UNDER PEER REVIEW

Original Research Article

Isolation, characterization and acid/alkaline tolerance of lactic acid bacteria present in fermented rye, wheat, oat and barley

ABSTRACT

Objective: This study determined the lactic acid bacteria (LAB) present in fermented rye, wheat, oat and barley grains, and evaluated their survival in simulated gastric juice and pancreatic juice.

Methods: Samples of rye, wheat, oat and barley grains were fermented for 72 hours. Lactic acid bacteria (LAB) strains were isolated using MRS agar and were enumerated. Isolated LAB strains were cultured with MRS broth and were characterized using API 50 CH kit (Biomerieux, France). Each isolated LAB strain was exposed to simulated gastric juice at pH of 2.0 for 80 minutes at 37^0C, followed by exposure to simulated pancreatic juice at pH of 8.0 for 120 minutes at 37^0C. Aliquots were taken at 0_a minute and 80_a minutes at pH of 2.0 and, 0_b minutes and 120_b minutes at pH of 8.0 for enumeration of LAB strains.

Results: The total LAB cell count ranged from $6.6 * 10^8 \pm 11$ cfu/ml in the rye sample to $9.5*10^9 \pm 7$ cfu/ml in the oat sample. 13 LAB strains were isolated from the four selected cereal grains and were characterized as six strains of *Lactobacillus plantarum*1, five strains of *L. brevis* 1 and one strain each of *L. collinoides* and *Leuconostoc citreum*. There was no significant difference in the LAB cell count between 0_a and 120_b minutes ($p > 0.05$). All strains were capable of surviving in simulated gastric juice at pH of 2.0 and simulated pancreatic juice at pH of 8.0.

Conclusion: LAB associated with fermentation of rye, wheat, oat and barley grains are likely to survive transport through the harsh acidic and alkaline conditions of the GIT.

Keywords: lactic acid bacteria, rye, wheat, oat, barley, fermentation

1.0 INTRODUCTION

Cereal grains are the edible seeds of plants which belong to the grass family *Poaceae* also known as *Gramineae* [1]. They are important staple foods both in the developed and developing countries [2] and represent up to 73% of the total world plant produce harvested annually [3]. The major cereals grown around the world include rice, maize, wheat, oat, barley, millet and sorghum [4].
Cereals are rich sources of protein, carbohydrate, vitamins and minerals as well as non-nutrients such as dietary fibre [2,5]. The protective roles of cereals such as lowering the risk of gastrointestinal diseases like hemorrhoids [6], diverticulitis [7], colorectal cancer [8], and constipation [9] are linked to the colonic fermentation of their dietary fibre content. These include oligosaccharides and non-starch polysaccharides [8]. These short chain carbohydrates referred to as prebiotics are resistant to digestion and absorption but are fermented by bacteria in the colon [10]. They also induce the growth and activity of beneficial bacteria in the colon [11]. These beneficial bacteria are known as probiotic microorganisms [12] and usually belong to *Lactobacillus* and *Bifidobacillus* species [11].
The organisms that are involved in fermentation of cereals include bacterial species such as *Lactobacillus*, *Streptococcus* and *Bacillus*, yeast species of *Saccharomyces* and mould such as *Cladosporium* and *Penicillium* species [13]. Some of these organisms possess probiotic potentials especially *Lactobacillus, Saccharomyces* and *Bifidobacillus* species [11]. Probiotics prevent the activities of pathogenic microorganisms by reducing the luminal pH [14], producing antimicrobial substances or by inhibiting the adherence and translocation of bacteria in the gut [13].
Ability to survive the passage to the active site of expected beneficial action is an essential selection criteria for a probiotic microorganism [15]. The stomach secretes up to 2.5 litres of gastric juice at a pH of approximately 2.0 everyday [16]. In contrast, about 0.7L of pancreatic juice at an average pH of 8.0 is secreted into the small intestine every day [16. The pH of these secretions in addition to bile (secreted into the small intestine) leads to destruction of majority of ingested microorganisms. Therefore any microorganism expected to have beneficial effects in the gut must possess the ability to survive the transport through the acid and alkaline conditions of the stomach and small intestine respectively [17].

Several studies have explored the prebiotic potentials of rye, wheat, oat and barley grains or their products fermented using a starter culture [18;19]. However, previous studies on the fermentation of cereal or cereal products did not evaluate the acid and alkaline tolerance of the isolated lactic acid bacterial strains. Therefore, this study was aimed at evaluating the survival of lactic acid bacteria isolated from spontaneously fermented rye, wheat, oat and barley grains in simulated pancreatic and gastric juices. Emphasis was placed on the tolerance of each strain to the harsh acidic and alkaline conditions in the gastrointestinal tract in view of determining if spontaneous fermentation of the selected cereal grains will lead to the growth of bacteria with probiotic potential.

2.0 MATERIAL AND METHODS

2.1 Sources of materials

Organically grown rye (*Secale cereal)*, wheat (*Triticum aestivum)* and pot barley (*Hordeum vulgare*) were purchased online from buy whole foods online.co.uk while coarse oat meal (*Avena sativa*) was obtained from Mornflakes.

2.2 Soaking, washing, milling and fermentation

Fifty grams of each cereal sample was soaked in 150mls of cold tap water for 24 hours (h) in order to soften the grains for easy milling. Rye, wheat and barley grains were washed three times with tap water, milled using a smoothie maker (Kenwood – model number: 0WSB26001) and were fermented for 72h. The oat meal was ground in the water in which it was soaked in as it has already been processed and was allowed to ferment.

2.3 Enumeration, isolation and characterization of lactic acid bacteria

Six decimal dilutions (0.1% balanced peptone and 0.85% sodium chloride at a pH of 7.0) of each fermented cereal was prepared. One hundred micro liters of each serial dilution was streaked on MRS (deMan Rogasa Sharpe) agar (Technical No. 3 Oxoid, Basinstoke, Uk) plates. The plates were incubated for 48h at 30^0C and the bacterial colonies were counted.

Each distinct LAB colony observed was streaked repeatedly on MRS agar plates until a pure culture was obtained [20]. The presumptive LAB strains were gram stained as described by [21] to ascertain their gram staining features. Pure culture of each isolated strain was inoculated into 20 ml of MRS broth (Oxoid, Basinstoke, Uk) and incubated for 24h at 30^OC [21]. Pure cultures in MRS broth were then kept in the refrigerator at 4^0C for a maximum of 3 days or until they were used. Isolated strains were cultured twice in 20mls of MRS broth for 24h at 30^0C to revive the cells for further characterization based on their carbohydrate fermentation pattern using API 50 CH kit (BioMerieux SA, France) as described by [17].

2.4 Preparation of fermented cereal media

The pH of each fermented cereal sample was adjusted to 2.0 using 1Mol HCl (hydrochloric acid). Fifty milliliters of each fermented cereal sample was dispensed into 100cm^3 medical flasks in batches and were autoclaved for 15 minutes at 121^{0}C and pressure of 15 psi (pounds per square inch).

2.5 Preparation of simulated gastric juice and pancreatic juice

Gastric juice was prepared daily by dissolving 60mg of pepsin (Sigma, MO, USA) in 20mls of sterilized peptone saline diluent. Pancreatic juice was prepared by dissolving 40mg of pancreatin (Sigma, MO, USA) and 200mg of bile bovine (Sigma, MO, USA) in 40ml of peptone saline diluent.

2.6 Evaluation of acid and alkaline tolerance of all isolated LAB strains

Isolated LAB strains were cultured twice in MRS broth for 24h at 30^{0}C, centrifuged (Alc centrifuge, model no: PK120) at 4000g for 20 minutes (mins) and the residues were collected aseptically. Five hundred micro milliliters of residue from each centrifuged LAB strain was inoculated into 50ml of corresponding fermented cereal medium from which it was isolated at pH of 2.0. 5mls of simulated gastric juice was added to each medium. Each solution was mixed thoroughly using a vortex mixer (Stuart, Staffordshire, UK) and was incubated at 37^{0}C for 80mins [22]. This was followed by increase in the pH of the fermented cereal medium to 8.0 using 1Mol of NaOH (sodium hydroxide). Ten milliliters of pancreatic juice was then added to each cereal medium and was incubated at 37^{0}C for 120mins [23]. One milliliter of aliquot from each cereal sample was collected at 0_a minute (min) and 80_a mins at pH of 2.0 containing simulated gastric juice before and after incubation respectively and at 0_b mins and 120_b mins at pH of 8.0 containing simulated pancreatic juice before and after incubation respectively for determination of total viable LAB count.

2.7 Data Analysis

Statistical analyses were conducted with SPSS version 21.0.Test for normality of the colony counts of the strains was conducted using Shapiro-Wilk test (due to sample size below 100) at $p<0.05$. Non parametric Friedman ANOVA was carried out to determine significant difference ($p<0.05$) in the colony count of the LAB strains at all times tested during the acid and alkaline tolerance test.

3.0 RESULTS AND DISCUSSION

3.1 Enumeration of LAB

The LAB cell count of the selected cereals ranged from 6.6 * 10^8 cfu/ml (colony forming units per milliliter) in rye to 2.48 * 10^9 cfu/ml in wheat. The total LAB count of each fermented cereal sample is presented in table 1

Table 1: Total LAB count of each selected cereal grain

Cereal	LAB count (CFU/g)
Rye	6.6 $\times 10^8 \pm 11$
Wheat	2.48$\times 10^9 \pm 16$
Oat	9.5 $\times 10^9 \pm 7$
Barley	6.7 $\times 10^8 \pm 10$

Each result is expressed as the mean of triplicate measures and their standard deviations.

The total LAB cell count observed in this study suggests that they are good substrates for the growth of LAB. The high LAB cell count obtained in these fermented cereal grains may be due to their high nutrient contents, especially carbohydrates and vitamins, which promote the growth and activity of LAB in the presence of moisture and other environmental conditions such as oxygen and temperature [22]. The total LAB cell count obtained in the selected cereal samples after fermentation are within the same range reported by [23; 18].

3.2 Characterization of the LAB strains

Four distinct LAB colonies were isolated from the barley sample (B_1, B_2, B_3 and B_4) and rye sample (R_1, R_2, R_3 and R_4), three colonies isolated from wheat sample (W_1, W_2 and W_3) while two colonies were isolated from oat sample (O_1 and O_2). Using API 50 CH characterization test, two LAB strains isolated from barley were characterized as *Lactobacillus plantarum*1 while others were characterized as *L. collinoides* and *Leuconostoc citreum.* The four strains isolated from rye were all characterized as *Lactobacillus brevis* 1 whilst two strains isolated from oat were both characterized as *Lactobacillus plantarum*1. Two of the wheat strains were characterized as *Lactobacillus plantarum*1 while the remaining as *L. brevis* 1.

All isolated LAB strains had different API 50 CH identification profile. Interestingly, none of the six *Lactobacillus plantarum* strains and five strains of *L. brevis* 1 isolated in this study shared the same fermentation pattern. The variations observed in the fermentation patterns of the isolated *L. plantarum*1 and *L. brevis* 1 strains may be due to differences in their subspecies [24]. *L. plantarum*1 dominated the spontaneous fermentation of the oat sample whilst *L. brevis* 1 dominated the spontaneous fermentation of the rye sample. *L. plantarum*1 and *L. brevis* 1 were both present in the wheat sample while *L. plantarum*1, *L. collinoides* and *Leuconostoc citreum* dominated the fermentation of the barley sample. *L. plantarum* and *L. brevis* are among the LAB species commonly isolated from fermented cereal foods [14]. *L. plantarum* and *L. brevis* also dominated the spontaneous fermentation of wheat sourdough in the work by [23] whilst [16] found *L. fermentum* in addition to *L. plantarum* and *L. brevis* in fermented wheat sourdough.

3.3 Acid and alkaline tolerance of all isolated LAB strains

All the isolated LAB strains from the four selected cereals survived in the simulated gastric juice at pH of 2.0 (before and after incubation at 0_a min and 80a mins) and after addition of simulated pancreatic juice at pH of 8.0 (before and after incubation at 0b mins and 120 mins respectively). There was significant ($p = 0.017$) increase in the colony counts of all the strains at all times. The mean viable counts of all the strains ranged from 2.0 *10^8 in R_3 at 0b mins to 1.54 * 10^{10} in B_4 at 0_b minutes as shown in figure 1.

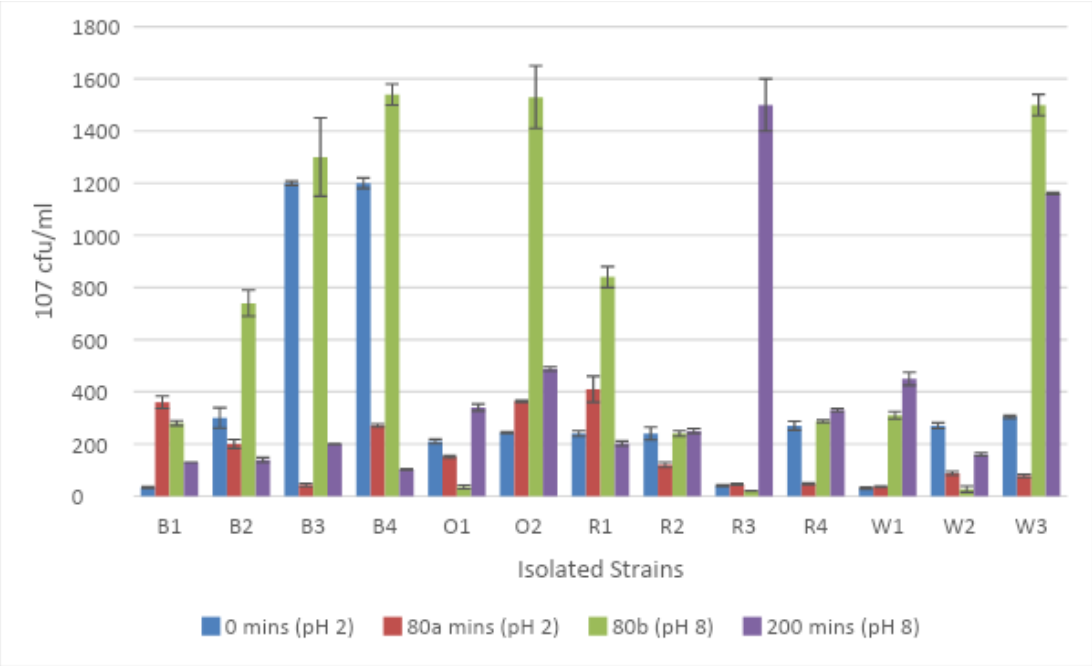

Figure 1: Viable cell count of isolated LAB strains during the acid and alkaline tolerance test. Each point shows the mean viable cell counts of triplicate measures and standard deviation of all the isolated strains when exposed to simulated gastric juice in acidic medium (pH of 2.0) before and after incubation (37^0C) at 0 min and 80a mins respectively and in alkaline medium (pH of 8.0) containing simulated pancreatic juice before and after incubation (37^0C) at 80b mins and 200 mins respectively.

All isolated LAB strains were capable of surviving in simulated gastric juice at pH of 2.0 and in simulated pancreatic juice at pH of 8.0 at all times tested in this study. The lowest viable cell count of 2.0 *10^8 cfu/ml obtained in R_3 at 0_b minutes before incubation in alkaline medium (at pH of 8.0) containing simulated pancreatic juice is higher than 1 * 10^7cfu/ml, which is the minimum viable count that any bacteria destined to confer a beneficial effect in the GIT is expected to have at the time of consumption [26]. This implies that the LAB strains are able to tolerate the effects of simulated gastric juice at pH of 2.0 and simulated pancreatic juice at pH of 8.0. Therefore, they are likely to survive the transport through the harsh acidic and alkaline conditions of the gastrointestinal tract.

4.0 Conclusion

All isolated LAB strains from the four selected cereals were capable of tolerating the activities of simulated gastric juice at pH of 2.0 and simulated pancreatic juice and bile at pH of 8.0. This shows that they are likely to survive the transport through the harsh acidic and alkaline conditions of the gastrointestinal tract. Therefore, consumption of fermented cereal foods and their products can be encouraged as a means of promoting the intake of microorganisms with probiotic potentials.

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Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.