International Food Research Journal 23(2): 889-893 (2016)
Journal homepage: *http://www.ifrj.upm.edu.my*

Effect of germinated Hang rice on growth and viability of probiotic *Lactobacillus* during refrigerated storage

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Article history

Received: 5 August 2015
Received in revised form: 9 September 2015
Accepted: 3 October 2015

Keywords

Lactobacillus
Probiotics
Germinated Hang rice
Viability
Refrigerated storage

Abstract

The ability of probiotic bacteria to grow in cereal substrates and viability during storage are important for the development of products supplemented with probiotic cultures. This study aimed to evaluate growth and viability of two probiotic *Lactobacillus* sp. in germinated Hang rice. *L. acidophilus* TISTR 450 and *L. plantarum* TISTR 875 were cultured in fermentation medium prepared from rice powder (3 and 5% w/v). After inoculation, the culture was incubated at 100 rpm and 37°C for 48 hr. Three and five percent (w/v) of germinated Hang rice supported the growth of *L. plantarum* TISTR 875 which cell population increased 4.5 and 4.10 $\log_{10}$CFU/ml, respectively. Survival of bacteria in germinated Hang rice during storage at 4°C was also observed. *L. plantarum* TISTR 875 remained at about 7.34 and 8.02 $\log_{10}$CFU/ml in 3 and 5% (w/v) germinated Hang rice, respectively, throughout the refrigerated storage period (22 days). In addition, the pH value and reducing sugar concentration of the culture medium reduced along the storage period.

Introduction

Probiotics are live microbial food supplements which are beneficial for health of consumers by maintaining or improving their intestinal microbial balance (Fuller, 1989). Probiotic bacteria are applied to balance disturbed intestinal microflora and related dysfunctions of the gastrointestinal tract (Salminen *et al*., 1998). The basic mechanisms associated with probiotic bacteria are the modulation of the intestinal microflora of the host and the capacity to interact with the immune system directly or mediated by the autochthonous microflora (de Vrese and Schrezenmeir, 2008). Microorganisms most commonly used as probiotics belong to *Lactobacillus* species, such as *Lactobacillus acidophilus, L. casei, L. reuteri, L. rhamnosus, L. johnsonii,* and *L. plantarum* and *Bifidobacterium* species, such as *Bifidobacterium longum, B. breve, B. lactis* (Shortt, 1999). Probiotic bacteria can be found worldwide in a variety of products, including conventional food products, dietary supplements and medical foods (Sanders, 2000). Traditionally, the incorporation of probiotic strains in food has been established in the dairy products (Rivera-Espinoza and Gallardo-Navarro, 2010). However, consumers nowadays are increasingly demanding non-dairy probiotic products due to vegetarianism, milk cholesterol content, and lactose intolerance (Granato *et al.*, 2010). Cereals offer another alternative for the production of probiotic foods. It has previously been reported that cereals, such as malt, barley, wheat (Charalampopoulos *et al*. 2002; Charalampopoulos and Pandiella 2010), brown rice and rice bran (Saman *et al.*, 2011), and germinated rough rice (Trachoo *et al*., 2006) can be used as fermentable substrates for the growth of probiotic microorganisms. Germinated Hang rice is rice product produced in the North Eastern Thailand. Hang rice is produced from harvesting rice grains in immature but fully formed stage (dough stage). The rice grains are then streamed, dried and partially polished. Hang rice is promoted as high nutritious food which rich of carbohydrate, vitamin and minerals (Kerdpiboon and Charoendee, 2012). Moreover, germination process of rice grains increased vitamin B, reducing sugar, total protein (Trachoo *et al*. 2006; Saman *et al.* 2008), and γ-aminobutyric acid (GABA) (Kayahara *et al*., 2000).

Apart from the good growth characteristics of the probiotic strain in cereal-based media, the viability of the strain in a product at the point of consumption is another important consideration for product development of probiotic foods. Probiotics are defined as "live microorganisms which, when

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administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). The microorganisms should maintain viability and be available in the food at a high number, typically at least 6-7 $\log_{10}$CFU/ml (Dave and Shah 1997; Gomes and Malcata 1999). Several factors particularly probiotic strains, pH, organic acid concentrations, storage temperature, presence of microbial inhibitors as well as dissolved oxygen have been shown to have their impact on survival of probiotic bacteria during processing and storage (Brunner *et al.* 1993; Gomes and Malcata 1999).

The aims of this study were to investigate the ability of germinated Hang rice to support the growth of *Lactobacillus* probiotics and evaluate the survival ability of the bacteria in germinated Hang rice medium during refrigerate storage.

Materials and Methods

Bacterial strains, culture medium and culture conditions

Lactobacillus acidophilus TISTR 450 (unknown source) and *L. plantarum* TISTR 875 (Source: pickled cabbage) were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Microbiological Resource Center (MIRCEN), Thailand. The isolates were maintained in MRS broth (De Man *et al*., 1960) and kept at -20°C with the addition of skim milk to 5% (v/v) final concentration. For cell propagation procedure, the stock cultures were taken from -20°C freezer, thawed at room temperature. Two hundred µl of each culture were inoculated into 2 ml of MRS broth. After incubation for 18 h in anaerobic jar (Merck, Germany) and 37°C, the culture was streaked onto MRS agar, and incubated under the same conditions for 48 h. Then, a single colony was inoculated in MRS broth (Hi media, India) for 18 h at 37°C. The cells were harvested by centrifugation at 5,000 g for 10 min at 4°C, wash twice with sterile phosphate buffer and re-suspended in the same solution. The bacterial suspensions were then used to inoculate to fermentation media at 1% (v/v). The initial cell concentration in fermentation media was approximately 7 $\log_{10}$CFU/ml.

Germinated Hang rice-based media and fermentation procedures

Germinated Hang rice was ground using a hammer mill equipped with a sieve of size 0.5 mm. Rice media were prepared using 500 ml Erlenmeyer flask. The resulting flour was suspended in 400 ml of distilled water to a final concentration of 3% (w/v) and 5% (w/v), and was then autoclaved at 121°C for 15 min. The sugar content, pH, and protein content and buffering capacity of the medium were analyzed.

Culture media were inoculated with cell suspension and incubated at 37°C with agitation at 100 rpm for 48 h. The fermentation media were then stored at 4°C. Samples from culture media were collected on days 0, 2, 4, 6, 10, 14, 18 and 22. Reducing sugar and lactic acid concentration, pH, and bacterial growth were then measured. All fermentations were performed in duplicate.

Chemical analyses

The fermented media were centrifuged at 8,000 g and 4°C for 10 min and then stored at -20°C until analysis. Reducing sugar concentration was determined by the 3, 5-dinitrosalicylic acid method (Miller, 1959), using glucose as a standard. The total sugar content was determined by the phenol-sulfuric acid method (DuBois *et al*., 1956), using glucose as a standard. Nitrogen content of the rice media was estimated by Kjeldahl method (AOAC, 1995). The factor used to convert nitrogen into crude protein was 5.95. The amount of the lactic acid produced in the fermentation media were determined by the standard titration procedure for total titratable acidity (TTA) according to AOAC (1990). The pH levels were also measured using a pH meter. The buffering capacity of the rice media was determined by titrating 100 ml of the medium with HCl (1 mole/l). The values were expressed as the amount of HCl (mmoles) required to drop 1 pH unit per unit volume (l) (Pai *et al*., 2001).

Bacterial enumeration

Viable cell counts (CFU/ml) were estimated by drop method (Collins *et al*., 1989) on MRS agar plate. Plates were incubated anaerobically at 37°C for 48 h, after which they were counted and expressed as $\log_{10}$CFU/ml.

Results and Discussion

Growth of Lactobacillus *in germinated Hang rice media*

The germinated Hang rice media were inoculated with 1% (v/v) starter culture suspension of *L. acidophilus* TISTR 450 and *L. plantarum* TISTR 875. The cell concentration was between 6-7 $\log_{10}$CFU/ml. Temperature of the cultivation medium were kept constant at 37°C. Results of bacterial growth in germinated Hang rice media after 48 h of fermentation are shown in Figure 1. The cell populations of *L. acidophilus* TISTR 450 increased and provided the highest bacterial cell numbers after 24 h of fermentation, then declined from 9.09 to

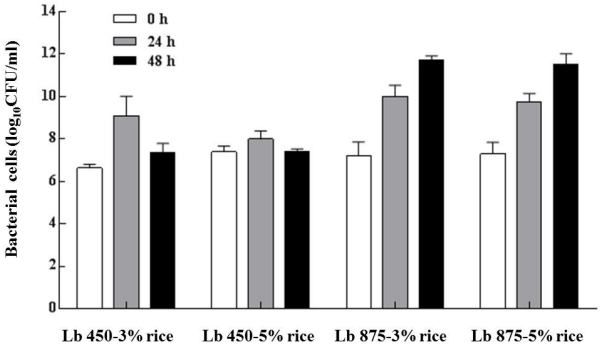

Figure 1. Growth of *L. acidophilus* TISTR 450 and *L. plantarum* TISTR 875 in 3 and 5% (w/v) germinated Hang rice media after 48 h of fermentation. Results shown as mean values ± standard deviation for duplicate samples. Lb 450 = *L. acidophilus* TISTR 450, Lb 875 = *L. plantarum* TISTR 875

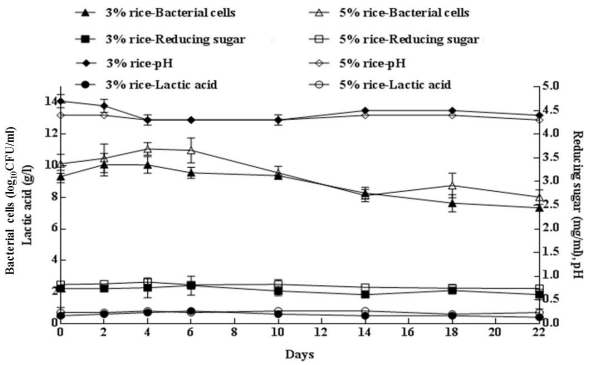

Figure 2. Survival curve of *L. plantarum* 875 in 3 and 5% germinated Hang rice media stored at 4°C. Results shown as mean values ± standard deviation for duplicate samples

7.36, and 7.99 to 7.41 $\log_{10}$CFU/ml in 3% and 5% germinated Hang rice medium, respectively, after 48 h. Germinated Hang rice supported growth of *L. plantarum* TISTR 875 which reaching a maximum cell concentrations of approximately 11.72 and 11.51 $\log_{10}$CFU/ml in 3% and 5% germinated Hang rice medium respectively after 48 h of fermentation. Lactobacilli are fastidious bacteria with complex nutritional growth requirements (e.g. for carbohydrate, amino acids, peptides, vitamins and nucleic acids derivatives) (Hammes and Vogel, 1995). Our study showed that the germinated Hang rice medium is appropriate substrate for the growth of *L. plantarum* TISTR 875. This could be ascribed to the presence of sufficient amounts of growth supplements in the rice medium. The chemical composition of germinated Hang rice medium was reported in Table 1. Charalampopoulos *et al.* (2003) suggested that the malt medium supported the growth of *L. fermentum, L. reuteri, L. acidophilus* and *L. plantarum* probably due to the availability of glucose, fructose, maltose, sucrose (approximately 15 g/l of total fermentable sugars) and free amino nitrogen (approximately 80 mg/ l). In this study, however, *L. acidophilus* TISTR 450 exhibited poor growth in germinated Hang rice medium. From this result, it seemed possible that the strain has a requirement for large amounts of growth supplements. Most strains of *L. acidophilus* have complex growth requirements (Mital and Garg, 1992). Substrate deficiency in specific nutrients, such as free amino acids, B-vitamins or minerals, contributed to growth limitation of *L. acidophilus* (Charalampopoulos *et al.*, 2003). The viable cell counts at the end of fermentation in germinated Hang rice remained above the suggested minimum limit of 6-7 $\log_{10}$CFU/ml (Dave and Shah, 1997). Previous studies have shown that cereals are good substrate for probiotic growth. Patel *et al.* (2004) reported a maximum growth of *L. plantarum* in malt, barley and wheat of 9.15, 8.46 and 8.39 $\log_{10}$CFU/ml, respectively. Kedia *et al.* (2008) reported a maximum growth of *L. plantarum* of 9.16 $\log_{10}$CFU/ml in white oat flour. Rice-based medium were also recorded that supported the growth of *L. plantarum* NCIMB 8826 with a biomass value of approx 10.4 $\log_{10}$CFU/ml (Saman *et al*., 2011). Malt medium supported the growth of *L. fermentum, L. reuteri, L. acidophilus* and *L. plantarum* (8.10-10.11 $\log_{10}$CFU/ml, depending on the strain) (Charalampopoulos *et al*., 2003).

Survival of Lactobacillus *in germinated Hang rice media during refrigerated storage*

After fermentation of germinated Hang rice media with *L. plantarum* TISTR 875 for 48 h and then stored at 4°C, the viability of *L. plantarum* TISTR 875 was monitored (Figure 2). At the end of the 22 days storage period, the viability of *L. plantarum* TISTR 875 in 3% and 5% germinated Hang rice medium was reduced by 1.98 and 2.10 $\log_{10}$CFU/ml, respectively. Although the loss of bacterial viability was found during cold storage, the viable cell counts of *L. plantarum* TISTR 875 in germinated Hang rice still remained at 7-8 $\log_{10}$CFU/ml after 3 weeks of cold storage at 4°C. Viability of probiotic bacteria in a product at the point of consumption is an important consideration. It has been suggested that probiotics should maintain viability and in sufficient numbers (approximately 6-7 $\log_{10}$CFU/ml) (Dave and Shah, 1997). The survival of *Lactobacillus* probiotics during storage has been shown in previous study. Martensson *et al*. (2002) reported high survival of *L. reuteri* ATCC 55730 in oat based non-dairy products after 30 days of storage at 6°C (10^8 CFU/ml). Gupta *et al*. (2010) reported the no significant reduction of 0.9 $\log_{10}$CFU/ml of *L. plantarum* in oat based drink during storage of 21 days. Several factors, including the pH, the lactic acid concentration, sugar

Table 1. Chemical composition of autoclaved 3 and 5% (w/v) germinated Hang rice media

	3% (w/v) germinated Hang rice	5% (w/v) germinated Hang rice
Protein content (%)	0.19±0.01	0.32±0.02
Total sugar (mg/ml)	17.42±0.12	23.43±0.22
Reducing sugar (mg/ml)	0.37±0.07	0.57±0.02
Buffering capacity	3.64±1.33	3.44±1.11
pH	6.52±0.11	6.25±0.09

concentration of the fermented products, have been reported to influence probiotic survival in cereal-based fermented products during fermentation and refrigerated storage (Charalampopoulos *et al*. 2002; Angelov *et al*. 2006; Charalampopoulos and Pandiella 2010). In our study, the slight reduction in pH value and reducing sugar concentration of the culture medium were observed during the storage period. In addition, there was no significant change in lactic acid concentration during the storage. Charalampopoulos and Pandiella (2010) studied the survival of *L. plantarum* in barley, wheat and malt extract during storage at 4°C for up to 70 days. The study found that both sugar and lactic acid influenced cell survival during storage. Survival of the bacteria progressively increased as the sugar increased (0 to 3 g/l), and as lactic acid concentration decreased (10 to 4 g/l). Viability of probiotic bacteria may be improved by the availability of micronutrients such as peptides and amino acids (Shah, 2000).

Conclusions

This study demonstrated that germinated Hang rice is suitable substrate for the growth of *L. plantarum* TISTR 875. The strain survived in germinated Hang rice during the refrigerated storage period (22 days) which remained at about 7.34 and 8.02 $\log_{10}$CFU/ml in 3 and 5% (w/v) germinated Hang rice, respectively. Further research will aim to study the protective effect of germinated Hang rice on viability of probiotic strains under gastrointestinal tract conditions to develop a novel delivery vehicle of probiotics. The information will be useful for product development of probiotics from germinated Hang rice.

Acknowledgements

The authors thank Research and Development Institute Chalermphrakiat Sakon Nakhon Province Campus for financial support. They are also grateful to Faculty of Natural Resources and Agro-Industry Kasetsart University for providing research facilities.

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