Growth Pattern of Lactic Acid Bacteria in Probiotic Rice Washed Water

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Abstract

Readily available rice washed water posed as potential medium for lactic acid bacteria, thus its utilization as probiotic drink for animals was explored. This study investigated the LAB growth pattern in rice washed water using factorial design following Completely Randomized Design. Highest bacterial count was from 1:3 rice-water ratio 1st washing (T5) at 12h (1.5 x109 cfu/ml) and 1:2 rice-ratio 1st washing (T3) at 30h (1.5x109 cfu/ml). The 1:3 rice-water ratio 1st washing (T5) and 1:1 rice-water ratio 2nd washing (T2) provided an early rapid growth environment. Logarithmic phase started at 18h with T5 having the highest microbial count. Decline phase ranged from 18h to 36h. Morphological and culture characteristics were identical to the activated LAB from the source. Results indicate that rice washed water can be used as probiotic drink within 12h to 42h after fermentation.

Keywords: microbial count, growth curve, logarithmic growth phase, LAB culture, morphological characteristics

1.0 Introduction

By and large, probiotics had already gained distinction in both human and animal health. The health benefits it offered had been proven effective, thus it obtained popularity in the market today. It is generally agreed that a probiotic is a preparation of live microorganisms which, when applied in adequate amounts to humans or animals, beneficially affects the host by improving the properties of the indigenous microbiota (Rush, undated) and intestinal balance (Fuller, 1991; FAO/WHO, 2001). The beneficial effects of probiotics include: modification of the intestinal microbiota, increase production of VFA (volatile fatty acid), stimulation of immune system, increased biomass and stool bulking, reduced inflammatory reactions, increased B vitamin synthesis, prevention of pathogen colonization, improved mineral absorption, enhanced animal performance, prevention of cancer, decreased carcass contamination, lower serum

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cholesterol, decreased ammonia and urea excretion, and lower skatol, indol and phenol (Patterson & Burkholder, 2003). The inhibition of pathogens by the intestinal microbiota has been called bacterial antagonism, bacterial interference, barrier effect, colonization resistance, and competitive exclusion. Mechanisms by which the indigenous intestinal bacteria inhibit pathogens include competition for colonization sites, competition for nutrients, and production of toxic compounds or stimulation of the immune system (Patterson & Burkholder, 2003).

Lactic acid bacteria (LAB) are among the probiotics used in the health industry. It has been used on foods for human consumption for the numerous benefits that it offers to consumers. LAB and lactic acid has also been used on plants and animals. Organic farming used LAB for the improvement of the quality of produce. Scientific studies revealed positive results on the use of lactic acid on broilers. Lactic acid bacteria have been used on the feed and drinking water. It had caused significant reduction of Salmonella and Campylobacter incidence in poultry product (Bryd et al., 2001). Administration of LAB likewise caused a significant reduction of salmonella recovered 24 hours after treatment of day-of-hatch broiler chicks (Higgins et al., 2007). Commercial producers of lactic acid products have promoted lactic acid feeds for poultry for the following reasons: improved performance of broilers, improved digestion, protection against microbial contamination, and increased food safety. These only show that production of lactic acid and use of lactic acid bacteria posed high potential for use as probiotic product.

LAB needs a good medium for growth and survival in order to maintain its integrity as probiotic. One potential medium is rice washed water which contain starch as source of LAB food. Being a rice-eating country, rice washed water as a waste product in cooking, is always available in the households. The prospects of LAB in rice-washed water necessitates thorough evaluation prior to its usage as probiotic drink, specifically for poultry, hence this study.

Generally, this study determined the growth pattern of lactic acid bacteria (LAB) in probiotic rice washed water. Specifically, the investigation focused to: determine the microbial count of the viable LAB at the different phases of its growth; determine the typical

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growth curve of LAB in probiotic rice-washed water; identify the logarithmic growth phase of LAB on probiotic rice-washed water medium; and examine the cultural and morphological characteristics of LAB growing on probiotic rice washed water.

2.0 Methodology

Research Design and Experimental Treatment

The study utilized factorial design employing Completely Randomized Design (CRD) in three replications. Independent variables included the rice-water ratio (1:1, 1:2, 1:3) used for washing and the number of washings (1st and 2nd washing). Table 1 shows the different treatments used in the study.

Preliminary Activities

Working area was cleaned and completely sanitized with chlorinated water and 70% ethyl alcohol. Tools and equipment were all washed, dipped in a 150 – 200 ppm chlorine solution while all glassware were sterilized (autoclave).

Preparation of microbial starter culture

Starter culture for LAB was obtained from a commercial yogurt. LAB culture from yogurt was activated using fresh carabao’s milk. Activated starter culture was inoculated into the probiotic rice washed water at constant

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Table 1. Experimental treatments of the probiotic rice washed water.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rice-water ratio</th>
<th>Number of washings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1</td>
<td>1st washing</td>
</tr>
<tr>
<td>2</td>
<td>1:1</td>
<td>2nd washing</td>
</tr>
<tr>
<td>3</td>
<td>1:2</td>
<td>1st washing</td>
</tr>
<tr>
<td>4</td>
<td>1:2</td>
<td>2nd washing</td>
</tr>
<tr>
<td>5</td>
<td>1:3</td>
<td>1st washing</td>
</tr>
<tr>
<td>6</td>
<td>1:3</td>
<td>2nd washing</td>
</tr>
</tbody>
</table>

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concentration, using 10% starter of the total volume of the water.

**Washing of Rice**

To obtain the probiotic rice washed water, the rice (red rice variety) was washed with distilled water following the rice-water ratios (1:1, 1:2, 1:3) and the number of washings (1st washing and 2nd washing). The rice was washed thoroughly and strained to separate the water from the rice. Final volume of the water was determined using the graduated cylinder. This determined the volume of the starter culture that was inoculated into the probiotic rice washed water.

**Pasteurization of Rice Washed Water**

The probiotic rice washed water was heated until it reaches the temperature of 72°C. This was maintained for 15 minutes to kill any microorganisms that might cause spoilage and unnecessary fermentation that may affect the growth of LAB. After heating, the probiotic rice washed water was cooled down to 37°C - 40°C for inoculation of starter culture.

**Preparation of Dilution Water**

Peptone water was used for the dilutions of the sample. The amount of peptone powder used was in proportion to the recommended amount of 20g of peptone powder to 1 liter of distilled water. Weighing of peptone powder was done using an analytical balance to obtain its exact weight. Diluted peptone water was then sterilized in an autoclave at 121°C for 15 minutes.

**Preparation of MRS Agar**

The amount of MRS agar powder used was in proportion to the recommended amount of 66g of MRS agar powder to 1 liter of distilled water. MRS mixture was
heated to completely dissolve the MRS agar powder. It was sterilized in an autoclave at 121°C for 15 minutes.

**Microbial Evaluation**

Samples from the different treatments of the probiotic rice washed water was subjected to microbial evaluation at 6 hours interval to determine the growth pattern of LAB. Serial dilution of the sample was prepared using the peptone water as diluents. The $10^{-7}$ dilution of the sample was pour plated on MRS agar using standard pour plating method. Standard plate count (SPC) of the microorganisms was evaluated and the growth pattern was analyzed by plotting the data on a graph. The cultural and morphological characteristics of the LAB growing on the probiotic rice washed water were also evaluated.

**Examination of the Cultural and Morphological Characteristics of LAB**

In order to examine the cultural and morphological characteristics of LAB, a glass slide was prepared and sterilized by applying alcohol and heating it until the alcohol evaporates. At the center of the glass slide, a drop of distilled water was poured. Afterwards, a loopful of colony taken from the petri plate where LAB is growing was spread in the drop of water. To detain the bacteria for examination, the glass slide was heat-fixed until the liquid evaporated. The slide is now ready for gram staining in order to be observed under an electronic microscope.

**Statistical Analysis**

Results of the study were subjected to Analyses of Variance (ANOVA) using SPSS to determine the effects between treatments following the Completely Randomized Design (CRD) with three replications. Treatments with significant difference were subjected to post hoc analysis using Duncan’s Multiple Range Test (DMRT).

**3.0 Results and Discussion**

**Microbial Count**

At twenty-four (24) hours after incubation, the microbial counting revealed less than $1 \times 10^7$ cfu/ml of all experimental treatments. The condition remained the same 6 hours thereafter except
for treatment with 1:3 rice-water ratio second washing ($T_6$) where microbial count reached $2.8x10^8$ cfu/ml (Fig. 6). It is interesting to note that the said medium offered the earliest high growth of LAB although the population dropped at 18 hours, regained maximum strength six hours after (at 24 hours), and gradually dropped in the succeeding hours. This growth behavior points out that the medium provided conducive environment for the multiplication of LAB in a longer duration compared to others. In one hand, $T_5$ and $T_2$ registered the first two highest microbial counts ($1.5x10^9$ and $1.3x10^9$ cfu/ml, respectively) at 12 hours after incubation (Figs. 5 & 2), while $T_4$ registered the lowest ($2x10^7$ cfu/ml) (Fig. 4). This indicates that both 1:3 rice-water ratio 1st washing and 1:1 rice-water ratio 2nd washing provided better media for early proliferation of viable LAB. On the other hand, highest count of viable LAB in $T_3$ and $T_4$ ($1.5x10^9$ and $1.4x10^9$ cfu/ml, respectively) occurred 30 hours after incubation (Figs. 3 & 4). This suggests that 1:2 rice-water ratio, whether 1st or 2nd washing, offered better probiotic medium but took longer lag duration. Of all the experimental treatments, $T_1$ had the lowest ($5.4x10^8$ cfu/ml) recorded microbial count throughout the duration (Fig. 1). It is basically due to its high starch content (1:1 rice-water ratio 1st washing) which is not readily consumable/fermentable by the LAB. At 42 hours, most of the LAB counts from various treatments dropped (<$1x10^7$) until finally at 48 hours, scanty LAB were left due to its death basically caused by acidic environment.

![Fig. 1. Growth trend of lactic acid bacteria from 0-48 hours](image1)

![Fig. 2. Growth trend of lactic acid bacteria from 0-48 hours after incubation of $T_2$.](image2)
Typical Growth Curve of LAB

During the first six hours after the incubation period, all experimental treatments exhibited a lag phase or adjustment period in their growth except for T6. At 6 hours after the incubation period, T6 showed 2.8x10^8 cfu/ml bacterial count which indicates that rapid growth of LAB already started. At 12 hours, all other experimental treatments entered logarithmic phase with T5 having the highest and T4 having the lowest microbial count (1.5x10^9 and 1.3x10^9 cfu/ml, respectively) (Fig. 7). It was observed that there was a variation in the peak growth of LAB from 12 hours (T5 and T2) to 30 hours (T3).
Logarithmic Growth Phase of LAB

In general, the logarithmic growth phase of LAB in various experimental treatments started at 12 hours after the incubation period. The stationary phase differs among treatments as well as the decline phase. Overall, the LAB in all treatments greatly dropped after 42 hours (Table 2). The rice-water ratio of 1:1 1st washing (T₁) attain logarithmic phase at 12 hours (3x10^7); at its highest (5.4x10^8) at 18 hours; and gradually declined thereafter. The highest bacterial growth was observed in the 1:3 rice-water ratio 1st washing (T₅) and 1:1 rice-water ratio 2nd washing (T₂). The rest in between (T₃ and T₄) had a growth period of 18 hours (T₅ and T₂) to 36 hours (T₃ and T₄). Beyond this, majority of the experimental treatments exhibited an estimated plate count (ESPC) of<1x10^7. The results specify that 1:3 rice-water ratio 1st washing (T₃) and 1:1 rice-water ratio 2nd washing (T₂) provided a growth environment which enhances early rapid growth while 1:2 rice-water ratio, whether 1st or 2nd washing, provided longer adjustment period prior to the attainment of rapid growth.
count of T$_1$ is the lowest among the peak bacterial counts of other treatments. The highest bacterial count attained among all treatments was that of T$_5$ at 12 hours ($1.5 \times 10^9$) and T$_3$ at 30 hours ($1.5 \times 10^9$) followed by T$_4$ at 30 hours ($1.4 \times 10^9$) and T$_2$ at 12 hours ($1.3 \times 10^9$) (Table 2).

The comparison of treatment means using one-way analysis of variance (ANOVA) unveiled highly significant difference (0.01 level of significance) from 12 to 36 hours; significant difference at 42 hours (0.05 level of significance) and no significant difference from 0, 6 and 48 hours (Table 2). The post hoc test (DMRT) result further revealed that at 12 hours after incubation period, T$_5$ and T$_2$ (first 2 highest microbial count) were not significantly different from each other ($1.5 \times 10^9$ and $1.3 \times 10^9$, respectively) but significantly different from the rest of the treatments. This suggests that, utilization-wise, either of the 1:3 rice-water ratio 1$^{st}$ washing or 1:1 rice-water ratio 2$^{nd}$ washing, can be employed as probiotic medium to grow and harvest viable probiotic earlier. Viable LAB can then be harvested and stored in the refrigerator to temporarily deactivate it while not yet used or directly use it as probiotic drink for animals.

Despite the similarity in the microbial count at 12 hours, T$_5$ and T$_2$ exhibited significantly different decline in microbial count ($7.7 \times 10^8$ and $4 \times 10^7$, respectively) at 18 hours. Treatment 5 generally showed higher viable bacterial count in longer duration (12 hours to 30 hours) (Table 2).

At 18 hours, T$_5$ remained as the treatment with highest microbial count ($7.7 \times 10^8$) followed by T$_1$ ($5.4 \times 10^8$). At 24 hours, T$_6$ posted the highest microbial count ($1.1 \times 10^9$). At 30 hours, T$_3$ ($1.5 \times 10^9$) and T$_4$ ($1.4 \times 10^9$) marked the significantly highest microbial count and were in their peak growth. The LAB population gradually declined at 36 to 42 hours, but still the two highest treatments excelled from the rest. This result manifests the longer lag period of T$_3$ and T$_4$ to attain the peak of LAB growth. Longer lag period means longer time of waiting prior to utilization thus, higher cost is needed. However, depending on the urgency of the need, appropriate rice-water ratio and number of washing can be employed to obtain the optimum viable LAB and maximize the use of this resource at lowest possible cost. At 48 hours, most of the LAB in all treatments were already dead (Table 2).
Table 2. Comparison of microbial count means at 6-hour evaluation interval.

<table>
<thead>
<tr>
<th>Trf</th>
<th>0 hr</th>
<th>6 hrs</th>
<th>12 hrs</th>
<th>18 hrs</th>
<th>24 hrs</th>
<th>30 hrs</th>
<th>36 hrs</th>
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<tr>
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<td>3.7x10⁴</td>
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<td>1.1x10⁷</td>
<td>2.5x10⁷</td>
<td>2x10⁷</td>
<td>5x10⁷</td>
<td>&lt;1x10⁷</td>
</tr>
</tbody>
</table>

Notes: <1x10⁷ is an estimated plate count (EPC).

**Morphological and Culture Characteristics of LAB in Probiotic Rice Washed Water**

Gram staining process of the LAB in various experimental treatments of probiotic rice washed water disclosed that their morphological and cultural characteristics such as shape, color and stain reaction were identical to the activated LAB from the source (Fig. 8), Nestle plain yogurt. This proved that the LAB from the source were the same LAB that grew in the different experimental treatments regardless of rice-water ratio and number of washing (Figs. 9 to 14).

Fig. 8. Lactic acid bacteria growing in activated yoghurt and fresh milk mixture (oil immersion).

Fig. 9. Lactic acid bacteria growing in 1:1 rice-water ratio 1st washing (oil immersion).
Based on the foregoing results, it can be deduced that:

1. Both 1:3 rice-water ratio 1st washing (T5) and 1:1 rice-water ratio 2nd washing (T2) provide better media for highest and early proliferation (12 hours) of viable LAB.

2. It takes at least 12 hours, after incubation, for the LAB to attain logarithmic growth phase and 42 hours and beyond for LAB to greatly drop in microbial count.

3. The rice washed water, as a probiotic medium, maintained the integrity of LAB within 12 to 42 hours after incubation.

**5.0 References Cited**


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\[3^3\text{Vol. 3:126-138(2015)}\]