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Full Length Research Paper

Effect of spearmint on the growth of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in probiotic milk and yoghurt

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In the present study, the potential of producing foods containing probiotic bacteria with the bases of milk, yoghurt and spearmint was evaluated. The goal was to determine the effects of different doses of spearmint (0, 0.3, 0.6 and 0.9%) on the growth of two probiotic bacteria, that is, *Bifidobacterium bifidum* and *Lactobacillus acidophilus*, in milk and yoghurt produced in the first and second phases, respectively. The products were then examined in terms of pH, acidity and microbe counting during the incubator setting period and their respective permanence. In day 10, the production quantities of the products were evaluated by sensory method. The results of the questionnaires filled by 50 people in statistical–descriptive test were analyzed using SPSS software version 16 system. In the samples with either *L. acidophilus* or *B. bifidum* and the sample containing both bacteria, it was observed that the increased concentration of spearmint did not create a favorable taste of yoghurt and of all the samples the yoghurt containing 0.3% spearmint and the testifier were with the best tastes. The increased concentration of spearmint had no effect on the yoghurt permanence and the yoghurt containing 0.3% spearmint, and the testifier containing 0% spearmint were of greater permanence than the other samples. The bioability of probiotic bacteria was measured by direct counting method. During the 15-day period, the number of bacteria decreased and no significant difference between the testifier sample and the samples containing different concentrations of spearmint was observed. Duration of the product permanence was determined within the same period. Upon examination of the results, it was revealed that the increased concentration of spearmint had a positive effect on the growth of the probiotic bacteria, *L. acidophilus* and *B. bifidum* in probiotic milk and yoghurt.

Key words: Probiotic, *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, spearmint.

INTRODUCTION

A functional food used by consumers is the one with additional basic nutritional properties, at least an established distinct healthful property and recommended are among functional foods. One of the significant points in relation to the selection and production of functional purposefully by producers or foods scientists. Milk and its products, particularly the respective fermentable products foods is their immune and safe consumption. Nowadays, because of confirmed undesirable results of imbalanced and bad eating in human societies, tendency toward the production and consumption of different functional foods have considerably increased. Probiotics, as a kind of new and favorite functional products are of great importance.

The distinctive feature of such products is their

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inclusion of some microorganisms, that is, bacteria rather than synthetic compounds in them (Mortazavian and Sohrabvandi, 2006). They serve as the food complements containing selective microorganisms, and are able to affect positively the metabolic and physiological functions of both humans and animals.

The most common cultures used in the production of such products include *Lactobacillus acidophilus, Bifidobacterium bifidum and Lactobacillus casei*. Once the bacteria within milk and yoghurt enter the digestive system; they strengthen and balance the beneficial bacteria in the intestines (Bassettis and Zimmeli, 1998). The emergence of probiotics and their related issues on the one hand and their respective complexities on the other hand, have brought about vast areas of research and investigations.

Recently, the design and production of plant-based probiotic products have received much attention chiefly due to their natural health benefits (protein, fiber, vitamin and salts) and also because of the variety in their production. Therefore, it seems that the issue of producing probiotic foods with appropriate qualities will be a major research topic for prospective researchers (Mortazavian and Sohrabvandi, 2006).

Although, in the past decades, the synthetic chemical drugs that make use of separation mechanisms have been much in demand, their corresponding side effects are being gradually observed so much so that their irregular and improper consumption has turned out to be a critical issue.

On the contrary, the benefits of medicinal plants and their little or zero side effects have made them a proper substitute, highly appreciated by physicians and patients. Iran possesses a very rich source of such plants and herbs in the world in terms of variety and amount. The essence plants play a critical role in human life, and have been used for long by the Iranians. Spearmint is one of the oldest and most important medicinal plants and spices, with 10,000 ton annual consumption worldwide and 2000 years background. The human consumption of spearmint in food products dates back to some 250 years ago. Leaves, barks and the essence of spearmint are used as medicine by the most accredited pharmacopeia.

MATERIALS AND METHODS

(i) Low-fat sterilized milk (1.5% fat).
(ii) Dried spearmint powder.
(iii) Low-fat yoghurt from supermarket.
(iv) Bacteria lyophilize *L. acidophilus* (CHR Hansen company, Denmark).
(v) Bacteria lyophilize *B. bifidum* (CHR Hansen Company, Denmark).

The effect of spearmint on the production of probiotic *Bifidobacterium bifidum* milk at first *passage*

In order to produce the milk containing probiotic bacterium *B. bifidum*, four containers each containing 1 L of low-fat sterilized milk (1.5% fat) were considered as our four groups. The starter (*B. bifidum*) was added directly to all the containers, followed by adding dried spearmint powder 0% (to the control), 0.3, 0.6, and 0.9% to all the containers, respectively and finally they were placed in incubator at 38℃. The acidity test was performed ap proximately every 2 h until reaching 42°Dornic (Standard anistit ue and industrial search of Iran, 2006).

The samples were then taken out of incubator and transferred to a refrigerator and stored at 2℃. The produced probiotic milk was evaluated once every 5 days by counting the microbes using direct counting method.

The effect of spearmint on the production of probiotic *Bifidobacterium bifidum* yoghurt at the second passage

To produce *B. bifidum* yoghurt in this stage, after providing four containers, 1 L of low-fat sterilized probiotic milk (1.5% fat) from the control group at first passage and the (1.5%) starter of low-fat yogurt (1.5%) were added to each container.

Different concentrations of spearmint (0, 0.3, 0.6 and 0.9%) were added, respectively to the containers and mixed properly so that spearmint was uniformly dissolved. Afterwards, all the containers were placed in the hot – house at 38℃. Approximate ly, every 2 h, the acidity and pH tests were done until the acidity reached 96°Dornic. Then, the samples were taken out of the i ncubator and transferred to a refrigerator and stored at 2℃. The pro duced probiotic spearmint yoghurt was evaluated every 5 days by counting the microbes using direct counting method and after 10 days the yogurt was evaluated for sensory properties, using questionnaires filled by 40 people. The respondents were asked to rate the factors of scent, taste and permanence on a scale ranging from very good, good, medium, to weak. The results were analyzed in a statistical descriptive test by SPSS version 16 software.

The effect of spearmint on the production of probiotic *Lactobacillus acidophilus* milk at first passage

In order to produce the milk containing the bacterium *L. acidophilus*, four containers each containing 1 L of low-fat sterilized milk (1.5% fat) as our four groups, were prepared and the starter (*L. acidophilus*) was added directly to all of them. Then, dried spearmint powder 0 (control), 0.3, 0.6 and 0.9% was added to them, and all the containers were placed in the hot-house at 38℃. Approximately, every 2 h, the acidity test was done until it reached 42°Dornic. The samples were then taken out of the inc ubator, transferred to a refrigerator and stored at 2℃. The pro duced probiotic milk was evaluated every 5 days by counting the microbes using direct counting method.

The effect of spearmint on the production of probiotic *Lactobacillus acidophilus* yoghurt at second passage

To produce *L. acidophilus* yoghurt in this stage, after providing four containers, 1 L of low-fat sterilized probiotic milk (1.5% fat) from the control group at first passage, and the starter of low-fat yoghurt (1.5%) were added to each container.

The different concentrations of spearmint (0, 0.3, 0.6 and 0.9%) were added to containers, respectively and mixed properly so that the spearmint was uniformly dissolved. Then, all the containers were placed in the incubator at 38℃. The acidity le vels of the samples were checked approximately every 2 h till reaching 96°Dornic, at which point the samples were taken out of the incubator, transferred to a refrigerator and stored at 2°C. The produced probiotic spearmint yoghurt was evaluated every 5 days by counting the microbes using direct counting method, and after

Table 1. The acidity level based on Dornic degree in the spearmint *B. bifidum* milk and yoghurt within 15-day storage in the refrigerator.

Acidity level in Dornic degree									
Spearmint milk (%)	**1 day**	**5 day**	**10 day**	**15 day**	**Spearmint Yoghurt (%)**	**1 day**	**5 day**	**10 day**	**15 day**
0	45	50	62	80	0	96	112	138	185
0.3	48	65	85	98	0.3	102	116	142	190
0.6	50	65	85	98	0.6	115	132	170	192
0.9	52	68	88	100	0.9	115	120	155	188

10 days, the yoghurts were evaluated with regard to sensory properties using the questionnaires. The factors including scent, taste and permanence were ranged on a scale from very good, good, medium, to weak. The results were subsequently analyzed in a statistical–descriptive test by SPSS software version 16.

The effect of spearmint on the production of Probiotic *Lactobacillus acidophilus* and *Bifobacterium bifidum* milk at first passage

As previously described, four containers containing 1 L of sterilized low-fat milk (1.5% fats) were considered as our four groups. The bacteria lyophilized *B. bifidum* and *L. acidophilus* were simultaneously added to all the containers. The first container was considered as the control group and dried spearmint powder 0.3, 0.6 and 0.9% were added, respectively to the other containers. The procedures were followed as we did in the first stage.

The effect of spearmint on the production of probiotic *Lactobacillus acidophilus* and *Bifidobacterium bifidum* yoghurt at second passage

To do so, the four containers each of which containing 1 L of sterilized low-fat milk were considered as four groups. Then, the starter of yoghurt and the probiotic milk from the control group in the previous stage were added to all the containers. Except for the first container which was considered as the control, the spearmint 0.3, 0.6 and 0.9% was added to the other containers. Procedures involved in the yoghurt production were the same as those for the yoghurt with the above–mentioned bacteria.

Having produced the above-mentioned products, we stored 1000 gr of each product in a disposable container placed in a refrigerator for 15 days. During this period, each sample was tested in days 1, 5, 10 and 15 for acidity, pH and sensory properties.

RESULTS

Table 1 shows the acidity degrees of spearmint milk and yoghurt *B. bifidum*, during storage time in the refrigerator and Table 2 shows the growth rates of microbes in spearmint *B. bifidum* milk and yoghurt at storage time. Table 3 shows the acidity degrees of spearmint *L. acidophilus* milk and yoghurt and Table 4 shows the growth rates of microbes in spearmint *L. acidophilus* milk and yoghurt at storage time in the refrigerator.

Table 5 shows the acidity degree of spearmint milk and spearmint yoghurt *B. bifidum* and *L. acidophilus* during storage in the refrigerator, and Table 6 shows the growth rate of microbes in spearmint milk and spearmint yoghurt *B. bifidum* and *L. acidophilus* during the same cooling period.

DISCUSSION

In recent years, the probiotic bacteria, as the food additives, have been introduced into numerous foods, of which the dairy products especially yoghurt has played an important role in carrying these bacteria (such as *B. bifidum* and *L. acidophilus*).

Eating regularly the sufficient amounts of the living cells called "the minimum treatment' is required if the consumer is to benefit from the probiotic products. The daily recommended amount of the yogurt containing10^6 CFU.ml^{-1} probiotic bacteria is 100 gr. It is also very important to investigate the survival of these microorganisms within the interval between storage in the refrigerator and consumption (Khosravi and Koshki, 2008).

Essence medicinal plants and herbs play a significant role in the human life and have been very popular for long among the Iranians. The spearmint, as a medicinal plant, has extensive effects of which the most notable include anticonvulsant, anti-cough, anti-spasm and anti-bloat effects. Spearmint is produced and consumed in the forms of syrup, candy, essence and also aromatherapy compounds. The other forms of available drugs from spearmint include the spearmint drop and topical spearmint lotion (Jahanara and Haerizade, 2001).

In the present study, the effects of spearmint on the growth of the bacteria *B. bifidum* and *L. acidophilus* in probiotic milk and yogurt were investigated. The acidity, pH and survival of the bacteria in the spearmint probiotic milk and yogurt were evaluated at 2 h intervals till reaching 42°Dornic acidity degrees for milk and 96°Dornicdegree for yogurt in the incubator at 38°C and also within 15-day period of storage in the refrigerator. The acidity- based Dornic index, remained almost constant for the first two-hours, suggesting the absence of probiotic activity within this period.

The probiotic spearmint milk 0.9% in the sample containing *B. bifidum* and the sample with *L. acidophilus*,

Table 2. Growth of microbes in the spearmint *B. bifidum* milk and yoghurt.

Spearmint milk (%)	1 day	5 day	10 day	15 day	Spearmint Yoghurt (%)	1 day	5 day	10 day	15 day
0	7.5×10^{10}	8.5×10^{10}	12.5×10^{10}	7×10^{10}	0	5.75×10^{10}	1.25×10^{10}	2.75×10^{10}	1.25×10^{10}
0.3	7.5×10^{10}	7.5×10^{10}	8.5×10^{10}	7×10^{10}	0.3	1.5×10^{10}	3.75×10^{10}	4×10^{10}	2×10^{10}
0.6	5.75×10^{10}	5.25×10^{10}	10.75×10^{10}	4.5×10^{10}	0.6	2.75×10^{10}	1.75×10^{10}	2×10^{10}	2.25×10^{10}
0.9	6.5×10^{10}	4×10^{10}	7.5×10^{10}	5.25×10^{10}	0.9	2.25×10^{10}	6×10^{10}	6.25×10^{10}	3×10^{10}

Table 3. The acidity level based on Dornic degree in the spearmint *L. acidophilus* milk and yoghurt within 15-day storage in the refrigerator.

Acidity level in Dornic degree									
Spearmint milk (%)	**1 day**	**5 day**	**10 day**	**15 day**	**Spearmint Yoghurt (%)**	**1 day**	**5 day**	**10 day**	**15 day**
0	55	91	96	110	0	97	116	136	141
0.3	64	110	115	125	0.3	102	120	157	160
0.6	70	113	125	134	0.6	110	125	165	170
0.9	96	116	128	136	0.9	112	126	167	174

Table 4. Growth of microbes in the spearmint *L. acidophilus* milk and yoghurt.

Spearmint milk (%)	1 day	5 day	10 day	15 day	Spearmint Yoghurt (%)	1 day	5 day	10 day	15 day
0	6×10^{10}	11.5×10^{10}	11.5×10^{10}	3×10^{10}	0	3×10^{10}	7×10^{10}	3×10^{10}	2.75×10^{10}
0.3	6×10^{10}	14.5×10^{10}	14.75×10^{10}	9.5×10^{10}	0.3	1.5×10^{10}	4.75×10^{10}	3.5×10^{10}	3×10^{10}
0.6	5×10^{10}	7.75×10^{10}	12.75×10^{10}	10×10^{10}	0.6	2.5×10^{10}	6.25×10^{10}	3.75×10^{10}	3×10^{10}
0.9	7×10^{10}	18×10^{10}	12×10^{10}	8.25×10^{10}	0.9	1.5×10^{10}	2×10^{10}	3.25×10^{10}	2.75×10^{10}

Table 5. The acidity level based on Dornic degree in the spearmint *L. acidophilus* and *B. bifidum* milk and yoghurt within 15-day storage in the refrigerator.

Acidity level in Dornic degree									
Spearmint milk (%)	**1 day**	**5 day**	**10 day**	**15 day**	**Spearmint Yoghurt (%)**	**1 day**	**5 day**	**10 day**	**15 day**
0	48	77	82	96	0	107	112	117	112
0.3	53	76	100	116	0.3	112	124	142	144
0.6	54	76	102	116	0.6	117	128	146	148
0.9	57	96	112	118	0.9	123	130	144	155

Table 6. Growth of microbes in the spearmint *L. acidophilus* and *B. bifidum* milk and yoghurt.

Spearmint milk (%)	1 day	5 day	10 day	15 day	Spearmint Yoghurt (%)	1 day	5 day	10 day	15 day
0	4.5×10^{10}	4.75×10^{10}	7.5×10^{10}	8×10^{10}	0	4.25×10^{10}	5×10^{10}	3.75×10^{10}	6.25×10^{10}
0.3	12.5×10^{10}	3.25×10^{10}	3×10^{10}	7×10^{10}	0.3	3.75×10^{10}	5.5×10^{10}	4.5×10^{10}	2.5×10^{10}
0.6	8.5×10^{10}	4.5×10^{10}	9.5×10^{10}	6.25×10^{10}	0.6	4×10^{10}	4.75×10^{10}	6.5×10^{10}	2.25×10^{10}
0.9	12.5×10^{10}	4.5×10^{10}	4×10^{10}	5.75×10^{10}	0.9	2.25×10^{10}	2.5×10^{10}	1.5×10^{10}	2×10^{10}

and the sample containing both bacteria reached the acidity degree of 42°Dornic much earlier than others, which were transferred to a refrigerator and stored at 2°C. The sample with 0.6% spearmint and subsequently the sample with 0.3% and finally the control sample reached 42°Dornic degrees and transferred to the refrigerator.

During the 15 days storage in the refrigerator, the acidity levels in the samples with mint milk 0.9% was higher in the products with either of the bacteria and their compound, compared to the other samples including the control one.

The samples containing probiotic yoghurt were transferred to the incubator at 38°C to let the acidity reach 96°Dornicdegrees. The level of acidity was similar in all samples within the first 2 h, but after 6 h, it suddenly exceeded 90°Dornic. In samples with either *B. bifidum* or *L. acidophilus* and the sample containing both the 0.9% yogurt spearmint sample reached the acidity level earlier than others, and transferred to the refrigerator.

The spearmint milk containing *L. acidophilus* and the one containing both bacteria needed shorter time to reach the desired acidity, compared to the spearmint milk with *B. bifidum* in the incubation. This seems to be due to the weak proteolysis activities in *B. bifidum* along with the insufficient non-protein organic nitrogen in the milk which makes it an improper condition for growth.

Observing the sample with both bacteria and the samples with each separate bacterium and comparing them, revealed that the milk with *B. bifidum* remained unchanged in appearance during storage in the refrigerator, but the other two samples were biphased.

Statistically, *B. bifidum* in comparison with *L. acidophilus* and the other lactic acid bacteria was lower in count, growth and proliferation. This could be attributed to greater sensitivity to oxygen, high acidity, low pH, need for growth complements including organic nitrogen micro molecules and vitamins, and need for potential low reduction (Khosravi and Koshki, 2008; Mortazavian and Sohrabvandi, 2006).

The *L. acidophilus* yogurt was sourer and thicker than the yogurts containing both microbes and the *B. bifidum* yogurt. Of the yogurts with *L. acidophilus*, the control yogurt was found to be thicker.

The number of the probiotic living cells per gr or ml of the products during consumption time, referred to as the product quality, should be 10^7. This is the minimum of biovalue (MBV) index, defined by International Dairy Federation (IDF) (Mortazavian and Sohrabvandi, 2006).

During the storage in the incubation, measuring the acidity and pH revealed that adding higher concentrations of spearmint to the probiotic milk and yogurt enhances the growth of the microbes but no remarkable effect on their counts is evident.

Although, the basic feature of the probiotic products consumption is their medicinal effects (biovalue), their associated sensory properties are also important. In other words, sensory properties rather than medicinal effects play the most important role in their daily consumptions. Among the probiotic products, fermented ones especially the probiotic yoghurt is popular worldwide for its unique sensory properties (Mortazavian and Sohrabvandi, 2006). In the present study, the sensory properties investigated in all the samples including the controls, were aroma, scent, color, thickness and taste. Non parametric methods were used for that purpose. There were significant differences among the spearmint yoghurts with *L. acidophilus* in terms of such sensory properties ($P< 0.05$). Increased spearmint concentration did not cause favorable properties in these yoghurts, and the samples with spearmint 0.3% and the control were with the best taste, aroma, color, and thickness. There were significant differences between the control 0 and 0.3% spearmint yoghurt with *B. bifidum* on the one side and the yoghurts with 0.6 and 0.9% spearmint concentration with the same bacterium on the other side in terms of sensory properties ($P<0.05$). The spearmint yoghurts with the bacteria compound were significantly different in taste, color and flavor ($P<0.05$), but no significant difference was detected between them in thickness. A significant difference was also observed between the spearmint yoghurt with *B. bifidum* and the sample with *L. acidophilus* in terms of thickness, and the increased concentration of spearmint caused the decease of thickness in both.

The bacterial growth in the yogurt with *B. bifidum* was slower than that in the products with *L. acidophilus* and tasted less sour with longer shelf life. This may be due to the low proteocaft activity of *B. bifidum* bacterium. However, when the two bacteria are present in the same product, the proteocaft activity is enhanced (Mortazavian and Sohrabvandi, 2006).

As the investigation of shelf life for the products over a 20-day period revealed, the shelf life was found to be 15 days. The probiotic yoghurts, especially 0.9% spearmint one, were tasted full fat, although low fat milk was used in them.

The results show that the starter bacteria count in the milk and yoghurt with *L. acidophilus* within a 15 day period was increasing while the respective count for the milk containing *B. bifidum* and the milk and yoghurt containing the compound of both bacteria was decreasing over the same period.

The high acidity and low pH in the probiotic fermented products is one of the most important factors responsible for the decreased survival of the probiotics. Therefore, the survival capability of these bacteria in the products such as non-sour milk is at least ten times greater than that in the fermented ones and *B. bifidum* enjoys longer survival in the ice cream and yoghurt than in the milk (Mortazavian and Sohrabvandi, 2006).

One way to avoid the probiotic decrease due to the increased acidity and decreased pH is to end the fermentation stage at pH level above the range of 4.7-4.9, because the final pH of the product in such a condition during storage period reaches about 4.5. It is suggested that the final pH during storage not be lower than 4.5. Although, the termination of fermentation in higher pH promotes the survival of the probiotics, the taste and texture of the products would not be favorable (Mortazavian and Sohrabvandi, 2006).

The refrigerator storage temperature directly affects the bioactivities of the probiotics through the survival of the cells in the products and indirectly affects them through the production of antimicrobial and the bio relationship created between the probiotics and the culture bacteria during the shelf life period.

It was late 1950s, that Shahani embarked on investigating the nutritional effects of *L. acidophilus*, *B. bifidum* and other lactic acid producing microorganisms on humans. He and his associates demonstrated that a particular species *of L. acidophilus*, called DDS-1, can greatly help digestion, health and recovery from illnesses.

Kaila and Rybka (1997) proposed that probiotics should be introduced into the food products prior to or simultaneously with the starter. This is indicative of fast growth probiotics during the initial stages of incubation because the yoghurt starter is of fast growth rate exceeding that of *L. acidophilus*. The otherwise condition happens if *L. acidophilus* is adapted to the condition during the first passage and has sufficient growth (Kaila and Rybka, 1997).

The results of the studies addressing the probiotic bacteria have demonstrated the following: The increased concentration of malt and soya caused increase in the microorganism growth and rising acidity level which in turn resulted in shorter incubation time for the desired acidity (Marhamatizade and Farokhi, 2008; Marhamatizade et al., 2009).

In a study on the effects of soya powder on the growth of the bacteria, *L. acidophilus* and *B. bifidum*, in probiotic products, it was demonstrated that the shelf life for the acidity reaching the desired level during incubation decreased for the milk with both bacteria and combined soya and malt, compared to the milk with only soya. As for the yoghurt with both bacteria, the same results yielded and incubation time for the yoghurt with malt and soya decreased (Marhamatizade et al., 2009).

The effect of honey on the growth of the above-mentioned bacteria introduced simultaneously into dairy products and drinks was investigated, and the results indicated that the yoghurt with only *L. acidophilus* tasted more sour than the yoghurt with both bacteria. The products containing *B. bifidum*, compared to those with *L. acidophilus*, were with slower growth rate and also tasted less sour and were of longer permanence. They were not of favorable taste when honey concentration increased and the control was of the best taste among all the samples (Marhamatizadeh and Kazeroonian, 2009; Marhamatizadeh and Mahmodi, 2009; Marhamatizade et al., 2010).

A study investigating the effect of thyme on the activities of the two bacteria, *L. acidophilus* and *B. bifidum*, in probiotic milk and yoghurt showed that the increased thyme concentration enhanced the growth of both bacteria in such products (Marhamatizade and Abbasi, 2009).

In another study addressing the effect of cinnamon on the bacterial growth, it was demonstrated that the increased cinnamon concentration promoted the growth of the bacteria in probiotic milk and yoghurt (Marhamatizade and Yaghtin, 2009).

Taking into account the results of the above-mentioned studies investigating the effects of malt, soya, honey, thyme and mint on the growth of *L. acidophilus* and *B. bifidum*, we can conclude that they all enhance the bacterial growth in dairy products, either when the bacteria are introduced separately or in combination into the products. Comparatively, spearmint proved to be more effective than thyme, cinnamon and honey and the low bacterial growth in the case of thyme and cinnamon might be due to their antibacterial and bactericidal activities.

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