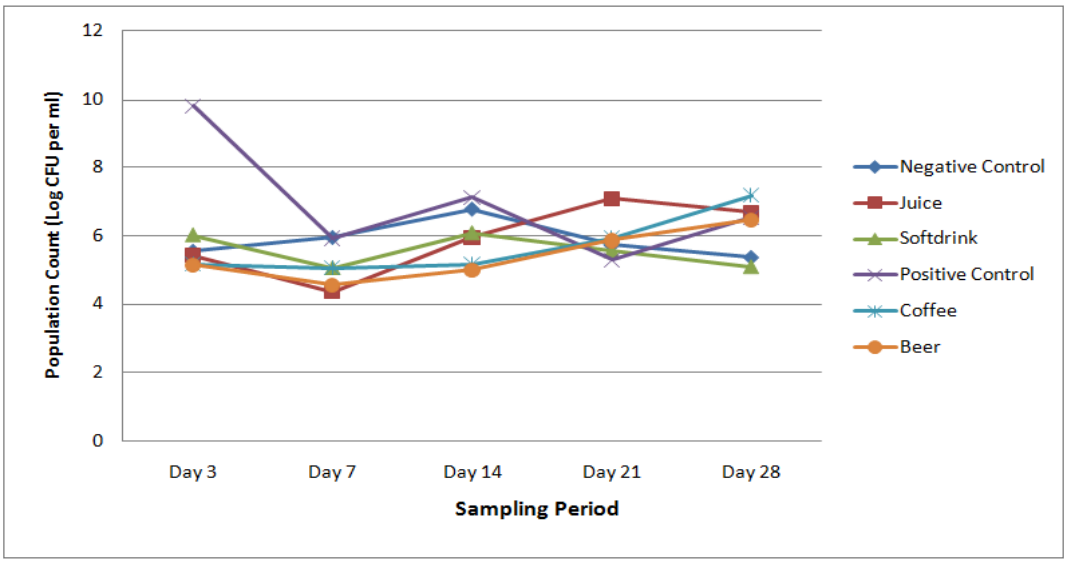

Figure 2. Growth of *Lactobacillus spp*. in different Beverages over a 28-day Sampling Period

3.3. pH of Different Beverages in 28-day Period

Figure 5 shows the pH of different beverages in a 28-day sampling period inoculated with *Lactobacillus spp.* from tuba. At day 0, all the initial pH of the beverages were the same (M=pH 4; SD = 0.000). After day 3 of sampling period, coffee has the highest pH (M = pH 4.33; SD = 0.000) while after the 28-day sampling period, water, probiotic drink, coffee and beer have the highest pH (M = pH 4; SD = 0.000).Water, juice, softdrink, probiotic drink and beer have lowest pH (M = pH 4; SD = 0.000) after day 3 sampling period while the juice and softdrink have the lowest pH (M = pH 3.33; SD = 0.08) after the 28-day sampling period.

Based on the combination of physicochemical factors (temperature and pH) on the growth rate of *Lactobacillus spp.* in different beverages, *Lactobacillus spp.* tolerates best in juice (M log CFU/ml=6.68; M pH=3.33), beer (M log CFU/ml=6.47; M pH=4) and the positive control, probiotic drink (M log CFU/ml=6.53; M pH=4) beverages over a 28-day sampling period. However, *Lactobacillus spp.* tolerates the least in softdrink (M log CFU/ml=5.08; M pH=3.33) after a 28-day sampling period.

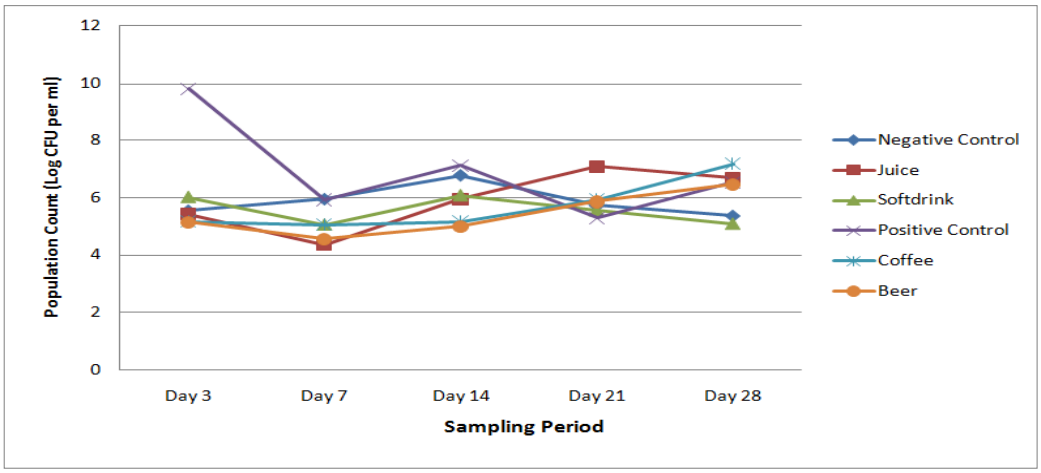

Figure 5. pH of Different Beverages with *Lactobacillus spp*. in a 28-day Sampling Period

3.4. ANOVA with Repeated Measures Tests of Within Subjects Contrasts

Table 1 shows the ANOVA with repeated measures tests of within-subjects contrasts as indicated by the sampling period and the interaction between time and treatment groups. In a 28-day sampling period, there is no significant difference in the population count of *Lactobacillus spp.* over a 28-day sampling period inoculated in different beverages, $F(1,12)=0.668$, $p=0.423>0.05$, partial eta squared=0.054. The effect size is small indicating the measure of the degree to which variability among the observations on the population count of *Lactobacillus spp.* in the different beverages can be attributed to conditions controlling for the subjects effect that were unaccounted. This may imply that the inoculated *Lactobacillus spp.* in different beverages at room temperature have almost identical growth rate in the entire sampling period. However, there is a significant difference in the interaction between time and treatment groups. This is supported by the Least Significant Difference (LSD) for pair wise comparison, a significant difference between softdrink and the positive control ($p=0.017$); between the positive control and coffee ($p=0.029$); between the positive control and coffee (0.029); positive control and beer ($p=0.011$), $p < 0.05$.

Table 1. ANOVA with Repeated Measures Tests of Within-Subjects Contrasts as Indicated by the Sampling Period and the Interaction between Time and Treatment Groups

Source	Time	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared
Time	Linear	1.175	1	1.175	0.688	0.423	0.054
Time*Group	Linear	34.957	5	6.991	4.095*	0.021	0.630
Error (Time)	Linear	20.490	12	1.708			

P< 0.05 is significant.

3.5. One-way Analysis of Variance with Repeated Measures Tests of Between-Subjects Effects

Table 2 shows the One-way Analysis of Variance with repeated measures tests of between-subjects effects of *Lactobacillus spp.* count in different beverages. It shows a no significant difference in the population count of *Lactobacillus spp.* over a 28-day sampling period in inoculated in different beverages, F(5,12)=2.339, p=0.106>0.05, partial eta squared=0.494. The effect size is medium indicating the measure of the degree to which variability among the observations on the population count of *Lactobacillus spp.* in the different beverages can be attributed to conditions controlling for the subjects effect that were unaccounted. This may imply that in general, a no significant difference *Lactobacillus spp.* count in different beverages.

This means that the population of *Lactobacillus spp.* in different beverages did not significantly vary. Thus, this may imply that the growth and survival rate of *Lactobacillus spp.* from tuba samples may be supported by the ingredients present in each beverage used. However, the best survival rate of *Lactobacillus spp.* from tuba best occurs in the positive control (probiotic drink), beer, and juice samples.

Table 2. One-way Analysis of Variance with Repeated Measures Tests of Between-Subjects Effects of *Lactobacillus spp*. Count in Different Beverages

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	3125.824	1	3125.824	1665.950	.000	.993
Group	21.944	5	4.389	2.339	.106	.494
Error	22.516	12	1.876			

P> 0.05 is not significant.

3.6. Correlation between *Lactobacillus spp.* Count and pH

Table 3 shows the relationship between pH and population count (log CFU per ml) was investigated using Spearmann rho correlation coefficient. There was a medium positive correlation between the two variables [r=.45, n=18, p> 0.05] with an acidic environment, low pH favors the growth of *Lactobacillus spp.*

The key characteristics for *Lactobacillus* from tubâ could be attributed to acidogenicity, acid tolerance and synthesis of water insoluble glucan from available sugar content from the medium [17]. A low pH would promote the growth of lactic acid

bacteria in a higher rate causing similar drop in pH [37]. The growth of *Lactobacilli* began to be stable and further declines when pH dropped to 4 or 3 and no significant (P>0.05) growth was observed in any fermentation experiments [42].

Table 3. Spearmann rho Correlation between Log CFU per Milliliter of *Lactobacillus spp.* Population Count and pH

Test Statistic			pH	CFU per ml
Spearman's rho	pH	Correlation coefficient	1.000	.450
		Sig. (2-tailed)	.	.061
	CFU per ml	Correlation Coefficient	.450	1.000
		Sig. (2-tailed)	.061	

P> 0.05 is not significant.

4. Discussion

In recent years, probiotic bacteria, as the food additives, have been introduced into numerous foods of which the dairy products have played an important role in carrying these bacteria (such as *Bifidobacterium bifidum* and *Lactobacillus acidophilus*). Eating regularly the sufficient amounts of living cells "the minimum treatment" is required if the consumer is to benefit from the probiotic products [28].

The purpose of this research study was to isolate and characterize potential probiotic bacteria from tubâ samples taken from Calinog, Iloilo and to assess their survival rate in different commercial beverages. Based on the morphological characteristics, *Lactobacillus spp.* from tubâ samples were able to grow in a selective medium, Rogosa agar. After the gram staining procedure the isolated bacteria were rod-shaped, convex, rough, smooth, shiny, circular, gram positive, facultative anaerobic, non-spore forming, and catalase-negative which indicate them to be the member of *Lactobacillus spp.*

An important factor that affects the survival of probiotic bacterial strains in beverage is pH. The survival is constrained at low pH values. This was observed in softdrink (M log CFU/ml=5.08; M pH=3.33), coffee (M log CFU/ml=5.87; M pH=4) and the negative control, distilled water (M log CFU/ml=5.37; M pH=4.00), and juice (M log CFU/ml=6.68; M pH=3.33) with a reduced pH and drastically dropped the population count of *Lactobacillus spp.* after the 28-day sampling period. Hence, different beverages should be a good food carrier for probiotic strains because it has a pH in the range of 4.7-5.8 and even it lowered to a pH of 4.5 after 28 days of storage except for softdrink and juice. The tubâ, which is the source of *Lactobacillus spp*. has a pH of 4. Upon evaluation of the cultured samples on Rogosa agar medium, the same correlation was revealed. The minimum required level of probiotic bacteria to be useful for the consumer's body is 10^6 CFU per ml(log 6 CFU per ml) of living bacteria and the level in the present study was found to be 10^7(log 7 CFU per ml) for the positive control, probiotic drink at day 14 (M log CFU/ml =7.12; pH=4) and juice at day 21 (M log CFU/ml = 7.08; pH=4), and 10^6 for water (M log CFU/ml =6.77; pH=4) at day 14; juice (M log CFU/ml =6.68; pH=3.33) at day 28; softdrink (M log CFU/ml =6.08; pH=4) at day 14; probiotic drink (M log CFU/ml =6.53; pH=4) at day 28; and beer (M log CFU/ml =6.47; pH=4) at day 28. Thus, it could be beneficial for the consumers. Upon evaluation of the samples on Rogosa agar, *Lactobacillus spp.* in juice beverage had the counts equal to logarithmic value of 6.68 until 28th day which had the highest count of bacteria.

Temperature and pH seemed to play the largest role in the organism ability to grow and thus, affecting its production of lactic acid [8,9,10]. The result agrees by using *Enterococcus flavescens* for production of lactic acid [1]. In their opinion, beyond a certain concentration lactic acid yield dropped due to high cell density resulting in fast depletion of essential nutrients, limiting further growth and reducing the yield [19].

A firm correlation between the presence of sugar and pH (lactic acid) was expected, but on the one hand it is known that the practical yield of sugars conversion to lactic acid of the strains of the group *Lactobacillus* such *L. acidophilus* is about 85% [6]. The selected beverages used in this study may have the sugar content which promoted the growth of *Lactobacillus spp.* In soft drinks, high fructose corn syrup (HFCS), also called glucose-fructose, is present [15]. In beer, maltodextrins are found and are digested in the form of glucose [40]. Juice may also contain high fructose corn syrup (HFCS), also known as glucose-fructose, or their naturally occurring sugars such as fructose [26]. On the other hand, starch broken down to simple sugar is commonly found in coffee beans during roasting, however, this may be lost along various processes [18]. Meanwhile, sucrose and other commonly added sweeteners may be present in commercial coffee [18].

The results of the experiment in this study showed that juice beverage was a suitable support for the intestinal bacterium, *Lactobacillus.* The juice beverage had the highest viability in all of the beverages investigated. The survival rate of tubâ *Lactobacillus* at room temperature condition for 28 days at 10^6 CFU/ml is very essential if a product should have probiotic properties. From the foregoing result, it can be implied that *Lactobacillus* from tubâ can be somehow used in the preparation of different probiotic beverages.

For probiotics to be effective, scientists have suggested that there be a minimum number of 10^6 to 10^7 CFU of probiotic bacteria /gram of product at the time of consumption [17, 20, 21]. While some reports have shown probiotic growth and survival numbers to be stable during the shelf life of the product, others have cited a rapid decline in the number of viable probiotic bacteria over the shelf life. Studies have shown that a number of factors can affect the growth and the survival of *Lactobacillus spp.* in different beverage products. These factors include strains of probiotic bacteria, pH of substance medium, presence of lactic and acetic acids, interactions with other microorganisms, storage temperature and manufacturing conditions [5, 36, 37].

In general, the results revealed different survival pattern of Lactobacillus spp. compared with that of the control group at room temperature. At the end of 28th day of storage, all beverage samples showed a significant decrease in viability remaining for some beverages above the critical level of 10^6 CFU/ml for over 28 days. In contrast, the control sample inoculated with commercial strain of *Lactobacillus*, despite its initial higher concentration, showed a significant decrease, reaching cell density of 10^6 CFU/ml up to 28 days of storage at room temperature.

Moreover, several studies have revealed that some commercial products do not sustain adequate populations of viable probiotic bacteria during their shelf life [34]. It is noteworthy that many intrinsic and extrinsic properties of food, such as pH, availability of nutrients, concentration of sugars (osmotic pressure), oxygen level, water activity, and storage temperature influence the viability of probiotic organisms [8,31].

Results of the present study clearly demonstrate that the presumptive *Lactobacillus* strains from tubâ were able to survive in the different beverages during storage at room temperature, highlighting that these media could be a good candidate as vehicle of probiotics. The strains showed better survival ability in juice and beer beverages than in the (positive or negative) control groups confirming that food formulation affects the viability of probiotics during storage [21]. In the present study, the formulation in the different beverages, which includes various ingredients, seemed to better support the

probiotic viability. This is in accordance with previous reports which asserted that solid matrices may protect bacteria during the storage of food [25,34].

Although lactobacilli have been considered as "difficult" microorganisms due to their demand for various essential amino acids and vitamins, some of them have been found to survive in different beverages at room temperature condition [7,36]. In our study, the tubâ *Lactobacillus* strain viability is in agreement with results obtained by other researchers who reported that cells of probiotic strains, produced in different ways, had comparative stability in milk, whereas in juice, sucrose-protected cells survived better than in reconstituted skim-milk protected cells [36].

As reported by other authors, the observed variations in strain stability may be due both to pH and storage temperatures [22]. With respect to pH, studies reported that in many fermented dairy-products, the loss of viability of probiotic bacteria is to be attributed to the decrease in pH values to 4 - 5 and to the accumulation of organic acids as a result of growth and fermentation [9, 31].

In the present study, tubâ *Lactobacillus spp*. showed good viability even at pH values lower than 4. In the present study, almost all *Lactobacillus spp.* remained viable above the critical level in the different beverages at 25 °C for 28 days. The relationship between pH values and viable counts showed that the viability of the tubâ *Lactobacillus* strain, in agreement with literature data, was strongly affected by the pH reduction.

5. Conclusions and Recommendations

Beer and juice beverages might be good candidates for producing a novel and tasty functional, non-dairy, probiotic beverage which could effectively deliver probiotic tubâ Lactobacillus strains at room temperature. Findings of this study showed that after the 28-day sampling period, juice has the highest colony count at 6.68 log CFU/ml while the softdrink has the lowest colony count of 5.08 log CFU/ ml. However, the colony count of *Lactobacillus spp.* in beer, 6.47 log CFU/ml and with the juice, 6.68 log CFU/ml are comparable to the positive control, probiotic drink with colony count of 6.53 log CFU/ml. The population count in the experimental groups (juice and beer) and the positive control containing an acceptable viable count of *Lactobacillus spp*. at 6 log CFU/ml. After the 28-day sampling period, the average pH of water, probiotic drink (positive control), and beer is 4 and the pH of juice and softdrink is 3.33. There is no significant difference in the population of *Lactobacillus spp*. from tubâ incorporated in different types of beverages, p(0.053)>0.05. This means that at the end of a 28-day sampling period, the population count of *Lactobacillus spp.* did not significantly vary when incorporated in different beverages. Furthermore, the inoculated *Lactobacillus spp.* in different beverages at room temperature have almost identical growth rate in the entire sampling period. There is a medium positive correlation between the population count of Lactobacillus spp. and pH in different types of beverages, rho (0.450). This means that pH of the different beverages strongly affects the population count of *Lactobacillus spp.* due to the accumulation of organic acids as a result of growth and fermentation.

The results of the study may imply the future use of commercial beverages incorporated with probiotic *Lactobacillus spp.* instead of the conventional probiotic products that is being marketed at present. In this regard, probiotics such as *Lactobacillus spp*. from tuba can be offered in a wider variety of products for consumers to choose from.

It is recommended to assess the safety of the tubâ *Lactobacillus spp.* beverage before sensory evaluation. The sensory evaluation should be performed for the tubâ *Lactobacillus spp.* beverage, after seven days in terms of scent, color and taste. It is recommended that manufacturers use a sensory analysis technique to evaluate and optimize consumer acceptability of new probiotic beverage formulations. Total sugar

content (brix) at the end of incubation should also be monitored. From an industrial point of view, the variability in survival at different storage temperatures should be considered as a major criterion for the selection of strains to be used in probiotic beverages stored at refrigerated or room temperature.

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Authors

Connie C. Durana, she is a graduate of BS Biology (Microbiology Track) at West Visayas State University, Iloilo City on April 2016.

Jacinth L. Legario, she is a graduate of BS Biology (Microbiology Track) at West Visayas State University, Iloilo City on April 2016.

Rey G. Tantiado, he is a graduate of BS Biological Sciences and Master of Arts in Biology at West Visayas State University, Iloilo City Philippines on March 2003 and March 2006, respectively. He graduated his Doctor of Philosophy in Education (Biology) from the University of the Philippines Open University, Los Banos, Laguna Philippines on May 2014. He is currently an instructor at West Visayas State University.