College of Tropical Agriculture and Human Resources
University of Hawai'i at Manoa

Natural Farming

Identification of bacteria in fermented cultures of rice water and milk

Abstract

The internet provides numerous sources to teach people how to make lactic acid bacteria (LAB) stock for agriculture uses. The most common is the fermentation of rice-water plus milk. The filtrate from this culture is said to contain high amounts of LAB.

The main purpose of this experiment was to identify the bacteria in filtrate of rice-milk fermentation. The secondary interest was to test the filtrate in cleaning up slaughterhouse wastewater. The bacteria isolated were identified using a 16S rDNA sequencing technique. Our study identified 16 bacteria in the filtrate. Three of the identified bacteria were LAB (18.75%). Only one of those LAB was contributed by the addition of milk. Preliminary testing of the filtrate suggested it could be used for the treatment of slaughterhouse wastewater. However, more studies are needed to determine its usefulness.

Beef cattle at the Mealani Research Station in Waimea.

Introduction

Regenerative agriculture practices have gained tremendous interests in recent years. The enthusiasm generated a slew of information in social media and publications on how to make various cultures for plants. These cultures have been touted to help with plant growth and seed germination, reduce odor, etc. One such popular recommendation is how one can make lactic acid bacteria (LAB) from the fermentation of rice water and milk (Ikeda et al., 2013). The internet also provides a ready source of videos from many practitioners of natural farming on how LAB is made. However, these sources showed no evidence that LAB is actually present or produced. Further, they lacked systematic documentation on how these cultures could enhance plant growth or fulfill other claims.

Curiosity led our group to ask the question of what LAB could be found in such fermented cultures. Unraveling this would be beneficial to the agriculture community, given the high price of fertilizers, especially since the Russian-Ukraine conflict began. For example, the price of inorganic fertilizer more than doubled in 2022. In addition, there were indications that LAB derived from such fermentation can reduce odors in livestock waste (DuPonte and Fischer, 2012). Hence, we set out to design some experiments to determine: 1) what LAB are present in the solute of rice-milk fermentation, and 2) can this solute be used in the treatment of livestock wastewater. Limitations at the onset of this study included limited funding and limited sources of fresh or raw milk, given that no dairy cows existed on the island of O'ahu when we began.

Materials and Methods

1. Preparation of the solute: fermented rice plus milk

The rice and whole milk (ultra-pasteurized, UHT) used in the culture were purchased from a local supermarket. We chose to use UHT milk over regular milk to avoid

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the potential increase of psychrophilic bacteria (He et al., 2010) due to the duration of transportation from the U.S. West Coast to Honolulu. The rice was washed with tap water in a ratio of 1:2 (cup/cup). The first two washing's cloudy rice water (200 mL) was collected in a clean glass jar (2/3 full). The jar was covered with tissue paper and secured with rubber bands to keep out pests. The covered jar was stored at room temperature (24°-26° C) away from direct light. The jar was stored for two days without shaking, at which time the rice water would emit a slightly sour odor. On day 3, a matting foam formed on the top layer. The cloudy liquid (fermented rinse water) on the bottom was collected by pouring off and discarding the mat layer. Then, about 200 ml (1 part) of the fermented rice water was mixed well with about 400 ml (2 parts) of whole milk in a new clean jar. The jar was covered with tissue paper like before. The jar was stored at room temperature, away from direct light. After four days, the contents of the jar were separated into a floating solid fraction and a yellow liquid fraction. The fermentation was stopped by collecting the yellow liquid into a new container and storing in the refrigerator. The process of collecting the fermented liquid was repeated three times.

2. Measurement of pH value

Five milliliters of fermented liquid samples were taken from the middle layer of the jar and transferred to a 10 mL beaker daily so that its pH could be measured. A laboratory digital pH meter was used to measure the pH values of the collected samples. The pH values were recorded every day during the whole process; seven days for each fermentation process.

3. Identification of selected strains

During the whole fermentation process, samples were collected every day. The samples were diluted 10-fold serially, and proper dilutes were spread on Plate Count Agar (PCA, Becton Dickinson) for quantification of total bacterial counts and Lactobacilli MRS Agar (Becton Dickinson) for the isolation of potentially probiotic LAB. All plates were incubated at 30°C for two days under aerobic conditions. The total bacterial counts on PCA and MRS agar were recorded after incubation. The viable cell counts on both agar plates were converted to Log CFU/mL. The bacterial counts on PCA agar were compared with that on MRS agar at each time point. The data were analyzed via analysis of variance (ANOVA) with a significance level of 0.05 utilizing Statistical Package for the Social Sciences (SPSS).

The colonies from each plate were purified three times by the streak plate method (Montville et al., 2012). The single colonies from plates were selected based on morphology (Figure 3) and identified by sequencing their 16S rDNA genes and aligning them to similar sequences in NCBI databases (Vliegen et al., 2006).

Results and Discussion

Figure 1 shows the fermented rice water and milk mixture. The pH of the liquid in the middle was taken daily for seven days.

Figure 1. The fermented solution of rice water and milk by day 7.

The pH values and bacteria count results are presented in Figure 2. The pH of the rice water on day 1 was 5.50±0.08. During the fermentation of rice water, pH decreased from 5.50±0.08 to 4.25±0.20. When milk was added to the fermented rice water, pH increased to 7.03±0.05. Subsequently, pH decreased to 5.02±0.03 after 24 hours. At the end of fermentation period, pH was 4.67±0.24. The low pH value of the final product could be explained by the presence of acid-producing bacteria in the ferment.

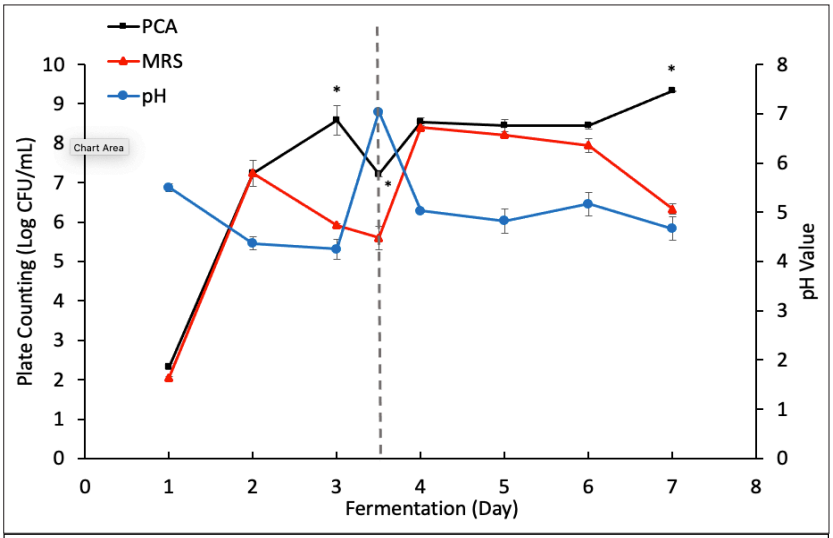

Figure 2. Changes in pH value of fermented rice water and milk and total bacteria counts in the culture on Plate Count Agar (PCA) and Lactobacilli MRS Agar (MRS) agar. The dashed line stands for the samples collected after milk was added to the rice water. The * denotes statistical differences in total bacterial counts between PCA and MRS media ($P<0.05$).

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The total bacterial count on PCA was 2.33±0.08 log CFU/mL in rice water on day 1. The count increased to 8.58±0.37 log CFU/mL on day 3. After the addition of milk, the total count in the mixture dropped to 7.22±0.06 log CFU/mL. This may be due to the dilution factor. Following further fermentation, the total bacterial count increased to 9.32±0.02 log CFU/mL This was the last day, day 7 of our study.

The total bacterial count on MRS agar was 2.05±0.04 log CFU/mL in rice water on day 1. The number increased to 7.24±0.33 log CFU/mL on day 2, which was similar to the total number of bacteria on PCA (7.23±0.33 log CFU/mL). However, the total bacterial count on MRS agar dropped to 5.92±0.03 log CFU/mL on day 3. This could be due to the lack of nutrients for *Lactobacillus* spp. to grow. This possibility was likely as the bacteria count on the MRS agar increased to 8.40±0.09 log CFU/mL after mixing with milk, a rich nutrient source. Another possibility for the decline in bacteria count is the antagonistic effect of dominant bacteria, which could favor the dominant bacteria in the fermentation and suppress the reproduction of other microorganisms (Chen et al., 2021). The number of bacteria on MRS agar was similar to the total count on PCA until day 6. By day 7, the number of bacteria on MRS was significantly lower than that on PCA (6.33±0.13 vs 9.32±0.02 log CFU/mL; $P<0.05$).

Table 1. The identification of selected colonies using 16S rDNA gene sequencing

Species	LAB[a]	No. of strains	Percent Identity	Accession	Origin[b]	
					R	R + M
*Acinetobacter baumannii**	N	17	98.30%	gi|1839332900|MT444986.1	√	√
*Enterobacter asburiae**	N	8	88.60%	gi|1775479350|MN691453.1	√	√
Enterococcus mundtii	N	7	98.36%	gi|1195569847|MF136796.1	√	√
Lactococcus lactis	Y	6	97.29%	gi|1173584870|KY883564.1	√	√
Leuconostoc mesenteroides	Y	4	97.69%	gi|459218611|KC456628.1		√
*Acinetobacter pittii**	N	3	99.36%	gi|2106443845|OK391188.1		√
Enterococcus italicus	Y	3	99.59%	gi|1407511716|MH509189.1	√	√
*Serratia marcescens**	N	3	94.37%	gi|114438991|DQ864990.1		√
*Bacillus cereus**	N	1	93.16%	gi|1421154618|MG548649.1	√	
*Enterobacter kobei**	N	1	96.79%	gi|1775479802|MN691905.1		√
*Klebsiella oxytoca**	N	1	97.65%	gi|1279497349|MG557812.1		√
*Lactococcus garvieae**	N	1	90.62%	gi|1852325204|MT597623.1		√
*Pantoea agglomerans**	N	1	95.07%	gi|1163245440|KY587408.1		√
*Pseudomonas putida**	N	1	95.37%	gi|1834981527|MT378398.1	√	
*Stenotrophomonas maltophilia**	N	1	97.68%	gi|1491966430|MG734142.1		√
*Weissella confuse**	N	1	68.99%	gi|1057718743|KX246791.1		√

[a] The identified strain was reported as a type of lactic acid bacteria (LAB). (N strands for No, Y stands for Yes)

[b] Colonies were isolated from fermented rice water (R) or the mixture of rice water and milk (R+M).

* indicates that the identified strain was reported as an opportunistic pathogen.

Individual colonies on PCA and MRS agar were subcultured and purified by streaking on agar plates. Among all isolated colonies, 110 isolates were selected based on visual morphology. Examples of morphology differences on the PCA media (left) and the MRS media (right) are presented in Figure 3.

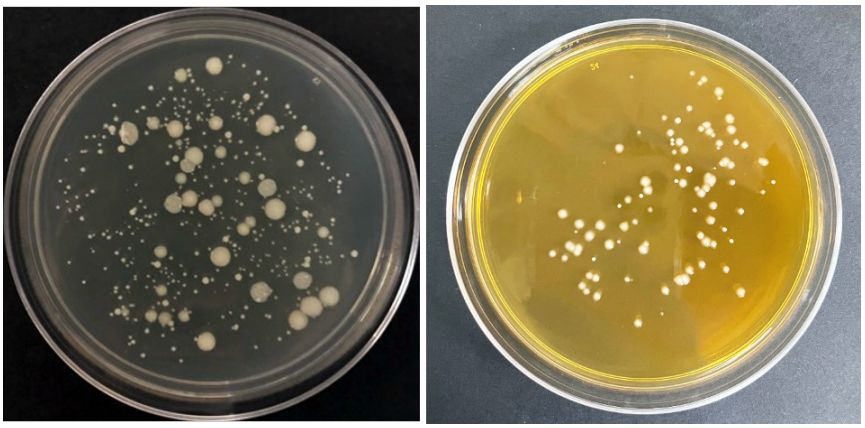


Figure 3. Differences in morphological appearance of bacteria colonies on PCA (left) and MRS (right) agar.

Due to financial constraints, 59 isolates were randomly selected for 16S rDNA sequencing. The results are shown in Table 1. Three strains that have been reported as Lactic Acid Bacteria (LAB) were identified: *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Enterococcus italicus*. *L. lactis* was found in both fermented rice water and rice-milk mixture in the present study. It has been used for centuries in food fermentation, especially cheeses and yogurts. It is generally regarded as a safe additive, (GRAS), a status granted by the Food and Drug

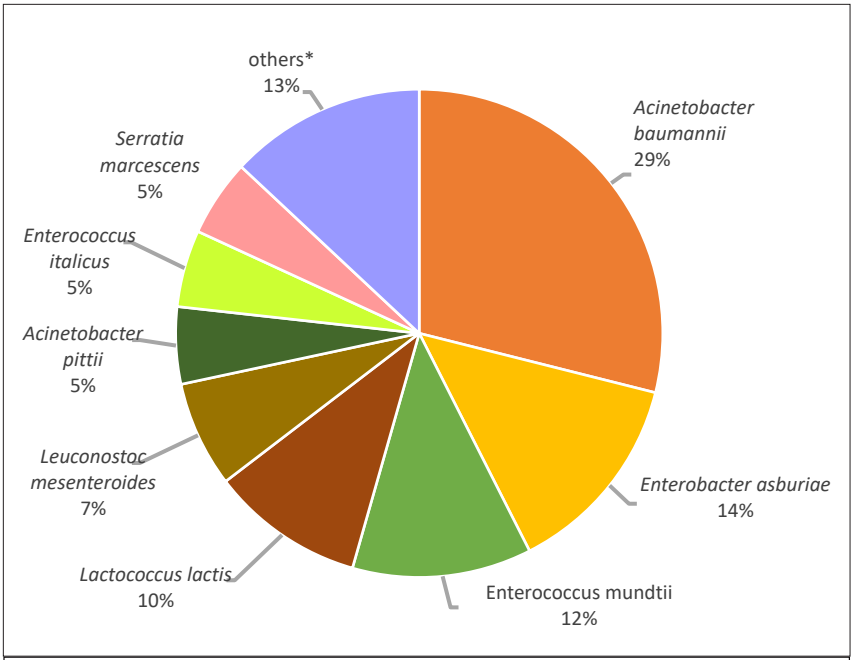


Figure 4. Percentages of major identified species in the final culture. *Includes all strains whose percentage is lower than 3%.

Administration (FDA) (Song et al., 2017). *L. mesenteroides* only presented in a mixture of rice water and milk. It is reported as the starter culture of various breads and dairy products, which is responsible for flavor development (Arakawa et al., 2016). *L. mesenteroides* is also known for the production of exopolysaccharides and bacteriocins, which exhibits anti-bacterial, antiviral and immunomodulatory activities (Arakawa et al., 2016; Chang-Liao et al., 2020). *E. italicu* is considered to play an essential role in ripening and aroma development in a variety of artisanal cheeses (Merlich et al., 2019). Moreover, the bacteriocins produced by *E. italicus* showed antibacterial activities (Merlich et al., 2019).

The percentage of the bacteria types found in this study is presented in Figure 4. The predominant bacteria found in both fermented rice water and the mixture was *Acinetobacter baumannii*. This is an opportunistic bacterial pathogen and commonly found in the environment, like in soil and water. *A. baumannii* is primarily associated with hospital-acquired infections (CDC, 2019).

Enterobacter asburiae was also found in both fermented rice water and the mixture. This is an opportunistic pathogen that has been isolated from a variety of clinical and environmental specimens (Mardaneh and Soltan, 2016). Nawaz et al. (2019) presented that the *Enterococcus mundtii* strain isolated from the indigenous fermented milk product, dahi, exhibited antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa*, and *Listeria monocytogenes*. They indicated that the fermented milk product containing *E. mundtii* could be used as functional food.

Acinetobacter pittii was found only in the mixture of rice water and milk, which is a significant cause of nosocomial infection (Chusri et al., 2014). *A. pittii* is widely distributed in the environment and may contaminate food and animals; thus humans could acquire skin and/or oral carriage that subsequently causes infection (Chusri et al., 2014). *Serratia marcescens* was found in the mixture of rice water and milk, which is an opportunistic, gram-negative, nosocomial pathogen (Khanna et al., 2013). *Bacillus cereus* was found in fermented rice water, which is an aerobic spore-forming bacterium that is associated mainly with food poisoning. It is commonly found in soil, on vegetables, and in many raw and processed foods (Bottone 2010). *Enterobacter kobei* infections were rarely reported, but cases indicated that the infections often occurred in patients with implanted devices (Hoffmann et al., 2005). *Klebsiella oxytoca* was found only in the mixture of rice water and milk. *Klebsiella oxytoca* was reported as an emerging pathogen causing hospital-acquired infection in adults and exhibiting resistance to commonly used antibiotics

Figure 5. The outflow of the wastewater from a local slaughterhouse prior to passing through a solid-liquid separator. This was the source of wastewater used in the current study.

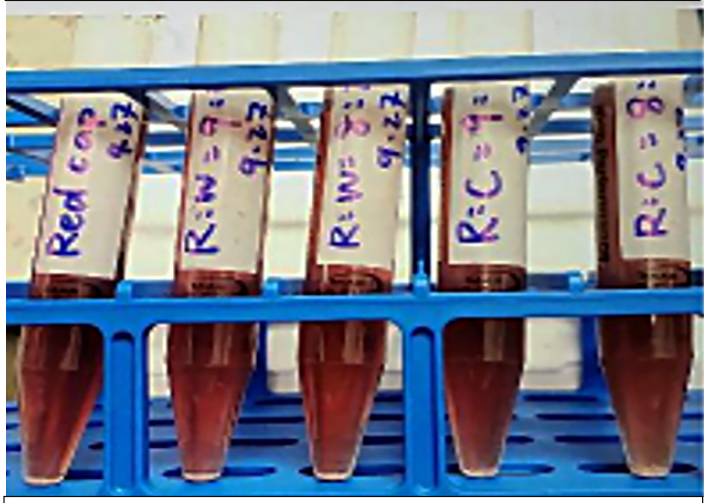
(Singh et al., 2016). *Stenotrophomonas maltophilia* is an environmental bacterium found in aqueous habitats and an emerging multidrug-resistant opportunistic pathogen. It is most commonly associated with respiratory infections in humans (Brooke 2012).

Other bacteria identified were *Lactococcus garvieae, Pantpea agglomerans, Pseudomonas putida* and *Weissella confusa*. *L. garvieae* was reported to cause human infections through the consumption of contaminated raw seafood (Malek et al., 2019). *P. agglomerans* could be a cause of opportunistic human infections, mostly by wound infection with plant material (Cruz et al., 2007). *P. putida* was only found in fermented rice water. It is a rare cause of infection in humans. However, lethal cases of bacteraemia and soft skin tissue infections have been reported to be associated with *P. putida* (Peter et al., 2017). This bacteria has also shown some potential to fight off diseases in plants (Lázaro et al., 2020). *W. confusa* was reported as an opportunistic pathogen and has been isolated from a variety of foodstuffs such as milk, acid-rich carbohydrate food, carrot juice, sugar cane, and fermented meat (Fairfax et al., 2014). Many cases of *W. confusa* infections were associated with medical procedures prior to the period of infection (Fairfax et al., 2014). Given that so many opportunistic pathogens were isolated from the samples, proper precautions, e.g. wearing disposal gloves, thorough washing of hands and utensils, etc. should be employed when handling this rice-milk solution.

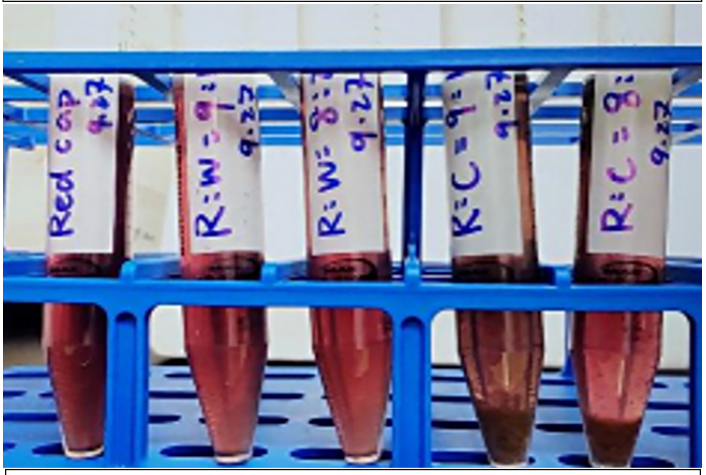
We are uncertain if the choice of milk we used, UHT, played a difference or limited the number of LAB found in the ferment. A previous paper (Ikeda et al., 2012) seems to imply that raw milk would be a preferred source for making LAB with this procedure. Several studies (Wouters, et al., 2002; Zhong, et al., 2016; Gao et al., 2017) suggest that the source of milk, method of harvest, temperature, and its location would influence the types of bacteria in the fermented milk. Some of the microbes found in these studies were *Lactococcus species, Leuconostoc species, Lactobacillus species*, Streptococcus species, *Acetobacter, Acinetobacter*, fungi and molds.

Several authors (Chantsayang et al., 1994; DuPonte et al., 2012; Ikeda et al., 2013) had suggested that the rice-milk ferment could be used to reduce odor in livestock pens. We performed a rapid trial (3x) using this ferment in treating slaughterhouse wastewater. The wastewater was collected at the site of discharge and transported back to the laboratory 45 minutes away. Figure 5 shows the wastewater discharge from a local slaughterhouse into a holding tank prior to it passing through the solid-liquid separator. Wastewater collected from this outflow was mixed with

Figure 6. Laboratory test of slaughterhouse wastewater treated with ferment from rice water–milk fermentation (RM).



Day 0. Left to right: slaughterhouse wastewater (SW), SW + water at 9:1, SW+ water at 9:2, SW + RM at 9:1 and SW + RM at 8:2.



Day 1 (24 hrs later). From left to right: slaughterhouse wastewater (SW), SW + water at 9:1, SW + water at 9:2, SW + RM at 9:1 and SW + RM at 8:2.

the ferment at 9:1 and 8:2 ratio in graduated test tube. Within the first hour, air bubbles could be seen in the tubes. By the end of 24 hours, the wastewater was clearer in the 8:2 treatment (Figure 6). It seems that the suspended solids had settled in the bottom of the tube. Further studies, with appropriate funding, would be needed to explore the use of this ferment in wastewater treatment.

Washed rice water (WRW) and milk are sources of nutrients to plants. They are known to support bacteria growth given that they are rich in carbohydrates and minerals. Nabayi et al., (2021a) reviewed the nutrient composition of WRW and cited work done in various laboratories on

Figure 7. A field of guinea grass (Panicum maximum), where fermented/spoiled milk was applied four days prior. Dark green strips are where the spoiled milk were applied.

the benefits of WRW on crops. Nabayi et al (2021b) identified 15 bacteria in soils applied with washed rice water but none were LABs. The work was further affirmed in another study with repeated WRW application on three different soil types, the accumulation of nutrients in the soil and yield of the crop studied, *Brassica chinensis*; commonly known as "choi sum" (Nabayi et al., 2022).

The coronavirus pandemic resulted in a surplus of fluid milk; several Extension articles on the use of "surplus milk" as fertilizer were posted by Land Grant universities. The estimated fertilizer of milk was presented in an Extension publication by Czymmek (2020). The beneficial effects of the application of milk on crops was reported by Fernando and Smith (2007), where pumpkin was the crop studied. The nutrients in milk are readily available to plants. The application of spoiled milk on the field must be done with caution so as not to contaminate the ground water or cause a nuisance due to odor. Evenly and appropriately applied, discard fermented milk can be beneficial to forage (Figure 7). If the ferment in the rice water-milk fermentation produces LAB beneficial to plants, then more defined qualitative experiments to determine such claims must be carried out in the future.

Summary

The current small study showed there are few LAB (n=3) in the ferment from rice water-milk fermentation. Two of these were already in the rice water at the onset. In order to ascertain the benefits of adding milk to the rice water to grow LAB, further research with more refined approach would be required. Similarly, the benefits of LAB on crops or ther remediation of livestock waste odor need further investigations. This study provided a glimpse of the potential of using some of the microbes to remediate slaughterhouse wastewater.

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