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

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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 at room temperature. Lactic acid bacteria (LAB) strains were isolated using MRS agar and were enumerated. Isolated LAB strains were cultured with MRS broth and the fermentation patterns of the isolated strains 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 minute and 80 minutes at pH of 2.0 and 0 minutes and 120 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*. 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 0min and 80a min) and after addition of simulated pancreatic juice at pH of 8.0 (before and after incubation at 80b min and 200min respectively). The mean viable counts of all the strains ranged from $2.0 *10^8$ in R_3 at 80b min to $1.54 * 10^{10}$ in B_4 at 80_b minutes.

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]. Cereals are rich sources of nutrients as well as non-nutrients such as dietary fibre [2,4]. The protective roles of cereals such as lowering the risk of gastrointestinal diseases like hemorrhoids [5], diverticulitis [6], colorectal cancer [7], and constipation [8] are linked to the colonic fermentation of their dietary fibre content which 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 beneficial bacteria in the colon [9]. These beneficial bacteria are known as probiotic microorganisms [10].

The major microorganism involved in the spontaneous/traditional fermentation of cereals is *Lactobacillus* [1] others include bacterial species such as *Streptococcus*, yeast species of *Saccharomyces* and mould such as *Cladosporium* and *Penicillium* species [12]. In the food industry, probiotics especially lactic acid bacteria are usually utilized as starter cultures during controlled fermentation in the production of fermented foods and beverages such as yoghurt, bread, saukraeut and pickled cucumber [13]. The increasing interest in functional foods that promote nutrition and health status has increased the demand for novel strains of probiotic microrganisms which have desirable qualities on food products and improves health and well-being [14]. In addition, probiotics from fermented plant foods could be of importance in developing probiotic food products for strict vegetarians. LAB isolated from spontaneously fermented foods which have probiotic characteristics can be utilized by food and pharmaceutical industries in developing functional foods and supplements [15].

Ability to survive the passage to the active site of expected beneficial action is an essential selection criteria for a probiotic microorganism [16]. The stomach secretes up to 2.5 litres of gastric juice at a pH of approximately 2.0 everyday [17]. In contrast, about 0.7L of pancreatic juice at an average pH of 8.0 is secreted into the small intestine every day [17. 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 [18].
Previous studies that explored the prebiotic potentials of rye, wheat, oat and barley grains or their products were carried out using cereals or their products fermented using a starter culture [19;20]. However, there is limited data on the acid and alkaline tolerance of lactic acid bacteria isolated from spontaneously fermented rye, wheat, oat and barley grains. 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 and milling and fermentation

The selected cereal grains were fermented using the method of Chalarampopoulos et al [21] with slight modifications.

Fifty grams each of rye, wheat and barley sample was soaked in 150ml of cold tap water for 24 hours (h) in order to soften the grains for easy milling. The selected cereal grains were then washed three times with tap water. 100ml of tap water was used for wet milling of the cereal samples using a smoothie maker (Kenwood – model number: 0WSB26001). In contrast, the oat meal was not washed prior to grinding, as it has already been processed. It was however, ground using the water in which it was soaked. The smoothie maker was rinsed five times with hot boiling water before milling each sample to reduce possible cofounders such as the transfer of microorganisms from one cereal sample to another. After milling, 100ml of each cereal sample was transferred into a covered plastic bowl and was allowed to ferment

spontaneously for 72h at room temperature in the Food Demonstration Laboratory of the University of Chester, United Kingdom.

2.3 Enumeration, isolation and characterization of lactic acid bacteria (LAB)

The enumeration, isolation and characterization of LAB were carried out in the Food Microbiology Laboratory, University of Chester, United Kingdom. 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 Rogosa 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 [22]. The presumptive LAB strains were gram stained as described by [23] 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 [23]. Pure cultures in MRS broth were then kept in the refrigerator at 4^0C for a maximum of 3 days. 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 [18].

2.4 Preparation of strains

Isolated LAB strains were cultured twice in 20mls of MRS broth for 24h at 30^0C [24]. Each MRS broth sample was transferred into two, 10ml sterile centrifuge tubes. The tubes were centrifuged (Alc centrifuge, model no: PK120) at 4000g for 20minutes and the residues were collected aseptically.

2.5 Preparation of simulated gastric juice and pancreatic juice

Gastric juice was freshly prepared 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 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 121^0C and pressure of 15 psi (pounds per square inch) in order to eliminate any live LAB or other microoganisms present in the fermented product, and the pH was adjusted to 2.0.

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

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 votex mixer (Stuart, Staffordshire, UK) and was incubated at 37^0C for 80min [25]. 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 37^0C for 120min [26]. One milliliter of aliquot from each cereal sample was collected at 0 min and 80 min at pH of 2.0 containing simulated gastric juice before and after incubation respectively and at 0 min and 120 min at pH of 8.0 containing simulated pancreatic juice before and after incubation respectively for determination of total viable LAB count on MRS Agar.

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 values 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 dietary fiber contents [9] which has been demonstrated to induce the growth and activity of probiotics [14]. The total LAB cell count obtained in the selected cereal samples after spontaneous fermentation are within the same range reported by Huttner et al, [26] and Saeed *et al.* [19].

3.2 Characterization of the LAB strains

The LAB strains isolated from this study were characterized using API 50 CH kit (Biomerieux SA, France) and the results were analyzed using apiweb^R software version 5.1 (Biomerieux). 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). 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*1while the remaining as *L. brevis* 1. The results of API 50 CH kit characterization test of the isolated LAB strains is presented in table 2.

Table 2: API 50 CH characterization result of the isolated strains showing the profile of each strain

Colony	**Classification**	**Percentage (%) of identification**	**Description of profile**
R_1	*Latobacillus brevis* 1	99.9	Good identification
R_2	*L. brevis* 1	99.9	Excellent identification
R_3	*L. brevis* 1	99.3	Very good identification
R_4	*L. brevis* 1	99.1	Very good identification
W_1	*L. plantarum* 1	95.8	Excellent identification
W_2	*L. brevis* 1	80.6	Acceptable identification
W_3	*L. plantarum* 1	97.0	Doubtful profile
B_1	*L. plantarum* 1	84.0	Very good identification
B_2	*L. collinoides*	98.6	Good identification
B_3	*Leuconostoc citreum*	96.7	Very good identification
B_4	*Lactobacillus. plantarum* 1	83.9	Doubtful profile
O_1	*L. plantarum* 1	97.3	Good identification
O_2	*L. plantarum 1*	96.4	Doubtful profile

All isolated LAB strains had different API 50 CH identification profile. Interestingly, none of the six *Lactobacillus plantarum*1strains 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 as suggested by Kao et al [27]. *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. Hemaiswarya et al [28] reported that *L. plantarum* and *L. brevis* are among the LAB species commonly isolated from fermented cereal foods. *L. plantarum* and *L. brevis* also dominated the spontaneous fermentation of wheat sourdough in the work by Huttner [26] whilst Saeed et al [19] 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

In this study, the gastrointestinal emptying, was mimicked by subjecting the isolated LAB strains to simulated gastric juice in acidic fermented cereal medium at pH of 2.0 and incubating at 37^0C for 80 minutes, followed by addition of simulated pancreatic juice in alkaline fermented cereal medium at pH of 8.0 and incubating at 37^0C for 120 minutes. The pH of the GIT ranges from 1.0 – 2.5 in the stomach and increases to 6.5 ± 0.5 in the proximal small intestine and up to a maximum of 7.5 ± 0.4 in the distal small intestine after the consumption of a meal [29]. In addition, it takes around 80 minutes for half of the stomach content to be emptied [30;31] whilst half of the ileal content will be emptied at approximately 2 hours after the consumption of a meal [32]

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 min and 80a min) and after addition of simulated pancreatic juice at pH of 8.0 (before and after incubation at 80b min and 200 min respectively). The mean viable counts of all the strains ranged from 2.0 $*10^8$ in R_3 at 80b min to 1.54 $* 10^{10}$ in B_4 at 80_b minutes as shown in figure 1. The lowest viable cell count of 2.0 $*10^8$ cfu/ml obtained in R_3 at 80_b minutes before incubation in alkaline medium (at pH of 8.0) containing simulated pancreatic juice is higher than 1 $* 10^7$cfu/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 [33].

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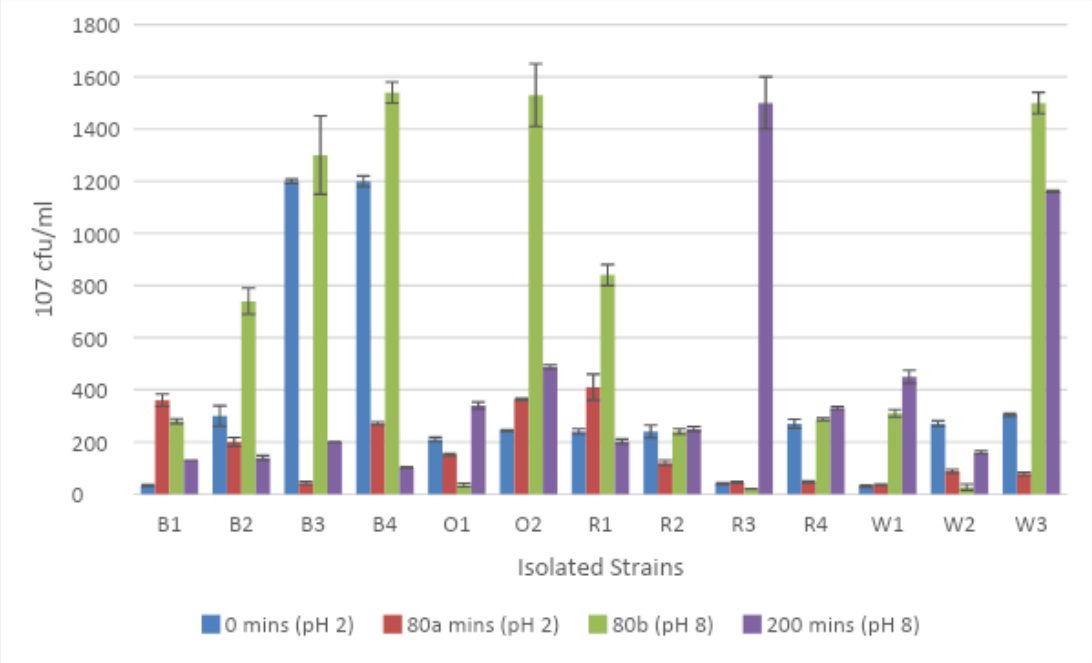

Figure 1: Viable cell count of isolated LAB strains during the acid and alkaline tolerance test. Each point shows the mean viable cell counts of triplicate measures and standard deviation of all the isolated strains when exposed to simulated gastric juice in acidic medium (pH of 2.0) before and after 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 strains had different viability profiles in acidic fermented cereal medium (pH of 2.0) containing gastric juice and in alkaline fermented cereal medium (pH of 8.0) containing pancreatic juice including *L. plantarum* 1 and *L. brevis* 1 strains

isolated from the same cereal. This supports the claim by Ramos [34] that the survival of probiotics in the GIT is strain dependent. Similarly, three *L. plantarum* strains (*L. plantarum* 2963, 2966 and 2833) in Haller et al [35] had different viability profiles at all times, after exposure to hydrochloric acid diluted to pH of 1.0, 1.5, 2.0 or 2.5 at 37^0C for one hour followed by addition of bile (at pH of 8.0) and incubating at 37^0C for one hour. In contrast, Ramos et al. [34] reported that two out of ten *L. plantarum* strains and one out of seven *L. brevis* strains survived in simulated bile for only a period of one hour while two *L. brevis* strains were capable of surviving in simulated bile after four hours of incubation at 37^0C.

The fermented cereal media used in the acid and alkaline tolerance test might have contributed to improved tolerance of R_1 and R_4, (*L. brevis* 1 strains) W_1, B_1 and O_2 (*L. plantarum* 1 strains) to simulated gastric juice at pH of 2.0. This is in agreement with Kos et al [36]. They observed that the survival of *L. acidophilus* M92 at pH of 2.0 in simulated gastric juice and pH of 8.0 in simulated pancreatic juice increased from 15% in direct transit from simulated gastric juice to simulated pancreatic juice to 45% when the medium was supplemented with whey protein concentrate.

The difference observed in the viable cell counts of the isolated strains between 80b min and 80a min may be due to the gap before incubation as a result of the time it took (about 10min) to increase the pH of the cereal medium to 8.0 using 0.1M NaOH and for addition of simulated pancreatic juice. The best result in terms of tolerance to simulated gastrointestinal conditions of the GIT was obtained in W_1. W_1 (*L. plantarum* 1) maintained a gradual increase in its viable cell count throughout the period of incubation in acidic medium and in alkaline medium although it thrived better in the alkaline medium.

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.

Competing interests

The authors do not have any conflict of interest

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among the authors. The authors read and approved the final manuscript.

References

1. Lemmens E, Moroni A, Pagand J, Heirbaut P, Ritala A, Karlen Y, Kim-Anne L, Hetty C B, Brouns FJPH, De Brier N, Delcour JA. Impact of Cereal Seed Sprouting on Its Nutritional and Technological Properties: A Critical Review. Comprehensive Reviews in Food Science and Food Safety 2018;18:305-328
2. Tasie MM, Gebreyes BG. Characterization of Nutritional, Antinutritional, and Mineral Contents of Thirty-Five Sorghum Varieties Grown in Ethiopia. 2020; 2020:1-11
3. Food and Agriculture Organization (FAO) (2020). Cereals. Accessed 4th April 2021. Available: www.fao.org/country-showcase/selected-product-detail/en/c/1287948
4. Ogodo AC, Ugbogu OC, Onyeagba RA Okereke HC. Microbiological quality, proximate composition and in vitro starch/protein digestibility of *Sorghum bicolor* flour fermented with lactic acid bacteria consortia Chemical and Biological Technologies in Agriculture 2019;6(7):1-9
5. Alonso-Coello P, Mills DHA, López-Yarto M. Fiber for the treatment of hemorrhoids complications: a systematic review and meta-analysis. The American journal of gastroenterology, 2006;101(1): 181-188.
6. Stollman N, Raskin JB. Diverticular disease of the colon. Lancet, 2004;363(9409), 631-639.

7. Bingham S, Day NE, Luben R. Dietary fibre in food and protection against colorectal cancer in the European prospective investigation into cancer and nutriction (epic): an observational study. Lancet 2003;361:1496-1501.
8. Anderson JW, Baird P. Health benefits of dietary fiber. Nutrition Reviews. 2009;67(4), 188-205. doi: 10.1111/j.1753-4887.2009.00189.x
9. Kolida S, Gibson GR. Prebiotic Capacity of Inulin-Type Fructans. The Journal of Nutrition 2007;137(11), 2503S-2506S.
10. Marteu P. Prebiotics and probiotics for gastrointestinal health. Clinical nutrition, 2001;20:41-45.
11. Lee YK, Salminen S. Handbook of probiotics and prebiotics. 2nd ed. New Jersey, United States of America; 2009
12. Blandino A, Al-Aseeri ME, Pandiella SS. Cereal-based fermented foods and beverages. Food Research International, 2003;36(6):527-543
13. Maraz A. (2011). Food fermentationsby yeasts and filamentous fungi. Acessed 6th June 2019. Available: http://www.mf.unimb.si/mf/instituti/IPweb/html/Food_fermentations_yeasts-Maraz.pdf
14. Grosu-Tudor SS, Zamfir M. Probiotic potential of some lactic acid bacteria isolated from romanian fermented vegetables, 2012;17(1)233-239
15. Somashekaraiah R, Shruthi B, Deepthi BV, Sreenivasa MY. Probiotic properties of Lactic acid bacteria isolated from Neera: A naturally fermenting coconut palm nectar. Fronters in Microbiology, 2019;10:1382
16. Fernandez M, Boris S, Barbes C. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. Journal of Applied Microbiology, 2003;94(3):449-455
17. Vinderola C, Reinheimer J. Lactic acid starter and probiotic bacteria: a comparative “in vitro” study of probiotic characteristics and biological barrier resistance. Food Research International, 2003;36(9):895-904.
18. De Vries MC, Vaughan EE. Lactobacillus plantarum—survival, functional and potential probiotic properties in the human intestinal tract. International Dairy Journal, 2006;16(9):1018-1028
19. Saeed M, Anjum F, Zahoor T. Isolation and characterization of starter culture from spontaneous fermentation of sourdough. International Journal of Agriculture and Biology, 2009;11(3):329-332.
20. Chen YS, Wu HC, Yanagida F. Isolation and characteristics of lactic acid bacteria isolated from ripe mulberries in Taiwan. Brazilian Journal of Microbiology, 2010;41(4):916-921.
21. Charalampopoulos D, Pandiella S, Webb C. Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates. Journal of Applied Microbiology, 2002;92(5), 851-859.
22. Brukner MZ. (2012) Gram staining. Accessed 6th June 2019. Available: http://serc.carleton.edu/microbelife/research_methods/microscopy/gramstain.html
23. Marteau P, Minekus M, Havenaar R. Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. Journal of dairy science, 1997; 80(6), 1031-1037
24. Gotcheva V, Hristozova E, Hristozova T, Guo M, Roshkova Z, Angelov A. Assessment of potential probiotic properties of lactic acid bacteria and yeast strains. Food Biotechnology, 2002;16(3), 211-225.
25. Axelsson L. Lactic acid bacteria: Classification and physiology. *Lactic Acid Bacteria:* Microbiological and Functional Aspects, 2009;139:1-8.
26. Hüttner EK, Dal Bello F, Arendt E K. Identification of lactic acid bacteria isolated from oat sourdoughs and investigation into their potential for the improvement of oat bread quality. European Food Research and Technology, 2010 230(6):849-857.
27. Kao YT, Liu YS, Shyu YT. (2007) Identification of *Lactobacillus spp*. in probiotic products by real-time PCR and melting curve analysis. *Food Research International, 40*(1):71-79.
28. Hemaiswarya S, Raja R, Ravikumar R. Mechanism of action of probiotics. Brazilian Archives of Biology and Technology, 2013;56(1):113-119
29. Ibekwe VC, Fadda HM, McConnell EL, Khela MK, Evans, DF, Basit AW. Interplay between intestinal pH, transit time and feed status on the in vivo performance of pH responsive ileo-colonic release systems. Pharmaceutical research, 2008;25(8):1828-1835.
30. Graff J, Brinch K, Madsen JL. Gastrointestinal mean transit times in young and middle-aged healthy subjects. Clinical Physiology, 2001;21(2):253-259.
31. Hellmig S, Schöning , Gadow C, Katsoulis S, Hedderich J, Fölsch UR, Stüber E. Gastric emptying time of fluids and solids in healthy subjects determined by 13C breath tests: influence of age, sex and body mass index. Journal of gastroenterology and hepatology, 2006;21(12):1832-1838.
32. Camilleri M, Greydanus MP, Brown ML, Proano M. Towards a less costly but accurate test of gastric emptying and small bowel transit. Digestive diseases and sciences, 1991;36(5), 609-615.
33. Charalampopoulos D, Pandiella S, Webb C. (2002) Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates. *Journal of Applied Microbiology, 92*(5):851-859. doi: 10.1046/j.1365-2672.2002.01592.x

34. Ramos C, Thorsen L, Schwan R, Jespersen L. Strain-specific probiotics properties of Lactobacillus fermentum, Lactobacillus plantarum and Lactobacillus brevis isolates from Brazilian food products. Food Microbiology, 2013;36(1), 22-29.
35. Haller D, Colbus H, Gänzle M, Scherenbacher P, Bode C, Hammes W. Metabolic and Functional Properties of Lactic Acid Bacteria in the Gastro-intestinal Ecosystem: A comparative in vitro Study between Bacteria of Intestinal and Fermented Food Origin. Systematic and Applied Microbiology, 2001;24(2), 218-226.
36. Kos B, Šušković J, Goreta J, Matošić S. Effect of protectors on the viability of Lactobacillus acidophilus M92 in simulated gastrointestinal conditions. Food Technology and Biotechnology, 2000;38(2)1-8