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Original Article

Effect of Storage in the Fortified Probiotic Corn Flakes Prepared by *L. plantarum* and *L. reuteri*

Mehrnaz Dadgar[1], Kianoush Khosravi-Darani[2*], Azadeh Tofighi[1], Mina Khanbeigi-Dogahe[1]

1. Department of Food Science and Technology, Varamin Branch, Islamic Azad University, Varamin, Tehran, Iran

2. Department of Food Technology Research, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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ABSTRACT

Background and Objectives: Nowadays corn flakes are the most common form of breakfast cereals; however, their vitamins are declined during the baking process. In this research, production of fortified corn flakes by *Lactobacillus plantarum* and *L. reuteri*, as well as incorporation of oat and malt fiber as prebiotics have been studied.

Materials and Methods: Plackett-Burman statistical design was used to evaluate the impact of eleven-process variables on the viability of both probiotics.

Results: The highest survival rate of *L. plantarum* (340×10^6 CFU.g^{-1}) was obtained by 2.5% w/w inoculation of a 24 h inoculum in a medium containing 10% w/v oat extract to enrich corn flakes (10% w/w malt fiber), packed in aluminum foil and kept at 20 °C in anaerobic conditions for 2 weeks. Also the highest survival rate of *L. reuteri* (123×10^6 CFU.g^{-1}) was achieved when the 48 h cultured bacteria grown in a medium containing 5% w/v malt extract and MRS broth was inoculated (2.5% w/w) to the flakes enriched by 20% w/w malt fiber, and kept in anaerobic conditions inside the polypropylene cover packaging at 20 °C for 2 weeks.

Conclusions: Sensory evaluation showed no significant difference between the treatments in terms of taste, odor and overall acceptability as compared to the control.

Keywords: Corn flakes, Probiotic, Prebiotic, Plackett-Burman design

Introduction

Functional foods fortified with probiotics, prebiotics and fiber have been established in the global food market (1), leading to the development and commercialization of numerous health beneficial products. According to the FAO/WHO reports probiotics are live microorganisms that confer benefits to their host when consumed in adequate amounts as part of food (2). Different strains of Lactobacillus Spp., *Escherichia coli*, *Streptococcus thermophilus*, Propionibacterium, Pediococcus and Leuconostoc could be considered as the main microbial species to be used as probiotics (3). There is significant scientific evidence, suggesting the potentially beneficial effects of probiotics (4). Prebiotics are non-digestible food ingredients (for host) that improve health by selectively stimulating the growth and/or activity of probiotics in the gastrointestinal track (5). There has also been an increased focus on synbiotics, a combination of pre- and probiotics in a single product (6).

Corn flakes are the most common form of cereals in breakfast; however, their vitamins may be declined during the baking process. Cereals are grown on 73% of the world's total harvested areas, and comprise over 60% of the world food production (4). Breakfast cereals, as a ready-to-eat product, are widely used in urban areas (7). Cereals contain water soluble fiber oligosaccharides and resistant starch. So it has been suggested that cereal extracts could serve as a good medium for cultivating probiotics (8). In fact, cereal fibers have been selected as a good carrier for probiotics to protect the viability and stability of lactobacillus bacteria after formulation into products and during the storage (9).

Viability of *B. lactis*, *S. thermophilus*, *L. johnsonii*, and *L. paracasei* added to junior cereal products reached to 10^8, 4×10^8, 39×10^8 and 3×10^8 CFU.g^{-1}, respectively (10). *L. rhamnosus* was added to chocolate-coated breakfast oat cereals fortified by sucrose, wheat dextrin and polydextrose. Reduction of probiotic's viability during the storage showed different range from 10^1 to 4×10^2 CFU.g^{-1} (1). Cereal extracts, especially malt extract, caused improvement in the viability of *L. plantarum* (10^3-10^4

***Address for correspondence:** Kianoush Khosravi-Darani (PhD), Associate Prof. Department of Food Technology Research, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran, Tel: +982122376473; Email address: k.khosravi@nnftri.ac.ir

CFU.ml^{-1}) (11). Viability of *L. plantarum* under acidic condition in the presence of malt and barley was increased to 54×10^5 and 68×10^3 CFU.ml^{-1}, respectively (4). *Saccharomyces cerevisiae* was added to corn grain, and the average percentag of the viable cells reached to 70% during 110 days (12). The average percentage of the viable cells during the storage and acidic condition with using inulin, oat bran, unripe banana flour and apple fiber increased to 55%, 79%, 76% and 64%, respectively (2). *B. lactis* was added to oat-based cereal bar with oat bran, which increased the viability to 69×10^9 CFU.g^{-1}(13).

The impact of several independent variables on the viability of fortified cereal products by probiotic has been evaluated by many researchers. Positive effect of cereal extracts on bacteria viability, especially malt and oat, has been proved due to the impact of their sugar content (9, 11). Fiber was found as protective agent on improving the viability of bacteria and sensory properties (14, 4). The influence of size and age of inoculum (15, 10), as well as the impact of storage, temperature and time on the viability of probiotics has been studied and reported (1). *L. plantarum* and *L. reureri* are known as facultative anaerobic bacteria, so oxygen barrier property of polypropylene can increase their viability (16, 17).

The aim of the present study was production of corn flakes containing *L. plantarum* or *L. reuteri* enriched with malt or oat fiber. So, cereal extract was used as a nutrient medium for the growth of probiotics, and cereal fibers were added as prebiotic. The impact of process and storage variables on the viability of probiotics was studied by Plackett-Burmann design (PBD). Fresh biomass was used instead of lyophilized bacteria, which usually had been used in the reported literature. Absorptive drying was used by addition of malt or oat fiber to avoid high temperature, so possible loss of microorganisms was decreased. Dried product absorbed the water accompanied by probiotic biomass. The stability of fresh *L. plantarum* and *L. reuteri* cells mixed with selected fiber carriers in the corn flakes was evaluated during the storage process.

Material and Methods

Microorganisms and Cultivation: The microorganisms used in this study were *L. plantarum* PTCC (Persian Type Culture Collection) 1745 (isolated from pickled cabbage), and *L. reuteri* PTCC 1655 (isolated from the intestine of an adult) which were purchased from Iranian Research Organization for Science and Technology (IROST). The strains were maintained at 4 ºC and subcultured monthly on MRS agar (Merk, Germany). Also the strains were precultured weekly in MRS broth (Merk, Germany) at 37°C for 16-18 h (stationary phase) under anaerobic conditions (18, 2).

Precultured bacteria were prepared by addition of 1 ml of the incubated tubes (with the estimated viable cells of *L. plantarum* 10^6-10^7 CFU.ml^{-1} and *L. reuteri* 5×10^6-10^7 CFU.ml^{-1}) to 8 and 9 ml of sterilized saline solution (0.9% w/v), before use at 10^{-1} and 2×10^{-1} dilutions. Then serial dilutions of 10^{-2} to 10^{-5} were prepared as mentioned, and their absorbance at 600 nm was measured. Cell concentrations were measured by staining and microscope direct counting, and calibration curve was plotted. The direct colony count method was used to determine cell viability.

Preparation of cereal extracts: Oat and malt grains were used for preparation of the culture media. These grains were ground in a home mill (Kenwood, China). Samples of 5 and 10 g of the resulting flours were mixed with 90 and 95 ml of distilled water at 80 ºC for 20 min. After centrifugation (5000×g, 10 min) of the media at room temperature and separation of solids, they were sterilized at 121 °C for 15 min before use as 5 and 10% (w/v) nutrient cereal media (4).

Transferring of bacteria cells to nutrient medium: *L. plantarum* and *L. reuteri* were grown overnight (16-18 h) in MRS medium at 37 ºC. Then they were transferred (1% v/v) into fresh cereal extract or MRS broth containing cereal extract (in two separate trials), and cultured for 24 or 48 h at 37 ºC (4).

Preparation of product: Corn flakes were weighted in 40 g packages, enriched by adding 10 or 20% w/w malt or oat flour as fiber, and sterilized at 180 ºC for 1 h.

As mentioned above, in this research, type of bacterium was selected as an independent variable, so the trials were conducted using single bacterium inoculation by *L. plantarum* or *L. reuteri*. The inoculum culture was prepared in a nutrient medium at 37 ºC after 24 or 48 h. The viable count of inoculla in the nutrient medium was reached to 3×10^7- 3×10^8 CFU.ml^{-1} and 10^7-10^8 CFU.ml^{-1} of *L. plantarum* and *L. reuteri*, respectively. After centrifugatin at 5000×g for 10 min at 4 °C, the pellet was washed twice with sterile saline (0.9% w/v). Then the cells were resuspended in sterile saline (0.9% w/v) (11, 4) and added to sterilized corn flakes (2.5 or 5% w/w). Viability of probiotics in product is dependent variable that is affected not only by the process variables but also by the storage conditions. Therefore, the products were packed in aluminum foil or polypropylene film and kept at 4°C or 20°C for 1 or 2 weeks for reaching optimum storage situation. Figure 1 shows a schematic diagram of fortified corn flakes' production.

Bacterial enumeration: The "pure plate" method was used to count viable cells. The samples (products) were diluted (10^4 to 10^6 g.ml^{-1}) with sterile saline (0.9% w/v) solution. Three aliquots of 1 ml were dropped into the plates containing MRS agar, and mixed. The plates were incubated at 37ºC for 48 h and individual colonies in the plates were counted. Viable cell counts were calculated as colony forming units per gram (CFU.g^{-1}) (2, 1).

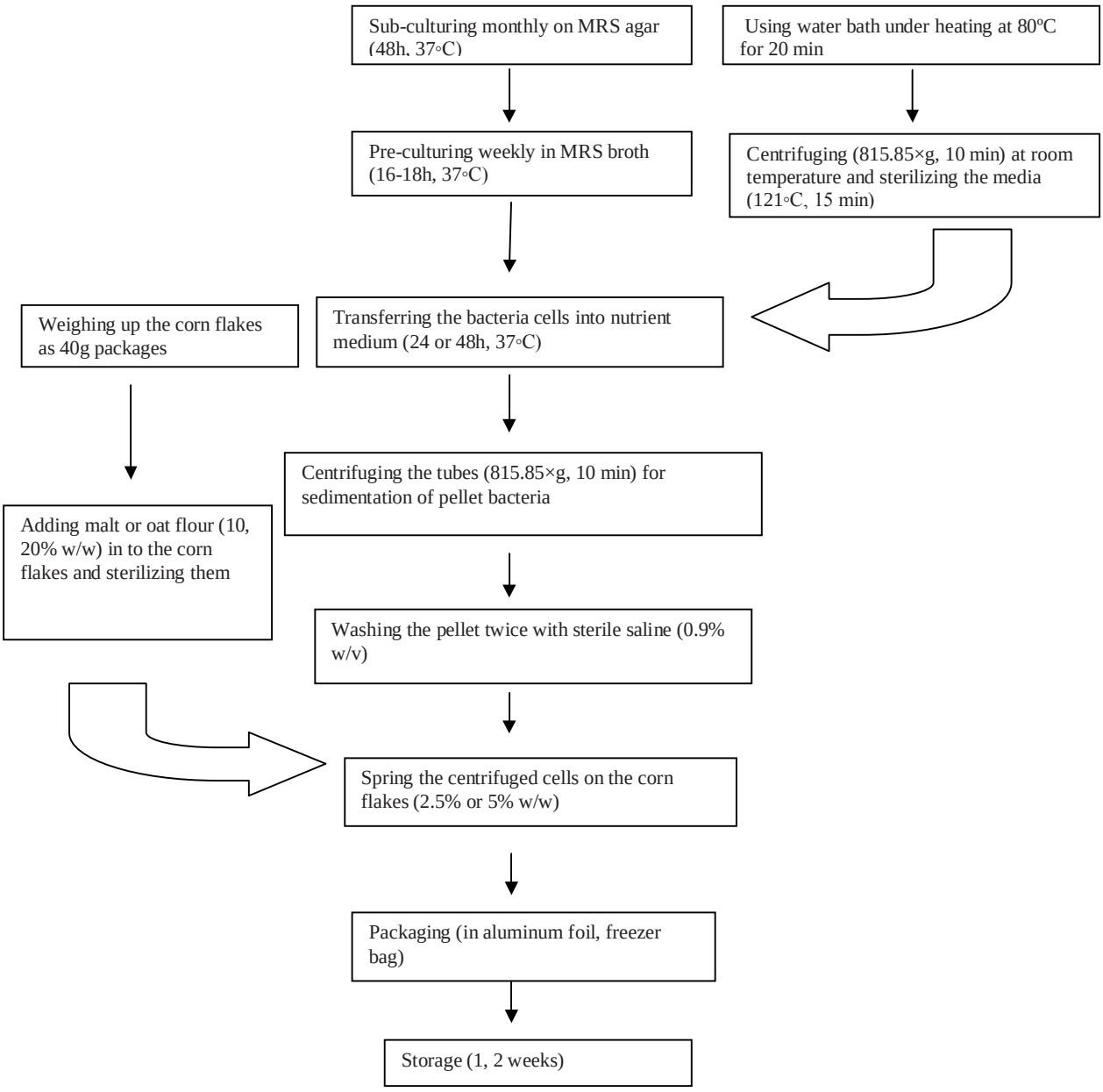


Fig 1. Schematic diagram of production of corn flakes containing probiotics *L. plantarum* and *L. reuteri*.

Water activity measurement: The water activity (a_w) of the corn flakes was measured before any treatments (control sample) and compared with samples which showed the highest viable cell count (water activity measurement Aqualab, Switzerland) (1).

Sensory evaluation of product: The taste, odor and overall acceptability of the simple corn flakes (not enriched) as control sample and the samples with highest viable cell count were determined by a panel consisting of 30 untrained tasters. A nine-point structured hedonic scale was used during the sequential presentation of the samples (with one= disliked very much and nine = liked very much). The resulted data were analyzed using Kruskal Wallis, and Duncan methods. P-value < 0.05 was considered significant (2).

Statistical analysis: The statistical design of PBD was used to determine the optimum condition for producing fortified corn flakes. The cultures were used individually in a single product. The effect of each factor was evaluated (α=1%). Statistical analysis was done using SPSS software (ver.16).

Since the experiments are designed to evaluate the relative effect of each variable on response, a significant level of 0.30 is acceptable (19). However, the tabulated *t*

value (degree of freedom 10) at P<0.1 and P<0.15 is equal to 1.2 and 0.69, respectively.

Selection of process variables and range finding: With the aim of determining the viability of both probiotics, choice of the factors and their range (in a wide but reasonable numerical range) was based on the literature review and also our previous experience (unpublished data). Some changes in the response (yield) were expected for each factor over the selected range (20, 21). Kind of the inoculla's medium, packaging material, aerobic/ anaerobic condition, kind and concentration of extract (malt or oat), and the inoculla's age, seed size, storage temperature, time, as well as the content and kind of incorporated fiber were selected as independent variables. Viability of the bacteria was measured as dependent variable.

High and low levels of variables, as well as the selected PBD for conducting 12 experimental trials are shown in Table 1.

The signs, + (high level) and - (low level) represent the two different levels of the independent variables examined. Therefore each experimental trial was performed according to the signs shown in trial number 1 of Table 2; e.g. the cereal extract (factor A^+) and oat were (B^-), the extract's concentration was 10% (w/v) (C^+), freezer bag for packaging (D^-) and aerobic atmosphere for storage were selected (E^-). Also 20%(w/w) (G^+) of oat fiber (F^-) was added to the corn flakes, probiotic bacteria incubated for 48 h in the nutrition medium (H^+) were inoculated to the corn flakes in the rate of 5%(w/w) (J^+), and the prepared product were stored at 4°C (K^-) for 2 weeks (L^+). The trials were experimented in three repetitions.

Table 1. Variables to be monitored in the fortified corn flakes' production

Code of factors	Variables	Low level (-)	High level (+)
A	Kind of medium	cereal extract+MRS broth	Cereal extract
B	Kind of extract	Oat extract	Malt extract
C	Extract concen (%w/v)	5	10
D	Kind of packaging	Freezer bag	Aluminum foil
E	Storage atmosphere	Aerobic	Anaerobic
F	Kind of fiber	Oat fiber	Malt fiber
G	Percent of fiber (% w/w)	10	20
H	Age of inoculum (h)	24	48
J	Inoculums concen (%w/w)	2.5	5
K	Storage temperature (◦C)	4	20
L	Storage time (week)	1	2

Results

Stability in corn flakes: The highest survival rate of bacteria was achieved in the trials number 4 and 11 for *L. plantarum* and *L. reuter*i, respectively (Table 2). After the addition of bacterial cells and malt or oat flour as fiber into the corn flakes, the cell viability was measured at baseline (in the beginning of the storage stability test, time=0), and changes in viability were estimated during storage time (1 or 2 weeks) at 4 °C or 20 °C (Fig. 2A and B). Cell viability remained within 0.02±0.7 $CFU.g^{-1}$ during the storage.

Viable *L. plantarum* cells were decrease in trials 1, 2, 3, 6 and 8 and increased in other samples after storage, though the viable cells were not changed in samples number 12 and 7 (Fig. 2A). Fresh *L. plantarum* cells were prepared by 24 h inoculum cultures in a medium containing 10% w/v oats extract, inoculated (2.5% w/w) to the corn flakes enriched by 10% w/w malt fiber (packed in aluminum foil) and kept at 20 °C in anaerobic conditions for 2 weeks.These cells showed highest survival rate (trial 4) (340×10^6 $CFU.g^{-1}$) (Fig. 2 A).

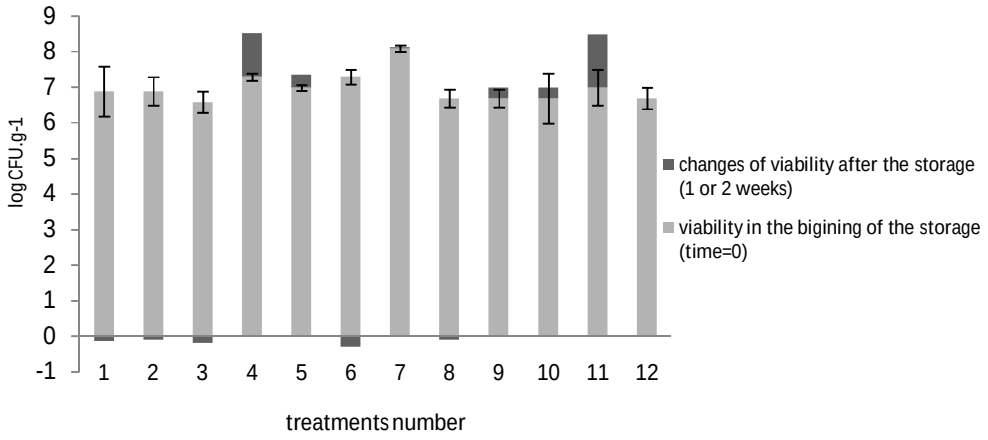
Viability of *L. reuteri* cells was decreased in the trials 1, 3, 6, and 8, and increased in other samples after storage. However, the viable cells did not change in the trials 12 and 7 (Fig. 2 B). The highest survival of *L. reuteri* was achieved in trial 11 when the 48h-cultured bacteria were grown in a medium containing 5% w/v malt extract and MRS broth. Then they were inoculated (2.5% w/w) to the corn flakes enriched by 20% w/w malt fiber, and kept in anaerobic conditions inside the polypropylene cover at 20ºC for 2 weeks (123×10^6 $CFU.g^{-1}$) (Fig. 2 B). All the growth requirements of bacteria have been met by the MRS broth medium enriched by 5% w/v malt extract (2, 4).

Table 2. Plackett-Burmann design to study eleven factors in fortified corn flakes production by L. plantarum and cereal fiber

Treatment No	Codded setting for factors											Response[(a)] (cfu.g^{-1})	
	A	B	C	D	E	F	G	H	J	K	L	*L. plantarum*	*L. reuteri*
1	+	-	+	-	-	-	+	+	+	-	+	$6.0\pm0.4\times10^6$	$1.0\pm0.02\times10^6$
2	+	+	-	+	-	-	-	+	+	+	-	$7.0\pm0.2\times10^6$	$4.0\pm0.2\times10^6$
3	-	+	+	-	+	-	-	-	+	+	+	$2.5\pm0.2\times10^6$	$1.5\pm0.1\times10^6$
4	+	-	+	+	-	+	-	-	-	+	+	$340.0\pm0.22\times10^6$	$1.0\pm0.1\times10^6$
5	+	+	-	+	+	-	+	-	-	-	+	$23.0\pm0.16\times10^6$	$7.0\pm0.6\times10^6$
6	+	+	+	-	+	+	-	+	-	-	-	$10.0\pm0.06\times10^6$	$3.0\pm0.3\times10^6$
7	-	+	+	+	-	+	+	-	+	-	-	$140.0\pm0.12\times10^6$	$50.0\pm0.2\times10^6$
8	-	-	+	+	+	-	+	+	-	+	-	$4.0\pm0.2\times10^6$	$1.0\pm0.07\times10^6$
9	-	-	-	+	+	+	-	+	+	-	+	$10.0\pm0.09\times10^6$	$5.0\pm0.2\times10^6$
10	+	-	-	-	+	+	+	-	+	+	-	$10.0\pm0.05\times10^6$	$4.5\pm0.3\times10^6$
11	-	+	-	-	-	+	+	+	-	+	+	$310.0\pm0.13\times10^6$	$123.0\pm0.05\times10^6$
12	-	-	-	-	-	-	-	-	-	-	-	$5.0\pm0.5\times10^6$	$2.0\pm0.2\times10^6$

[(a)]: response shows as mean±standard deviation for 3 replications. P-values are resulted from t-test.

(A)



(B)

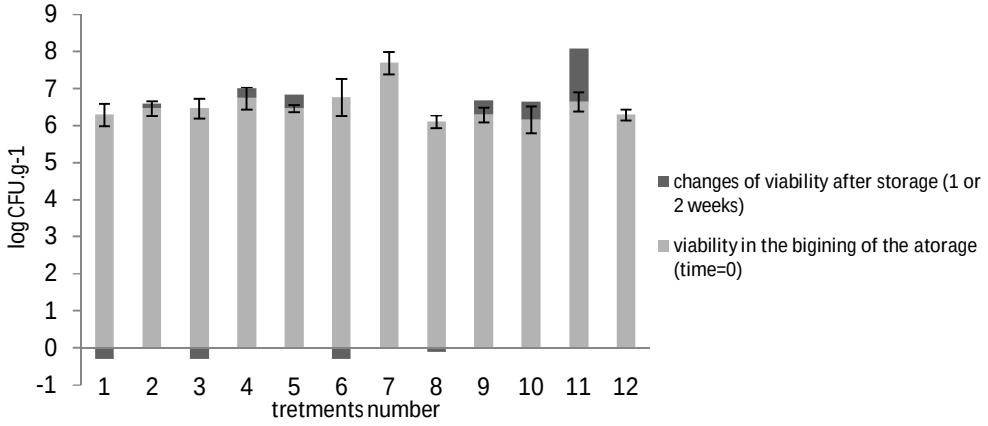


Fig. 2. Changes in viability of *L. plantarum* (A) and *L. reuteri* (B) in fortified cornflakes during storage time in 12 treatments in different conditions according to the Plackett-Burmann design

Statistical evaluation of cell viability: PBD was used to evaluate the impact of 11 process and storage variables (in 2 levels) on the viability of probiotics. The results for *L. plantarum* and *L. reuteri* are shown in (Fig. 3 A and 4 A) and (Fig. 3 B and 4 B), respectively. As shown, significant effect of all variables on the viability of both *L. plantarum* and *L. reuteri* is clearly observable. Type of fiber used in the product, incubate atmosphere (aerobic or anaerobic), inoculum concentration (% w/w), and storage time and temperature were found to be more effective than other variables for *L. plantarum.* Regarding *L. reuteri*, effective factors were type of fiber, incubate's atmosphere (aerobic or anaerobic), kind of extract, percent of fiber (%w/w) and kind of medium (Fig. 3). According to Fig. 4A and B, in the case of medium, the graphs show that the medium containing cereal extract and MRS broth is appropriate for both bacteria.

Water activity of product: The results of a_w measurement for the control sample and the samples of number 4 and 11 (as the best examples) were 0.022± 0.01, 0.37± 0.01 and 0.29± 0.01, respectively. No considerable increase of a_w was observed (P>0.05), and insignificant increasing might have occurred due to the addition of inoculation. In the trial number 11, the lower increase in a_w was observed may be due to high percent of the fiber.

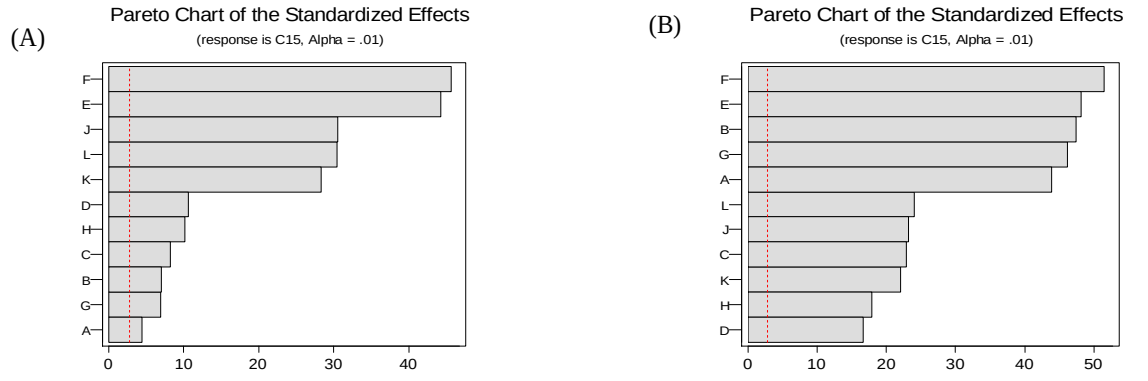


Fig. 3. The effect of eleven variables on the survival of *L. plantarum* (A) and *L. reuteri* (B) in the corn flakes during the storage time (significant level: α= 0.01)

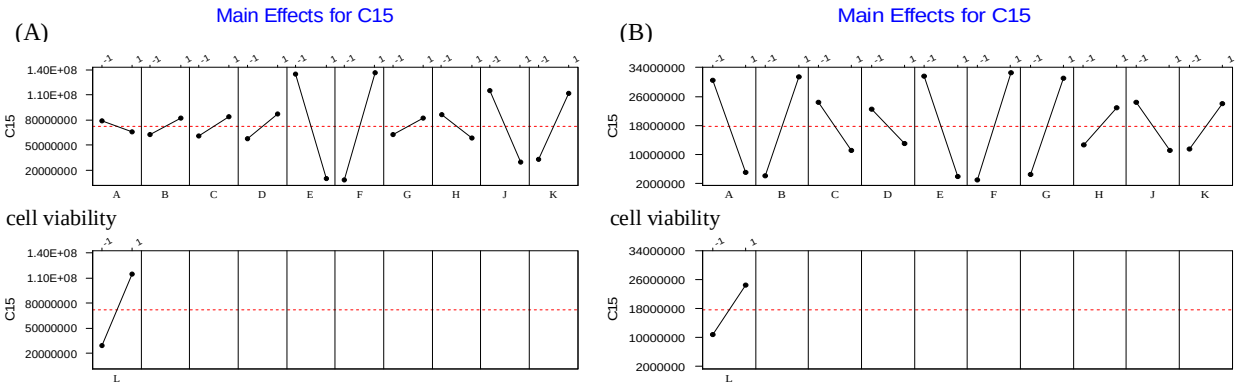


Fig. 4. The main effect of different levels (low and high: -1 and 1) of variables (code of factors:A-L) in the Plackett-Burmann design on the survival of *L. plantarum* (A) and *L. reuteri* (B) in the corn flakes during the storage time

Sensory analysis of product: The results of taste, odor and overall acceptability of the control and the samples h having the highest viable cells count (samples number 4 and 11) are shown in Table 3. ANOVA and F-test were applied to the data ($F< 0.05$), and no significant difference were observed between the treatments in terms of taste, odor and overall acceptability.

Table 3. Sensory indicators for the fortified corn flakes' production (in thecontrol, and the samples of trials 4 and 11 as the best products having the highest live bacteria)

Sensory indicators				Sample
Total score	Acceptance	Smell	Taste	
18	6.5	5	6.5	control
16.1	5.6	4.8	5.7	Trial no. 4 [(a)]
14.7	4.9	4.5	5.3	Trial no.11[(b)]

(a): sample that contains the highest number of live *L. plantarum.*
(b): sample that contains the highest number of live *L. reuteri.*

Discussion

It seems that oat extract, which includes vitamins, minerals, sugar and FAN (Free Amino acid Nitrogen), can provide necessities for bacterial growth (9, 11). Also bacterial growth has been supported by malt fiber with high reducing sugars during storage and protected against the environmental stress (2, 4). Aluminum is known as a barrier for gas transfer, so optimal condition for *L. plantarum* was achieved by the use of aluminum foil with anaerobic storage at 20 °C. Originally, optimum growth temperature for this anaerobic bacterium has been found between 35-40 °C.

L. reuteri as an anaerobic bacterium that is usually cultivated in limited oxygen condition. As Fig. 2 B shows, suitable condition is achieved by keeping the bacteria in anaerobic condition inside the polypropylene film. Oxygen penetration into package explains this observation. MRS broth is used as appropriate culture for all probiotics, and the cereal extract is also known as a good source of glucose, maltose, and FAN. So the combination of cereal extract and MRS broth was led to improve the cell growth and viability (4). Also Charalampopoulos et al. (2002, 2003) found that slow metabolizing energy sources such as arginine, fructose, citric acid, and malic acid, which could be present in cereals, enhance the viability of lactic acid bacteria (LAB) by providing energy. Sugar was used during the stationary phase, and additional ATP was produced (adenosine three phosphates) that was found necessary to protect viability of the cells. Malt extract has been introduced appropriate for both bacteria because of having higher total reducing sugar than oats, which occurs during the starch breakdown in the process of malting (11, 22). Ten percent (w/v) of the extract has been found effective on *L. plantarum* viability for having higher nutrient while the 5% (w/v) of extract has been shown to have suitable effect on *L. reuteri*. For packaging, applying aluminum foil for the products containing *L. plantarum,* and polypropylene film for the products fortified with *L. reuteri* showed an appropriate result. Aluminum foil has been determined as a net barrier that prevents mass transfer (gases, radiation and food ingredients) but polypropylene film is a functional barrier, which is a good barrier for moisture, thought oxygen can pass it (17). *L. reuteri* has been introduced as an anaerobic bacterium that is usually cultivated in limited oxygen condition, so polypropylene film acts as suitable packaging for it. In the case of incubate atmosphere, the products, stored in the form of anaerobic, caused increase in the number of bacteria in both probiotics. Survival of both bacteria was increased by the presence of 20% (w/w) malt fiber in the product due to its more reducing sugars. Immobilized probiotic on fiber could stay viable during the storage time (23). In addition, it is expected that this probiotic is protected under gastrointestinal condition. About *L. plantarum,* appropriate age of the inoculla was found to be 24 h. But for *L. reuteri*, it was realized 48 h. The use of cereal extract as carbon source in a soluble medium containing galacto- and fructo-oligosacharids led to prolonging the delay phase of LAB. But a good growth rate of *L. plantarum* was achieved at 37°C (24, 15). The results showed that cereal extracts (malt and oat) were more influencing on the growth of *L. plantarum* in compare to *L. reuteri*. So, suitable inoculla age for *L. reuteri* and *L. plantarum* was found 48 h and 24 h, respectively. The 2.5% w/w inoculations stored at 20 °C for 2 weeks showed positive impact on the survival of both bacteria. pH of traditional corn flakes is 4.9-5.38 that was increased to 5.5-5.9 after enriching with malt or oat fiber; therefore, as this is not an acidic condition for LAB, then pH of the product was not controlled during the storage time.

Conclusions: Corn flake is a nutrient\common\popular food, which can be functional by addition of probiotics and prebiotics. In this study, the survival rate of two probiotic bacteria (*Lactobacillus plantarum* and *L. reuteri*) was investigated during the storage time in corn flakes containing fiber by a 12-trial PBD. The results showed that malt flour was more effective on both bacteria than oat fiber (oat flour) due to its high sugar content. The highest survival rate of bacteria was achieved in the trials number 4 and 11 for *L. plantarum* and *L.reuteri*, respectively.

Synergistic effect on the viability of bacteria was observed between the cereal extracts in medium and cereal fiber in product, which led to adaptation of bacteria. It is expected that thebacteria will have good stability against gastric acid and bile juice due to the presence of sugars like sucrose and prebiotic fibers such as glucan in the fortified product. No significant difference in terms of sensory characteristics and water activity was observed in compare to the control samples. Resuted formulation containing

cereal fiber not only increases the nutritional value of product and supplies the ability of creating fortified product, but also is a good solution for adding cereal bran to the human meal.

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