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Microbial Vitality of Probiotic Milks Supplemented With Cereal or Pseudocereal Grain Flours

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Abstract

Probiotic products containing *Lactobacillus rhamnosus* GR-1 have been shown to help decrease the risk of urogenital infections, delay the decline of CD4 lymphocytes in patients with human immunodeficiency virus, and survive in the intestinal tract without stimulating immune or inflammatory responses. Cereal and pseudocereal grains can act as prebiotic agents and could be an economical way of improving the nutritional value, functional qualities, and overall health benefits of probiotic products. The purpose of this study was to measure the growth and survival of *L. rhamnosus* GR-1 in fermented probiotic skim milk supplemented with a grain flour over a 28-day storage period. The objective was to determine if supplementation of the grain flours would have a positive effect on the microbial vitality of *L. rhamnosus* GR-1 in the final product and during storage. Five probiotic skim milk samples supplemented with a grain flour were prepared: oat (3% weight to volume ratio), lentil (3% w/v), rice (3% w/v), barley (3% w/v), and quinoa (3% w/v) along with one control probiotic skim milk sample. Results showed that the oat, rice, barley and quinoa flours supported the growth of *L. rhamnosus* GR-1 at viable levels (10^8CFU/mL) during the first 14 days of storage; however, only skim milk supplemented with rice flour sustained the growth over the 28-day storage period. Future studies should conduct a sensory evaluation of this fermented probiotic rice flour milk as well as study rice milk inoculated with probiotics to produce a dairy-free and possible gluten-free product.

Keywords: barley, cereal grains, flour, lentil, oat, prebiotics, probiotic milk, quinoa, rice

1. Introduction

1.1 Introduction to Probiotics

Probiotic bacteria can provide numerous therapeutic effects such as moderating the immune system, lowering blood cholesterol, reducing lactose intolerance, diminishing the effects of Crohn's disease and alleviating diarrhea (Hekmat, Soltania, & Reid, 2009). Probiotic food products should contain a minimum of 10^6-10^9 colony-forming units (CFU) per 100g in order to provide health benefits; however, doses below 10^8 CFU per day should not be considered effective unless the product proliferates the probiotic effects in the digestive tract (Reid, 2001). The use of the probiotic strain *Lactobacillus rhamnosus* GR-1 in yogurt has been found to lower the risk of some urogenital infections and yeast infections, and to delay the decline of CD4 lymphocytes in patients with human immunodeficiency virus (HIV) (Anukam, Osazuwa, Osadolor, Bruce, & Reid, 2008; Hekmat et al., 2009; Hemsworth, Hekmat, & Reid, 2012; Reid et al., 2003). This strain is bile resistant and can survive passage through the human gastrointestinal tract without stimulating immune or inflammatory responses (Gardiner et al., 2002; Reid, Burton, Hammond, & Bruce, 2004). When probiotics are incorporated into food products, microbial culture viability tends to decrease (Douglas & Sanders, 2008).

1.2 Cereal or Pseudocereal Grains as a Prebiotic

In contrast, prebiotics are food ingredients such as certain carbohydrates that positively modify the function or growth of probiotic bacteria strains in the intestinal tract (Figueroa-González, Quijano, Ramírez, & Cruz-Guerrero, 2011). Recent studies have shown interest in using cereal or pseudocereal grains as a prebiotic due to their carbohydrate and nutritional value (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003;

Charalampopoulos, Pandiella, & Webb, 2003; Espirito-Santo et al., 2014; Gokavi, Zhang, Huang, Zhao, & Guo, 2005; Helland, Wicklund, & Narvhus, 2004; Kabeir, Abd-Aziz, Muhammad, Shuhaimi, & Yazid, 2005; Lazaridou, Serafeimidou, Biliaderis, Moschakis, & Tzanetakis, 2014; Rosburg, Boylston, & White, 2010; Saman, Fuciños, Vázquez, & Pandiella, 2009). The literature shows fermented dairy or cereal-based products such as yogurts, porridges, and gruel, being produced and inoculated with various probiotics to be tested for treating specific diseases, aside from having general health benefits (Agil et al., 2013; Angelov, Gotcheva, Kuncheva, & Hristozova, 2006; Bianchi, Rossi, Gomes, & Sivieri, 2015; Casarotti, Carneiro, & Penna, 2014; Charalampopoulos et al., 2003; Coman et al., 2013; Espirito-Santo et al., 2014; Helland et al., 2004; Kabeir et al., 2005; Kedia, Vázquez, & Pandiella, 2008; Lazaridou et al., 2014; Luana et al., 2014; Rosburg et al., 2010; Saman et al., 2009; Singh, Kim, & Liu, 2012). These studies yielded mostly significant results for microbial growth but more research is needed to test other probiotic strains and alternate dairy products such as milk.

1.3 Grain Flours

1.3.1 Oat

Oats are a source of dietary fiber that have been found to reduce the risk of certain cancers, decrease constipation, lower cholesterol, and control diabetes (Gokavi et al., 2005). Oat bran contains the fiber beta-glucan which contributes to health benefits such as reducing the risk of cardiovascular disease and stroke (Coman et al., 2013; Lazaridou et al., 2014). Beta-glucan has also been found to aid in enhancing the human immune system's response to bacterial infection (Coman et al., 2013). Studies which tested fermented dairy products containing oat flour mainly found that the beta-glucan increased the microbial count of different Lactobacillus probiotic strains (Angelov et al., 2006; Coman et al., 2013; Gokavi et al., 2005; Kedia et al., 2008; Lazaridou et al., 2014; Luana et al., 2014; Singh et al., 2012).

1.3.2 Rice

Rice is a source of carbohydrates, protein, iron, calcium, thiamine, riboflavin and niacin (Saman et al., 2009). Bran rice, a particular component of rice, has been shown to maintain normal blood pressure, glycemia, and serum cholesterol levels (Saman et al., 2009). Antioxidants in bran rice may act as a prebiotic that can help control colitis by managing microbial colonies in the digestive tract (Saman et al., 2009). Studies that have tested rice flour in fermented milk-based gruels, porridges, and puddings showed good growth and survival of various Lactobacillus strains such as *L. rhamnosus* GG (Espirito-Santo et al., 2014; Helland et al., 2004; Kabeir et al., 2005; Saman et al., 2009).

1.3.3 Lentil

Lentils contain carbohydrates, proteins, minerals, vitamins, antioxidants, and small amounts of unsaturated fats (Zare, Orsat, Champagne, Simpson, & Boye, 2012). Associated health benefits include lowering the risk of diabetes, colon cancer, and cardiovascular disease (Agil et al., 2013). Research so far has found that milk supplemented with lentil flour increased the microbial count of yogurt starter cultures and the strain *L. rhamnosus* AD 200, compared to controls (Agil et al., 2013; Zare, Champagne, Simpson, Orsat, & Boye, 2011; Zare et al., 2012).

1.3.4 Barley

Barley is a source of beta-glucan fiber and the health benefits from beta-glucan have been previously described in section 1.3.1. Current research concluded that barley extracts have a positive effect on the viability of various Lactobacillus strains in fermented cereal-based probiotic beverages (Charalampopoulos et al., 2003; Kocková, Dilongová, Hybenová, & Valík, 2013; Lazaridou et al., 2014; Rathore, Salmerón, & Pandiella, 2012). Interestingly, Kocková et al. (2013) found that barley flour produced the highest probiotic culture density for *L. rhamnosus* GG when compared to other cereal-grain flours while Vasiljevic, Kealy and Mishra (2007) obtained no measurable effects from barley flour on a Bifidobacterium probiotic strain.

1.3.5 Quinoa

Quinoa is classified as a pseudocereal and has a higher protein content compared to many cereal grains while containing all the essential amino acids along with fiber, iron, vitamins, and antioxidants (Bianchi et al., 2015; Casarotti et al., 2014). So far, studies examining quinoa in the form of flour or water extract found no significant effect on probiotic bacteria microbial counts (Bianchi et al., 2015; Casarotti et al., 2014; El-Deeb, Hassan, & Hassanein, 2014).

1.4 Importance of Study

More research is needed in producing food products with grains such as a milk beverage, testing microbial vitality for different probiotic strains, as well as testing the overall health benefits from these products. Nutrient content of probiotic beverages will vary due to the type of grains used for supplementation. The growth capacity for each probiotic strain will differ based on the various nutrients in the fermented milk (Gokavi et al., 2005). Thus, it is necessary to study different strains of probiotics to create the most effective and nutritious product in combination with various grains. Research examining the effects on *L. rhamnosus* GR-1 will be important because there is limited research as of present that has specifically investigated producing probiotic milk products fermented with grains and *L. rhamnosus* GR-1.

For this study, six probiotic skim milks were developed. Five of the milk samples were supplemented with a different cereal or pseudocereal grain flour, while one milk sample remained as the control. The purpose of this study was to measure the growth and survival of the probiotic *L. rhamnosus* GR-1 in the fermented, flour-supplemented, skim milks. The objective was to determine if supplementation of the grain flours (oat, white rice, green lentil, barley, and quinoa) would have a positive effect by increasing the microbial vitality of *L. rhamnosus* GR-1 in the final skim milk products and during storage.

2. Materials and Methods

2.1 Probiotic Culture Stock Preparation

10%(weight to volume ratio) of *L. rhamnosus* GR-1 was inoculated into sterilized de Man, Rogosa and Sharpe (MRS) broth (EMD Chemicals Inc., Gibbstown, NJ) and was incubated anaerobically (BDGasPak^{TM} EZ Anaerobe Container System, Becton Dickinson & Co., Sparks, BD), for 24 hours at 37.5°C. Stocks were prepared on a routine basis in MRS broth.

2.2 Milk Samples Preparation

2.2.1 Skim Milk (Control)

Skim milk (0.1% milk fat) (Neilson® Trutaste® Microfiltered Skim Milk, Saputo Inc., Montreal, QC) was autoclaved for 15 minutes at 15 psi and then cooled to 37°C.

2.2.2 Oat Flour Milk

Milled oat flour (Oat Flour, Bulk Barn #690, Aurora, ON) was supplemented into skim milk (0.1% milk fat) (Neilson® Trutaste® Microfiltered Skim Milk, Saputo Inc., Montreal, QC) at a 3% weight to volume (w/v) ratio. The sample was autoclaved for 15 minutes at 15 psi and then cooled to 37°C.

2.2.3 Rice Flour Milk

Milled white rice flour (White Rice Flour, Gluten-Free, Bulk Barn #690, Aurora, ON)was supplemented into skim milk (0.1% milk fat) (Neilson® Trutaste® Microfiltered Skim Milk, Saputo Inc., Montreal, QC) at a 3% w/v ratio. The sample was autoclaved for 15 minutes at 15 psi and then cooled to 37°C.

2.2.4 Barley Flour Milk

Stone ground barley flour (Barley Flour, Stone Ground, Bulk Barn #690, Aurora, ON) was supplemented into skim milk (0.1% milk fat) (Neilson® Trutaste® Microfiltered Skim Milk, Saputo Inc., Montreal, QC)at a 3% w/v ratio. The sample was autoclaved for 15 minutes at 15 psi and then cooled to 37°C.

2.2.5 Quinoa Flour Milk

Quinoa flour (Organic Quinoa Flour, Gluten-Free, Bulk Barn #690, Aurora, ON) was supplemented into skim milk (0.1% milk fat) (Neilson® Trutaste® Microfiltered Skim Milk, Saputo Inc., Montreal, QC) at a 3% w/v ratio. The sample was autoclaved for 15 minutes at 15 psi and then cooled to 37°C.

2.2.6 Lentil Flour Milk

Dried green lentils laird (Cedar Phoenicia^{TM}, Montreal, QC) were ground into a fine flour and sifted for particle consistency. The lentil flour was then supplemented into skim milk (0.1% milk fat) (Neilson® Trutaste® Microfiltered Skim Milk, Saputo Inc., Montreal, QC) at a 3% w/v ratio. The sample was autoclaved for 15 minutes at 15 psi and then cooled to 37°C.

2.3 Probiotic Milk Samples Production

At 37°C, the skim milks (control, oat, rice, lentil, barley, and quinoa) were inoculated individually with 2% (w/v ratio) stock solution of *L. rhamnosus* GR-1 and then anaerobically incubated (BDGasPak^{TM} EZ Anaerobe Container System, Becton Dickinson & Co., Sparks, BD) for 24 hours at 37.5°C. The probiotic milk samples were then stored at 4°C for 28 days.

2.4 Enumeration of Probiotic

Selective MRS agar plates were prepared containing 52.2g/L (5.22%) MRS broth (EMD Chemicals Inc., Gibbstown, NJ), 15g/L (1.5%) agar (EMD Chemicals Inc., Gibbstown, NJ), and 0.015g/L ($1.5x10^{-3}$%) fusidic acid (Enzo Life Sciences Inc., Farmingdale, NY) to enumerate *Lactobacillus rhamnosus* GR-1. The six milk samples were diluted to factors of 10^{-1}, 10^{-3}, 10^{-5}, 10^{-6}, and 10^{-7}in sterile 0.85% saline solution on days 1, 14, and 28 of refrigerated storage. For each milk sample, 0.1mL of the 10^{-6}, and 10^{-7} dilutions were then plated in duplicates on selective MRS agar plates and anaerobically incubated (BDGasPak™ EZ Anaerobe Container System, Becton Dickinson & Co., Sparks, BD) for 48 hours at 37.5°C. After incubation, viable microbial counts of *L. rhamnosus* GR-1weremeasured by averaging the two duplicate plates for each milk sample. The results were recorded in colony-forming units (CFU) per milliliter. Two replications of the milk samples were produced.

2.5 Statistical Analysis

Statistical analyses were conducted using SAS® 9.4 Software (SAS Institute Inc., Cary, NC). A one-way repeated measures analysis of variance (ANOVA) and Tukey's tests adjustment ($P<0.05$) were used to analyze the numbers of the probiotic bacteria within each milk sample and between milk samples, over time.

3. Results

3.1 Microbial Counts and Stability

All of the skim milk samples were able to support the survival of the probiotic *Lactobacillus rhamnosus* GR-1 at viable levels of at least $1x10^8$ CFU/mL over the 28-day storage period. Specifically, the samples oat, rice, barley, and quinoa supported an increase in the microbial count by day 14, but the sample containing rice flour was the only sample that did not significantly decrease in microbial count between days 14 to 28. Figure 1 illustrates the enumeration of *L. rhamnosus* GR-1 for all five samples and the control over a 28-day storage period.

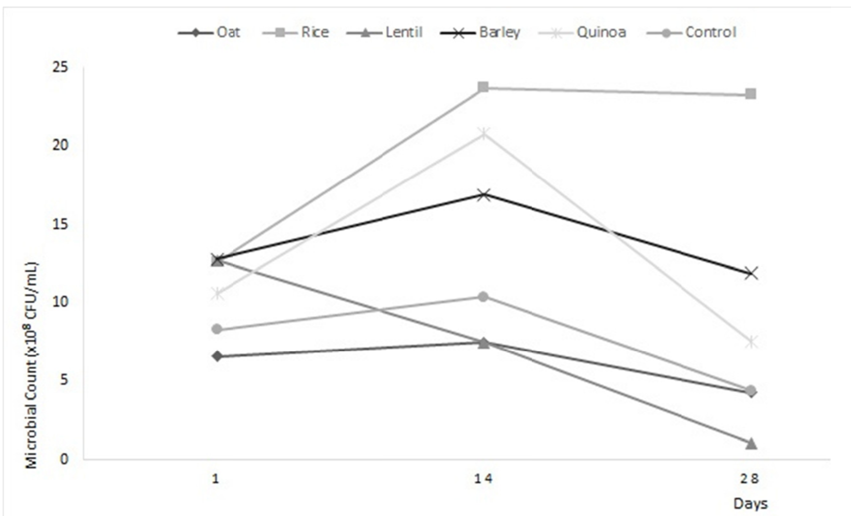

Figure 1. Enumeration ($x10^8$ CFU/mL) of skim milk samples over time (days)

Note. Mean microbial counts of *L. rhamnosus* GR-1 in probiotic skim milk samples supplemented with grain flours, and control (probiotic skim milk) over a 28-day storage period. Average of both replications. Where differences occur can be identified using Tukey's test for multiple comparisons.

The initial mean counts ($x10^8$ CFU/mL) of *L. rhamnosus* GR-1 for oat, rice, lentil, barley, quinoa, and control samples were: 6.6 ± 0.2, 12.6 ± 3.0, 12.7 ± 2.5, 12.8 ± 3.8, 10.6 ± 7.8, and 8.3 ± 3.9, respectively. Mean microbial counts ($x10^8$ CFU/mL) on day 14 were: 7.5 ± 2.2, 23.7 ± 1.8, 7.5 ± 2.5, 16.9 ± 2.9, 20.8 ± 6.5, and 10.4 ± 3.2, for oat, rice, lentil, barley, quinoa, and control samples, respectively. By day 28, the mean microbial counts ($x10^8$ CFU/mL) were: 4.3 ± 3.1, 23.3 ± 4.6, 1.1 ± 0.5, 11.9 ± 3.7, 7.6 ± 2.6, and 4.4 ± 3.5 for oat, rice, lentil, barley, quinoa, and control samples, respectively. The rice flour milk sample was found to have the highest mean count of 23.3 ± 4.6 $x10^8$ CFU/mL at day 28 of storage. Table 1 shows that the rice flour milk was significantly different overall in comparison to the other samples ($p=0.025$). The rice flour sample was significantly different between days 1 vs 14 ($p=0.029$) and days 1 vs 28 ($p=0.032$). As seen in Figure 1, the microbial count for the rice flour milk sample plateaued between days 14 to 28. This result is favorable in comparison to the remaining milk samples which saw a decrease in microbial count between days 14 to 28. Table 1 shows that the barley flour milk sample was also significantly different overall when compared to other milk samples (p=0.017). The barley flour milk sample was significantly different between days 1 vs 14 (p=0.026) and between days 14 vs 28 (p=0.018); however, the microbial count decreased by day 28.The lentil flour milk sample was significantly different in comparison to the other milk samples (p=0.019) as seen in Table 1. This is due to the decrease in microbial vitality over time (Figure 1); thus, this result is not favorable.

Table 1. P-Values (p<0.05) for within samples comparison of days (x10^8 CFU/mL)

	Overall	Day 1 vs Day 1	Day 1 vs Day 28	Day 14 vs Day 28
Oat	0.343			
Rice	**0.025**	**0.029**	**0.032**	0.947
Lentil	**0.019**	**0.082**	**0.018**	0.057
Barley	**0.017**	**0.026**	0.352	**0.018**
Quinoa	0.072			
Control	**0.008**	0.056	**0.016**	**0.007**

Note. Analysis completed on the average of both milk sample replications. The conventional approach is to not report pairwise comparisons if the overall comparison is not statistically significant. Significant p-values are bolded.

3.2 Comparison Between Milk Samples

By day 28 of storage, significant differences in the microbial counts for *L. rhamnosus* GR-1 were found between the following milk samples (Table 2): rice and control (p=0.009), rice and oat (p=0.010), rice and lentil (p=0.005), and between rice and quinoa (p=0.028). This suggests that overall, white rice flour has the potential to act as a prebiotic to significantly grow the probiotic *L. rhamnosus* GR-1 in skim milk and maintain this growth during the storage period.

Table 2. P-Values (p<0.05) for pairwise comparisons between samples for Day 28 (x10^8 CFU/mL)

	Control	*Oat*	*Lentil*	*Barley*	*Quinoa*
Oat	>0.999				
Lentil	0.966	0.938			
Barley	0.329	0.376	0.143		
Quinoa	0.796	0.852	0.428	0.900	
Rice	**0.009**	**0.010**	**0.005**	0.082	**0.028**

Note. Analysis completed on the average of both milk sample replications. There are statistically significant differences between the following samples: rice and control, rice and oat, rice and lentil, and between rice and quinoa. Significant p-values are bolded.

4. Discussion

The objective was to determine if supplementation of the different grain flours would have a positive effect on the microbial vitality of *Lactobacillus rhamnosus* GR-1 in the final product and during storage. Although all milk samples were able to maintain the probiotic at levels of at least 1x10^8 CFU/mL, only the rice flour milk sample significantly sustained growth over 28 days, reaching a microbial count of 2.3x10^9 CFU/mL. This result is similar to Helland et al. (2004) which found that *L. rhamnosus* GG exhibited the highest viable cell count in fermented milk-based puddings containing rice flour by growing to over 1x10^9 CFU/mL during a 28-day storage period. The growth of various Lactobacillus strains during less than 30 hours of fermentation in rice flour gruel and rice flour broth reached levels between 7.0-10.4 log CFU/mL for Espirito-Santo et al. (2014), and Saman et al. (2009), respectively. Kabeir et al. (2005) found significant growth of a Bifidobacterium strain in fermented porridge made of rice flour and skim milk, reaching a count of 9.9 log CFU/mL, and decreasing by 0.9 log CFU/mL during a two-week storage period (Kabeir et al., 2005). These studies, including our own, demonstrate that rice flour allows significant probiotic growth; however, it is difficult to compare due to various time frames and probiotic strains.

Studies testing fermented products containing oat flour or flakes found significant results with strains of *Lactobacillus plantarum* and *L. paracasei* which were found to remain at levels of at least $1x10^6$ CFU/mL over a 28-day storage period in fermented yogurt or water-based oat beverages (Angelov et al., 2006; Coman et al., 2013; Gokavi et al., 2005; Kedia et al., 2008; Lazaridou et al., 2014; Luana et al., 2014). While *L. rhamnosus* GR-1 remained above $1x10^8$ CFU/mL in our oat flour sample, significant microbial growth was not observed. Some studies suggest that beta-glucan found in oat flour protects probiotic strains which may explain why the probiotic remained at a viable level (Coman et al., 2013; Lazaridou et al., 2014; Rosburg et al., 2010).

Milk supplemented with lentil flour increased the microbial count of *L. rhamnosus* AD 200 to over $1x10^8$ CFU/mL over a 28-day storage period (Agil et al., 2013; Zare et al., 2011, 2012). The results of Zare et al. (2011, 2012) contradicts our study in which the lentil flour probiotic milk significantly decreased over time; however, the lentil milk in our study was at a level of $1.1x10^8$ CFU/mL by the end of the storage period. This difference could possibly be due to a difference in the type of lentil flour used or the alternate probiotic strain.

Barley flour was found to have a positive effect on the microbial viability of *Lactobacillus plantarum* and *L. acidophilus* strains in fermented cereal-based probiotic beverages (Charalampopoulos et al., 2003; Kocková et al., 2013; Rathore et al., 2012). Our results are similar to Kocková et al. (2013) which found that barley flour fermented in water produced the highest probiotic culture density for *L. rhamnosus* GG when compared to other cereal-grain flours.

Growth of various Bifidobacterium and Lactobacillus strains in fermented milk with quinoa flour or aqueous extracts of quinoa, respectively, were not found to be significant in past studies (Bianchi et al., 2015; Casarotti et al., 2014). Quinoa did not affect microbial growth or provide protective benefits over a 28-day period (Bianchi et al., 2015; Casarotti et al., 2014) while El-Deeb et al. (2014) found that the probiotic bacteria slightly decreased in count after seven days. These results coincide with our study which saw a decrease in the microbial vitality after 14 days.

5. Conclusion

Skim milk supplemented with white rice flour supports the growth and survival of *L. rhamnosus* GR-1 beyond 10^8 CFU/mL over a 28-day storage period to provide health benefits. This study exhibits the potential for production of this fermented probiotic milk product as an alternate food product to deliver the health benefits of probiotics to consumers. Further studies should conduct sensory evaluations, test alternate probiotic strains, and study the microbial vitality of probiotics inoculated in rice milk.

6. Implications, Limitations, and Future Proposals

The intention was to create a novel food product that can benefit the general population, but more specifically, individuals seeking relief from yeast infections or urogenital discomfort, as well as to help strengthen the immune system of patients with HIV/AIDS. The possible health benefits of the specific grain flours may also make the fermented probiotic milk products of interest to consumers with or at risk of certain diseases or nutritional deficiencies. The consumption of probiotic yogurt has increased significantly over the past few years in North America (Granato, Branco, Nazzaro, Cruz, & Faria, 2010; Hekmat et al., 2009). The possibility of a probiotic milk beverage may attract industry and consumer interest as an alternate product for consumption. This study showed that the addition of a grain flour, particularly white rice flour, acted as a prebiotic to increase the microbial vitality of *L. rhamnosus* GR-1. This would ensure a product that offers a viable number of probiotics at consumption, even once the product reaches the end of its shelf life. Besides the known therapeutic effects of the specific probiotic strain, the grain flour may contribute additional health benefits to the product; thus, further research should be conducted to determine this.

A limitation of this study was that the amount of each grain flour (3% w/v ratio) and the probiotic culture (2% w/v ratio) to be supplemented and inoculated, respectively, were estimated based off of previous studies which were similar but used alternate probiotics, grain flours, and fermented food products (yogurt, gruels etc.). These amounts may not have achieved the best possible results for microbial vitality. Another limitation was that only two replications were conducted. Although the results were similar for each replication, further replications can account for any possible errors during the procedure of the experiment as well as demonstrate if the results remain consistent.

Future studies with animal models and human clinical trials are necessary to determine any health-related properties of these probiotic, grain-supplemented milks; specifically the rice flour supplemented probiotic milk. Sensory panels with humans should also be conducted using this fermented probiotic rice flour milk to determine consumer acceptance and production of this product at the industry level. Further studies should test the microbial vitality of *L. rhamnosus* GR-1 in rice milk to obtain a lactose-free and possible gluten-free beverage as well as test the microbial vitality of other probiotic strains.

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