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The Effect of Biofilm-like Structure, Sub-lethal Acid Stress, and Plant Gums on Culturability of Entrapped *Lactobacillus Plantarum*

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Abstract

Introduction: To increase *lactobacillus* cells stability, different methods based on entrapment used and their effect on the culturability of entrapped cells was studied.

Materials and methods In the present study, sub-lethal acid stress, biofilm-like structure, and the combination of plant gums were used to assess the culturability of entrapped *Lactobacillus plantarum* during different production phases.

Results: Based on the obtained results, incubation of alginate entrapped cells in MRS broth containing acetic acid increased the survival rate by 29% compared to beads treated in MRS broth. In addition, incubation of alginate entrapped cells in acidic medium resulted in 5% higher survival of biofilm-like cells after the freeze-drying process. To increase the stability of cells during storage, cells were entrapped in alginate plus different plant gums. Incorporation of tragacanth gum and salep gum to alginate showed higher culturability of cells (4.6 and 3.1 folds, respectively) in comparison to alginate treated beads. Additionally, the lowest inactivation constant rate (*k* value), 0.09, and highest *D* value, 25 days, obtained for tragacanth gum at 4°C during storage indicating that treatment with tragacanth gum could preserve the cells better in comparison to other treatments under storage condition.

Discussion and conclusion: The incubation of *Lactobacillus plantarum* beads in acidic MRS medium can result in increased culturability especially after freeze-drying. This can be due to cross-protection effect. Additionally, due to tragacanth gum traits, this plant gum could protect the entrapped cells better compared to other plant gums in the storage condition. In conclusion, we can use tragacanth gum as a second material for increasing the stability of entrapped *L. plantarum* cells.

Key words: Acetic Acid, Freeze-drying, Probiotic, Salep Gum, Sodium Alginate, Tragacanth Gum

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Introduction

Lactobacillus plantarum is one of the most important lactic acid bacteria. Its widespread niches accompanying safety approval and probiotic effects have made it a suitable lactic acid bacterium (1). However, one of the basic drawbacks of lactic acid bacteria utilization is their sensitivity to different stress conditions such as, low pH, cold, heat, and salt which results in their decreased viability (2). Because at least 10^7 CFU/ml bacteria should be contained in a probiotic product (3), numerous methods have been proposed to maintain cell viability during food processes. Among them, entrapment based techniques, are among the most effective and modern approaches.

Entrapment techniques benefit from the formation of beads loaded with bacteria. The beads usually have two main parts, core and matrix (4). The most widely-used matrix material is sodium alginate. This polysaccharide can form strong interaction with divalent ions e.g. calcium ions which leads to hydrogels' formation (5). Although some authors indicated a positive effect of calcium-alginate hydrogel loaded with lactic acid bacteria (6, 7), these hydrogels have some crucial disadvantages. Lactate can disrupt their structure (8). Also, as sodium alginate is a porous gel, harsh conditions can have a partial effect on the viability of entrapped cells (9). To overcome the mentioned downfalls, using the second protective material has been suggested. The range of materials, from proteins and polysaccharides, can be used. One of the recently-used material is plant gums (FAO, 10October 2017). Considering our research, there are some papers regarding using plant gums such as Arabic gum, Gellan gum, Locust bean gum and the combination of gums for increasing the viability of hydrogels (11-15). However, there is a considerable gap for other kinds of plant gums such as Tragacanth gum and Salep gum. Its geographical distribution includes Southwest Asia, especially Iran and Turkey (16, 17). It has the safety code from FDA under title 21 section 184.1351 (FDA, 18October 2017). Although Tragacanth gum has not been used for entrapment of lactic acid bacteria before, it's been used as a stabilizing agent (19) and dressing agent in desserts due to its high acidity tolerance and long shelf time (20) in the food industry (21). Salep gum is another gum which is obtained from *Orchis* spp. and has been found in a lot of parts of the world including Iran (22). This gum can improve the aroma and flavor of a product and can also act as a stabilizing agent (23). One other novel approach to increase the efficiency of entrapment is utilizing biofilm-like cells instead of planktonic ones. In other words, confinement of bacteria in beads and incubation under the favorable condition can increase the number of the cells and form a biofilm-like structure (24). Formation of biofilm-like bacteria can increase their tolerance to some stress such as thermal stress and freeze-drying process (25).

In order to achieve a high-quality product which agrees with the customer's taste, the entrapped cells should be in the powder form. One way to achieve this is by using the freeze-drying process. Also, the freeze-dried cells experienced more stability compared to normal cells. The combination of these two methods has mentioned in some papers with a positive effect on the survival of probiotic bacteria.

Regarding the given introduction, we assessed the ability of *Lactobacillus plantarum* to form biofilm-like cells. Then, we entrapped *L. plantarum* in the gum-alginate matrix. Based on our knowledge, there is no paper indicating the effect of sub-lethal stress induced by acetic acid on biofilm-like cells. On the other hand, we could not find any study using Tragacanth gum, Salep gum and the combination of these two gums as a matrix for the entrapment of lactic acid bacteria. Hence,

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as using plant gums can decrease the costs of production (due to their low costs), we chose these two gums. As an example, as we used one to three ratio of tragacanth gum to alginate, the overall cost was 8 dollars while using alginate as a replacement, results in 149 $ overall cost. On the other hand, as we followed commercial goals, we decided to form powder from the encapsulated bacterium by the freeze-drying process as an available method. In the end, culturability of the cells by colony count and the total count of the cells by direct enumeration were determined during the storage time period.

Materials and methods

Microorganism: *Lactobacillus plantarum* AS101 which was isolated from the dairy product at microbiology department of University of Isfahan, was used in this experiment. The bacteria have been kept as stock at -80°C in glycerol 20% (v/v) (Merck).

Entrapment of *L.plantarum* in sodium alginate

Preparation of harvested cells: In order to assess the culturability of the cells, three different procedures were conducted which are shown in Fig. 1 drawn by the authors.

Prior to use, glycerol stock culture was retrieved on MRS agar. To prepare pre-culture, a loopful of bacteria transferred to a flask containing MRS broth and incubated at 37° C for 24 h under aerobic condition (140 rpm, Sanyo). After the incubation time, an amount of the pre-culture was added to the main-culture flask so that the initial OD_{600} reaches to 0.2±0.02. Medium pH was monitored and maintained at 4.8 for 48 h using 1N KOH (Merck).

For culturing cells under sub-lethal acid stress, the same procedure was used. Briefly, the retrieved bacteria were cultivated on MRS broth supplemented with 0.2% ((v/v), Merck) acetic acid. pH was adjusted to 4.8 ± 0.1 by KOH (1N, Merck). Thereafter, bacteria inoculated to the main culture with the same concentration of acetic acid.

Next, to harvest cells, the bacterial suspension was centrifuged (822 *g*, 10 min, Eppendorf) and washed with PBS (100 m mol/l, pH 7) twice. Then, the cells were counted by the direct method (Thoma slide, Boeco) based on Hao-Yu *et.al* and Shafiei *et.al* studies (26, 27). In addition, culturability was determined by colony count method on MRS agar medium. For the latter, the serial dilution of bacteria was prepared in peptone water 0.1% (v/v) (Merck). The spread plate method was used to enumerate single colonies. The washed and concentrated cells were then entrapped.

Preparation of alginate-calcium beads: To entrap the cells in sodium alginate, the whole procedure corresponds to Fig. 1(a) was done. The overall procedure with some modifications was used as described by Kiew *et al* (28) .Briefly, one ml of harvested cells, according to 'Preparation of harvested cells' section, added to nine ml of alginate sodium 3% (w/v) (Sigma), and mixed completely to make a uniform alginate-bacterial suspension. Next, the droplets of the suspension dropped into a beaker containing calcium chloride (0.1 mol/l, Merck) as a gelling agent through a three mm syringe. Afterward, the beads were washed with sterile distilled water and air-dried for 30 minutes. All of the procedures were accomplished under aseptic conditions.

Two groups of cells were produced: cells grown in MRS and cells were grown in MRS plus acetic acid. They were then entrapped in sodium alginate (a). A part of the beads was incubated in MRS with/without acetic acid, and then underwent a freeze-drying process (b, c). A part of the beads grown in MRS broth was entrapped in different plant gums, freeze-dried and stored (d). Abbreviations: SA: sodium alginate, Tra: tragacanth, Sal: salep gum, Comb: salep gum and tragacanth gum combination.

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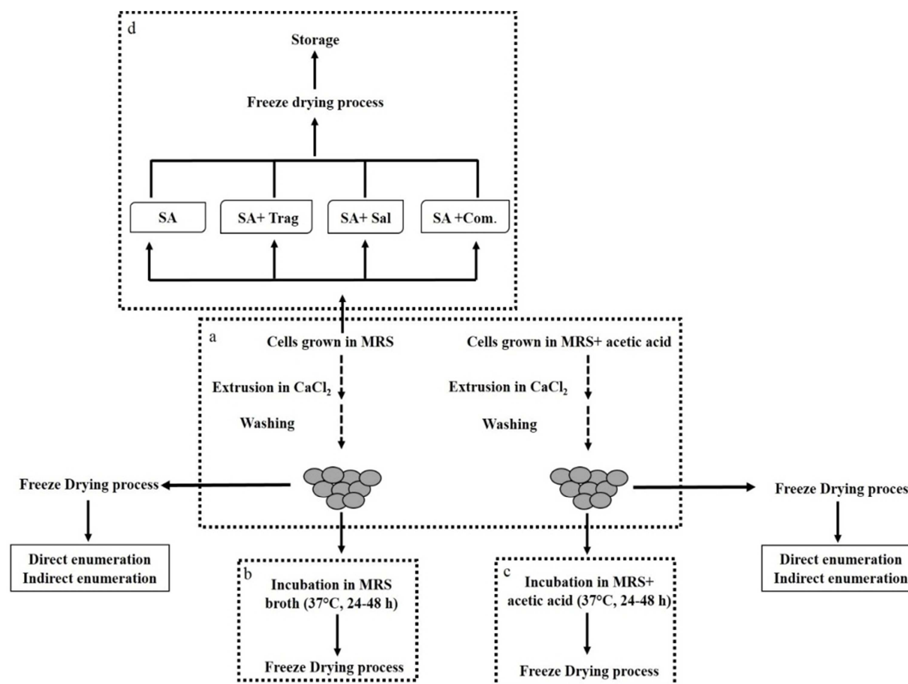

Fig. 1- Design of experiment for the evaluation of different treatments on culturability of *Lactobacillus plantarum*.

Freeze drying the entrapped cells: The entrapped cells were frozen at -20°C and freeze-dried up to 24 h (Heto dry winner, Denmark).

Releasing and determination of culturability of the entrapped cells: In all the experiments which needed assessment of cell culturability, the cells were released in sodium citrate (30 g/l, Merck) supplemented with MRS (3 g/l, Quelab) and incubated at 37°C under aerobic condition (140 rpm for 20 minutes). The total number of cells and culturability of released cells was assessed as described in 'Preparation of harvested cells' section by direct and indirect methods.

Preparation of biofilm-like *L. plantarum*: The appropriate inoculum was prepared according to 'Preparation of harvested cells' section. The biofilm-like cells were formed as Cheow et. al (24) described with some modifications. The overall procedure is depicted in figures 1b and c. Briefly, after obtaining alginate calcium beads as described in section 'Preparation of alginate calcium beads', the beads were incubated in MRS supplemented with $CaCl_2$ (0.1 mol/l, Merck) for 18, 48 and 72 h (Fig. 1b). As it was needed to assess the effect of acetic acid on the beads, another treatment was done in MRS supplemented with 0.1 mol/l $CaCl_2$ and 2% (v/v) acetic acid for the same time (Fig. 1c). Subsequently, the biofilm-like entrapped bacteria was freeze-dried according to 'Freeze drying the entrapped cells' section.

Preparation of biofilm-like cells lateral section: In order to determine the formation of biofilm-like cells, the lateral section of the beads was prepared by Razi medical diagnostic laboratory, Isfahan (Leitz, model LNG 1515) and observed by a light microscope (Olympus, Japan).

Releasing and determination of culturability of the entrapped cells: Thereafter, for the determination of cells' culturability, the cells were released and the culturability of the biofilm-like cells was determined as mentioned in 'Releasing and determination of culturability of the entrapped cells' section.

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Entrapment of *L.plantarum* in plant gums: To entrap cells in plant gums, different combinations of plant gums were used to entrap cells (Fig. 1d). The combinations were as follows: a) sodium alginate (3% w/v, Sigma), b) sodium alginate (3% w/v, Sigma) plus Tragacanth gum (3% w/v, local supplier), c) Sodium alginate (3% w/v, Sigma) plus Salep gum (3% w/v, local supplier) and, d) sodium alginate (3% w/v, Sigma) plus Tragacanth gum (3% w/v, local supplier) plus Salep gum (3% w/v, local supplier). For the hydration of Tragacanth gum and Salep gum, they were dissolved in deionized water. Both of them were incubated at 25°C with shaking (140 rpm, Sanyo). All of the materials were sterilized at 121°C for 20 min.

To prepare concentrated inoculum, the same procedure as 'Preparation of harvested cells' section was used. The harvested cells were washed with PBS (100 mmol/l, pH 7) twice. After that, one gram of bacterial suspension was mixed with nine ml of the all four mentioned combinations. The extrusion method was used for bead formation as explained in 'Preparation of alginate-calcium beads' section. The beads were then undergone the freeze-drying process as described in 'Freeze drying the entrapped cells' section.

Storage stability of plant gums' entrapped bacteria: To evaluate the effect of different temperatures on culturability of dried bacteria, the beads were incubated in incubators at four distinct temperatures including 4, 25, 37, and 55°C. The storage time was defined as 10, 20 and 30 days after the initial enumeration. To check the effect of gums on bacterial culturability, the cells were released and counted as described in 'Releasing and determination of culturability of the entrapped cells' section.

For assessing the effect of plant gums on entrapped cells in four temperatures, the kinetic measurements were conducted based on the *Arrhenius* equation. Cell inactivation during storage was calculated by equation 1.

$$\ln N/N_0 = -kT \qquad \text{equ 1}$$

Where N_0 is the initial number of culturable cells, N is the number of culturable cells after treatment, T is the storage temperature (based on Kelvin scale) and k is the inactivation rate constant at the given temperature (29).

The linearization of equation 1 results in equation 2.

$$\ln k = -E_a/RT + \ln A \qquad \text{equ 2}$$

Where E_a is activation energy, R is gas constant and A is frequency factor in a unit of time.

Also, the *D value* was calculated based on equation 2. This number is used to determine the loss of bacteria during treatment (30).

$$k = 2.303/d \qquad \text{equ 3}$$

Statistical analysis: All experiments were carried out in duplicate. The results were analyzed by SPSS (IBM®SPSS® statics, version 21) and *p*-value defined below 0.05 by univariate analyze.

Results and discussion

In the present study, the culturability and stability of *Lactobacillus plantarum* were assessed under some conditions such as sub-lethal acid stress, biofilm-like structure formation, and entrapment in plant gums.

The effect of sub-lethal acid stress on culturability of alginate calcium entrapped cells: As the main goal of this study was to conduct a method to increase the culturability of LABs, different methods were used to accomplish this. One way was to use sub-lethal acid stress in the experiment (31-33). Up to our knowledge, there is no paper regarding the effect of sub-lethal acetic acid stress on the entrapped lactobacilli cells. There is just one report in 2017 in which gelatin prepared beads in acetic acid (10% v/v) used for coaxial electrospray drying of *L.*

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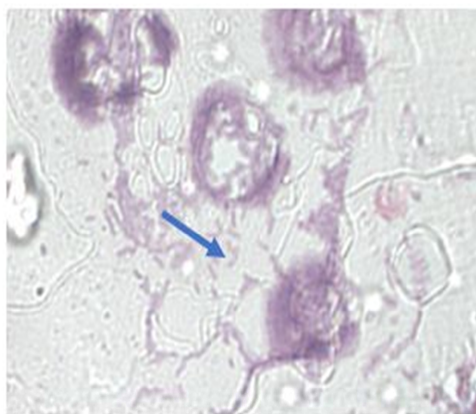
plantarum. It was mentioned that this treatment could maintain the culturability of the cells (34). However, a number of researchers mentioned using MRS with acidic pH, in the range of 5, for enumeration of lactic acid bacteria (35, 36). In this regard, our bacterial species was also able to tolerate and grow in low pH. At the first step, cells grown in MRS medium with/without acetic acid were entrapped in calcium alginate. The entrapped cells were then released in sodium citrate to determine the culturability of the cells. As Table 1 shows, comparison of direct and indirect counts in each condition indicates that 14% of cells grown in MRS plus acetic acid were able to tolerate extrusion, entrapment and releasing procedures while just a small fraction of the cells (0.8 %) grown in MRS was culturable after releasing from calcium alginate beads. In other words, growth in MRS plus acetic acid could resist cells to the entrapment condition.

The culturability of biofilm-like cells with or without sub-lethal acid stress: The other promising way was using biofilm-like cells instead of planktonic ones to improve the stability of the cells. This condition has been shown to have a positive effect on the survival of the encapsulated probiotics such as *Lactobacillus rhamnosus* (24, 25). In the present study, at first, it was needed to prove the ability of *Lactobacillus plantarum* to grow and form a biofilm-like structure in beads. Observation of stained lateral section of beads incubated in mentioned culture media by light microscope showed that *L. plantarum* cells were able to grow as biofilm-like cells inside the beads (Fig. 2a). On the contrary, most of the cells appeared as single cells in non-incubated beads (Fig. 2b) meaning that single cells grew and formed larger population after incubation of beads in an appropriate medium. In addition, some of the beads floated on top of the culture media during incubation which can be a consequence of gas production by the cells.

Table 1- The direct and indirect enumeration of the cells entrapped in alginate calcium

	Enumeration methods		
	Direct enumeration (Total number)		Indirect enumeration (Culturability)
Cells were grown in MRS and entrapped in calcium alginate beads	$1.2\times10^{8}\pm0.56$ (Cell/mL)		$1.06\times10^{6}\pm0.45$ (CFU/mL)
Cells were grown in MRS plus acetic acid and entrapped in calcium alginate beads	$8.16\times10^{7}\pm0.41$ (Cell/mL)		$1.15\times10^{7}\pm0.51$ (CFU/mL)


a

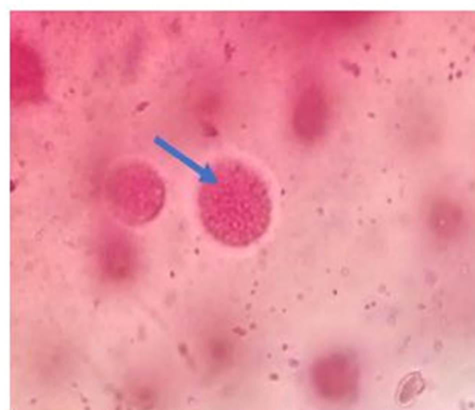

b

Fig. 2- Lateral stained sections of non-incubated beads (a) and incubated beads (b) in MRS broth.

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As it is seen, most of the cells in non-incubated beads are single (a) while in incubated beads, most of them formed biofilm-structures (b). The arrow in panel (a) indicates the single cells and in panel (b), shows biofilm-like cells.

In the next step, the cells fromed during incubation of beads in MRS were released in sodium citrate. The results indicated that the population of the cells increased dramatically, from 2.5×10^5 CFU/ml to 2.6×10^6 CFU/ml, after 18 h of incubation of the beads in MRS broth. In addition, as the increase of the cell population was not significant after 48 h, 2.9×10^6 CFU/ml, meaning that incresae of time to 48 hours had no significant effect on cells' culturability increasre. Henceforth, incubation up to 18 h was used for further analyses. Surprisingly, the number of culturable cells decreased sharply after 72 h incubation, 2.31×10^4 CFU/ml, meaning that extensive incubation is not appropriate for the biofilm-like structure formation of *L. plantarum*. The time needed for biofilm-like cells formation was the same in the other two studies (24). Kubota *et.al* showed that lactobacilli have a higher resistance in the concentration of 8% and 11% of acetic acid in form of biofilm compared to planktonic cells (37, 38). In the present study, we showed that the culturability of *L. plantarum* could be augmented in presence of acetic acid (0.2% v/v). However, the strain used in the present study was more susceptible to acetic acid as the higher concentration of acetic acid (0.5% v/v) inhibited the growth of the bacteria (data not shown). Furthermore, there is no research on the biofilm-like cells of *L. plantarum* incubated in medium containing acetic acid as well as its effect on cell culturability after the freeze-drying process. The potential of *L. plantarum* to form biofilm-like cells in this study agrees with Cheow *et.al* on the potential of other lactobacilli species, *L. rhamnosus,* to form a biofilm-like structure. As the culturability of the cells matters, the biofilm-like cells in normal MRS showed 0.2 log CFU/ml increase while this number was 0.4 log CFU/ ml for acidic treated biofilm-like cells. These numbers are in close relation with the numbers obtained by Chew *et al.* (25). Therefore, we can demonstrate that using acetic acid as one stress factor had a more substantial effect on the culturability of the cells. This effect that is known as 'cross-protection' has been proposed by other authors as well (39, 40). Nevertheless, the effect of cross-protection accompanied biofilm-like *L.plantarum* has not been studied.

To assess the biofilm-like cells' culturability, the harvested cells were first entrapped in calcium alginate beads and then incubated in MRS broth or MRS broth supplemented with acetic acid (Fig. 1b and Fig. 1c). In the third step, the culturability of 18-hour incubated beads was determined. Non-incubated beads were considered as control (Fig.3 (1 and 3)). Although the number of culturable cells was always lower than direct cell count, the incubation of beads and formation of the biofilm-like structure had a positive effect on the culturability of entrapped *L.* plantarum (Figure 3). The survival rate of the cells increased notably after incubation of beads in MRS broth by 17% (Fig. 3 (2)). Incubation in acidic condition also increased the survival rate by 37% (Fig. 3 (4)). Overall, the incubation of beads in MRS supplemented with acetic acid resulted in better survival rate compared to MRS.

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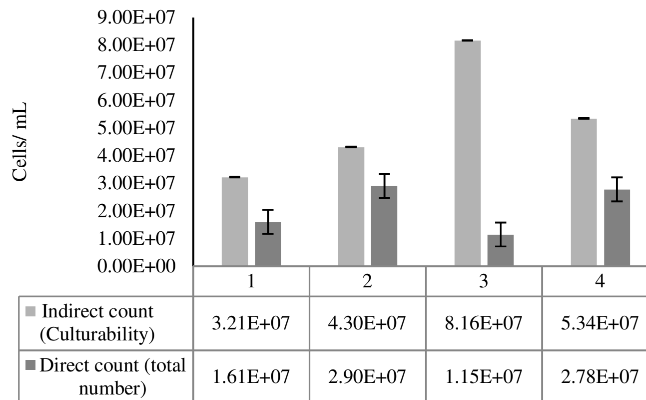

	1	2	3	4
Indirect count (Culturability)	3.21E+07	4.30E+07	8.16E+07	5.34E+07
Direct count (total number)	1.61E+07	2.90E+07	1.15E+07	2.78E+07

Fig. 3- Comparison of the total number (direct count) of released cells with their culturability (indirect count) before and after incubation of beads in culture media. 1, Non-incubated beads (Normal control). 2, Beads incubated in MRS broth up to 18 h; 3, Non- biofilm-like treated beads in acidic condition (Acidic control); 4, Beads incubated in MRS broth plus acetic acid up to 18 h. All of the trends have been compared with the control. The uppercase letter indicates the meaningful difference between values in direct enumeration while lowercase letters indicate the meaningful difference between indirect enumerations. The values are mean of independent duplicate enumerations.

The culturability of biofilm-like freeze-dried beads: In order to increase the stability of beads incubated in MRS broth or MRS broth plus acetic acid, they were freeze-dried, and then the culturability of released cells was assessed. As it is seen in Fig. 4, the beads incubated in MRS broth plus acetic acid showed a better survival rate than the beads incubated in MRS broth. The overall results indicated that the survival rate of cells formed a biofilm-like structure in MRS broth plus acetic acid is slightly (5%) higher than beads incubated in MRS broth. Interestingly, it was observed that incubation of beads in MRS broth plus acetic acid improved cell culturability after the freeze-drying process. This can be due to cross-protection effect which was mentioned earlier in this article and other ones (39, 40)

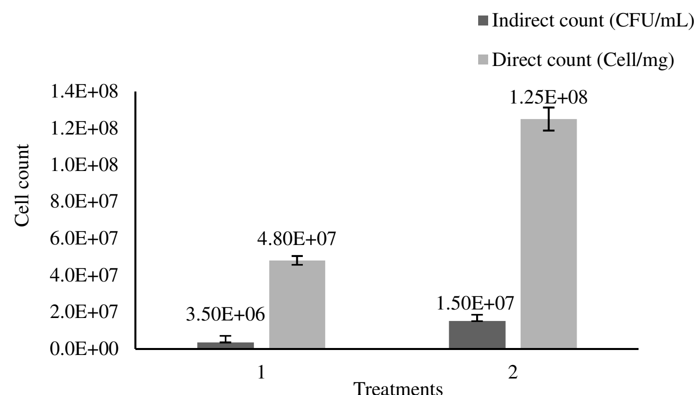

Fig. 4- Comparison of the total number (direct count) of released cells and their culturability (indirect count) before and after freeze-drying of biofilm-like beads. 1, freeze-dried beads incubated in MRS and 2, freeze-dried beads incubated in MRS plus acetic acid. The uppercase letter indicates the meaningful difference between values in direct enumeration while lowercase letters indicate the meaningful difference between indirect enumerations. The values are mean of independent duplicate enumerations.

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The effect of different plant gums on culturability of freeze-dried *L.plantarum*: Using the second material to increase the firmness of formed beads is another recommended method. This material can have proteinaceous or polysaccharide nature (41). Plant gums are one novel divergence of polysaccharides that have been utilized in a handful of research papers. In the present study, tragacanth gum and salep gum used as the second materials. To the best of our knowlegde, these gums have not been used for stabilization of lactic acid bacteria. In addition, freeze-drying used for obtaining bacterial powders.

To evaluate the effect of plant gums on culturability of the entrapped cells, the cells were entrapped in plant gums according to the procedure explained in 'Alginate-gum mixture formation' section and freeze-dried afterward. In the next step, the cells were released in sodium citrate, and direct and indirect counting was done. As it can be seen in Fig. 5, the culturability of the cells entrapped in a mixture containing salep gum, tragacanth gum, showed 4.6, 1.4 fold increase while the combination of gums entrapped cells showed 0.55 fold decrease. The number of cells entrapped in a mixture containing tragacanth gum was considerably higher than other treatments. Alginate-fenugreek gum-locust bean gum mixture can affect the viability of lactic acid bacteria after freeze-drying and storage period in a strain-specific manner. Regarding *L. plantarum,* the viability of freeze-dried cells was 97.41% (42). In the present study, the highest culturability obtained using Tragacanth gum improved by 4.6 fold compared to alginate beads. This difference can be due to the nature of the plant gums and the different enumeration procedure. In another study, gum acacia and spray-drying method improved the viability of *L. paracasei* at 4°C compared to higher temperatures (12). Chun *et al.* also reported improved viability of *L. plantarum* after freeze-drying process using alginate as the coating material by 0.18 CFU/g which is contrary to our results which indicate a reduction of cell count after entrapment in alginate. However, improvement of the cell culturability is in accordance with both studies with different ratios. In the mentioned study, the incorporation of alginate and gellan gum conferred lower protection than the incorporation of alginate and gum arabic (43). Up to our knowledge, there is one paper on using tragacanth gum for entrapment of *L. casei* through the extrusion method. In this article, this fact was mentioned that the viability of the bacterial cells are better supported by alginate-tragacanth mixture in comparison to entrapped cells in alginate pectin-chitosan and alginate-gumarabic-chitosan mixtures (44). The better survival of bacteria in alginate-tragacanth mixture is in accordance with our results, althought the strains were different. Regarding the effect of plant gums and freeze-drying on culturability of the cells, as can be inferred from Fig. 5, the incorporation of tragacanth gum and salep gum had a more positive influence on the culturability improvement of freeze-dried cells. In the present study, using the combination of plant gums, salep gum, and tragacanth gum, and alginate decreased the culturability 1.7 log while the incorporation of salep gum and tragacanth gum increased the culturability 0.2 log and 0.7 log, respectively. This positive effect can be attributed to the high molecular weight of these two plant gums. It seems that high molecular weight compounds have a positive effect on both the morphology of the capsules and the viability of the cells (45).

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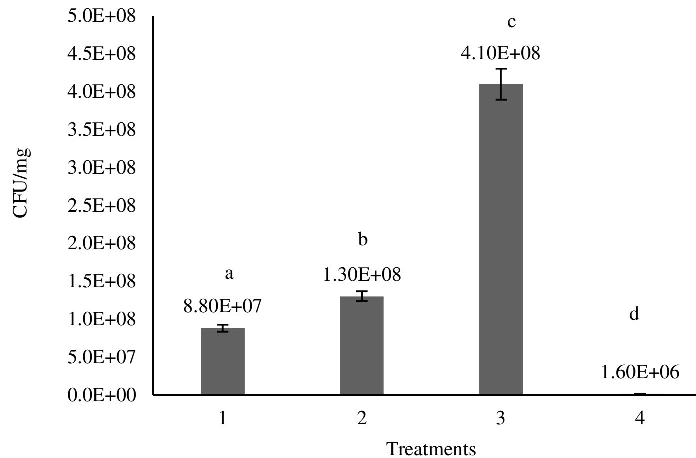

Fig. 5- Comparison of culturability of freeze-dried entrapped cells in different plant gum. 1, Alginate sodium; 2, Salep gum; 3, Tragacanth gum; 4, Combination of gums (tragacanth gum and salep gum). Alginate was used as a gelling agent in all treatments. Different letters indicate the meaningful difference between data. The values are mean of independent duplicate enumerations.

Storage stability of entrapped cells in plant gums: According to the guideline of FDA/WHO, the number of probiotic cells should be above 7 log/CFU in a product so that its probiotic trait can be assured (FAO,46, 2001). With respect to the storage at 4°C and 25°C, the number of viable *L. acidophilus* cells remained above 7 log/CFU for tragacanth gum beads for 20 days and 30 days, relatively. Incorporation of Gellan gum in the encapsulation process resulted in 1.84 times improvement which is lower than 4.65 times improvement of *L. plantarum* in the present study. This can be due to gum difference and the method of entrapment (47). Zhao *et al.* in 2017 showed that the incorporation of gum arabic into *Lactobacillus plantarum* microcapsules resulted in the high inactivation constant rate (*k* value) after spray drying process in storage at ambient temperature. They also concluded that by incorporating sucrose, this value decreases (49). In the present study, to evaluate the effect of the plant gums on the culturability of the cells during storage, the released cells in citrate buffer were cultivated on MRS agar, and the CFU/ml value of each treatment was determined. The overall results are illustrated in Fig. 6 and Table 2. There are some considerable results that can be inferred from Table 2: i) The highest *D value*, the time needed to inactivate 90% of the microbial population (48), and lowest *k* value, the inactivation constant rate (49) were 25 days and 0.09, respectively at 4°C for tragacanth gum beads. This indicates that tragacanth gum could protect the cells better compared to other treatments at 4°C. ii) The lowest *D value* and the highest *k* value was 3 days and 0.75, respectively at 55°C in beads containing the combination of two plant gums. iii) The *D* value of alginate containing beads was 3 days at 4°C while the highest *k* value for this treatment was 0.616 at 55°C. iv). The incorporation of salep gum and the combination of two gums into the beads formula also increased *k* value to 0.143 and 0.23, respectively,

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and reduced *D value*, 16 days and 9 days, accordingly. As a result, the culturability of the cells reduced in all four treatments, however, this reduction was less perceptible in the treatment of beads with tragacanth gum and salep gum at 4°C. Henceforth, the incorporation of salep gum and tragacanth gum could have more positive effects on maintenance of culturability of the beads in comparison to other treatments at 4°C. Also in another study, the incorporation of this plant gum into microcapsules increased the viability of *L. paracasei* by 10-fold after spray drying process which is higher than the effect of tragacanth gum in the present study. Henceforth, the incorporation of this plant gum can be considered as a protective agent for storage of *L. plantarum* at room temperature and refrigerator temperature.

Table 2. The effect of plant gums on kinetic parameters of entrapped cells

Treatment	*T* (°C)	K^* (day^{-1})	$t_{1/2}$ (day)	D (day)	R^2
Alginate	4	0.68	1.00	3.35	0.89
	25	0.65	1.05	3.51	0.86
	37	0.63	1.1	3.60	0.89
	55	0.61	1.12	3.73	0.79
Tragacanth	4	0.09	7.60	25.18	0.92
	25	0.19	3.50	11.52	0.82
	37	0.29	2.32	7.72	0.86
	55	0.51	1.34	4.48	0.89
Salep	4	0.14	4.90	16.04	0.93
	25	0.24	2.84	9.46	0.94
	37	0.31	2.17	7.23	0.60
	55	0.45	1.50	5.01	0.60
Combination*	4	0.23	2.90	9.62	0.68
	25	0.40	1.71	5.70	0.90
	37	0.52	1.31	4.37	072
	55	0.75	0.91	3.03	0.72

*Combination of tragacanth gum and salep gum

k: the inactivation constant rate; *D* :the time needed to inactivate 90% of the microbial population; *T* : absolute temperature; $t^{½}$: the half-life ; R^2: the coefficient of determination. (*k* value was different p<0.05).

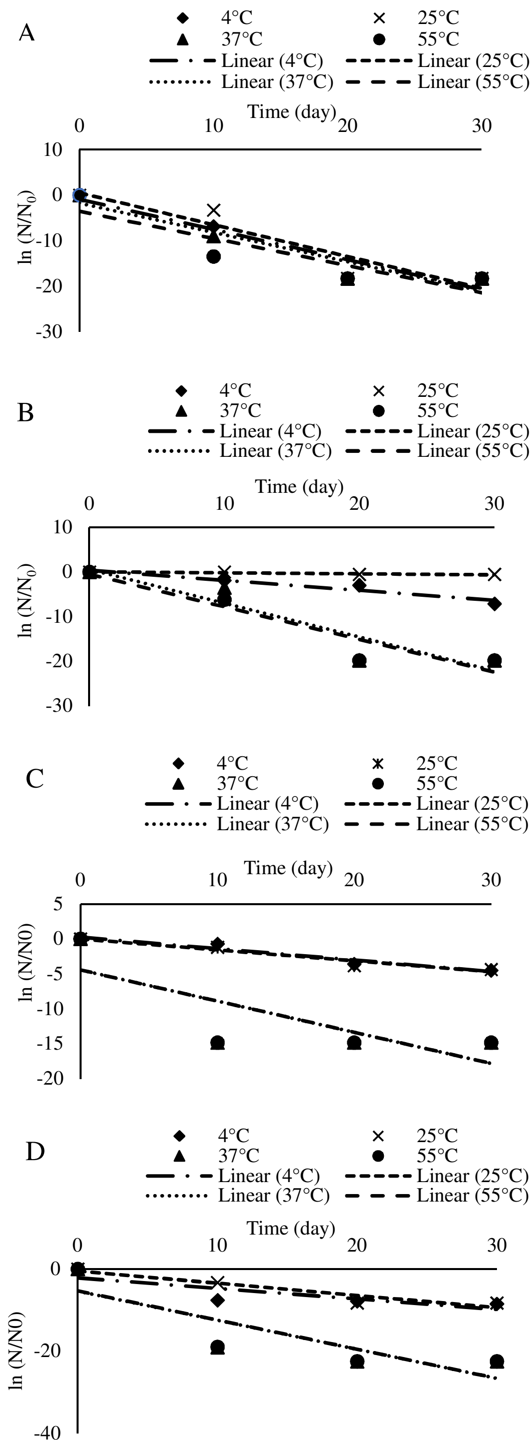

Fig. 6- The effect of plant gums on the culturability of *L.plantarum* during storage (30 days at different temperatures). A, alginate treatment; B, tragacanth gum; C, salep gums; D, a combination of plant gums (tragacanth gum and salep gum). Tragacanth gum and salep gum protect the cells better compared to alginate and combination of gums.

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Conflict of interests

Authors have no conflict of interest to declare.

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Abbreviations

k: inactivation constant rate

D: the time needed to inactivate 90% of the microbial population

T: absolute temperature

$t_{½}$: half-life

R^2: coefficient of determination

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تاثیر ساختار شبه بیوفیلم، استرس اسیدی تحت کشنده و صمغهای گیاهی بر روی قابلیت کشت لاکتوباسیلوس پلانتاروم به دام افتاده

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چکیده

مقدمه: برای افزایش پایداری سلول‌های لاکتوباسیلوس، روش‌های مختلفی بر مبنای به دام‌اندازی استفاده و اثر آنها بر روی قابلیت کشت سلول‌های پایدارشده مطالعه شده.

مواد و روش‌ها: در مطالعه‌ی حاضر، استرس اسید تحت کشنده، ساختار شبه بیوفیلم و ترکیب صمغ‌های گیاهی استفاده شد و سپس قابلیت کشت لاکتوباسیلوس پلانتاروم با استفاده از این مواد اندازه‌گیری شد.

نتایج: بر مبنای نتایج به دست آمده، انکوباسیون سلول‌های به دام افتاده در آلژینات در محیط مایع دارای MRS و اسید استیک، میزان زنده‌مانی را ۲۹٪ در مقایسه با سلول‌های انکوبه شده در محیط مایع MRS افزایش داد. به علاوه، انکوباسیون سلول‌های به دام افتاده در آلژینات در محیط دارای اسید استیک منجر به بهبود زنده‌مانی پنج درصدی سلول‌های شبه بیوفیلم بعد از فرآیند خشک کردن انجمادی شد. برای افزایش پایداری سلول‌ها در طی دوره انبارمانی، سلول‌ها در آلژینات به همراه صمغ‌های گیاهی متفاوت به دام انداخته شدند. استفاده از صمغ کتیرا و صمغ ثعلب منجر به افزایش (۴٫۶ و ۳٫۱ برابر به ترتیب) قابلیت کشت سلول‌ها در مقایسه با سلول‌های تیمارشده با آلژینات شد. به علاوه، پایین‌ترین ثابت غیرفعال سازی، مقدار k، 0.09، بوده و بالاترین مقدار D، 25 روز، برای سلول‌های تیمار شده با صمغ کتیرا در دمای 4°C طی دوران انبارمانی بدست آمد که نشان‌دهنده‌ی تاثیر بهتر صمغ کتیرا در نگهداری سلول‌ها در مقایسه با سایر تیمارها در شرایط انبارمانی بود.

بحث و نتیجه‌گیری: انکوباسیون گوی‌های لاکتوباسیلوس پلانتاروم در محیط MRS اسیدی می‌تواند منجر به افزایش قابلیت کشت خصوصا بعد از خشک کردن انجمادی شود که ممکن است به علت اثر حفاظت متقابل باشد. همچنین، به علت خصوصیات صمغ کتیرا، این صمغ گیاهیمی‌تواند سلول‌های به دام افتاده را در شرایط انبارمانی بهتر نسبت به سایر صمغ‌ها حفظ کند. درنتیجه، می‌توانیم از صمغ کتیرا به عنوان ماده ثانویه برای افزایش پایداری سلول‌های به دام افتاده لاکتوباسیلوس پلانتاروم استفاده کنیم.

واژه‌های کلیدی: استیک اسید، خشک کردن انجمادی، پروبیوتیک، صمغ ثعلب، سدیم آلژینات، صمغ کتیرا

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