Effect of Yogurt Treated with Probiotics and *Chlorella Vulgaris* on Levels of Liver Functional Enzymes in Dosed Rats

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Abstract

The study included culturing Chlorella vulgaris for 30 days, extracting and drying it for use with yogurt starter and Bifidobacteria + Lactobacillus for producing yogurt and studying C. vulgaris effect on stimulating the growth of probiotic bacteria. The results showed that the addition of C. vulgaris powder at a concentration of 0.6% w/v led to significant effects in stimulating the growth of the probiotics (Bifidobacteria + Lactobacillus) and increasing the number of cells with significant differences compared to the treatment of yogurt with bacteria in the absence of C. vulgaris. In general, the highest number of CFU for the growth of bacterial cells was found in A6 treatment, full fat milk treated with yogurt starter, Lactobacillus, Bifidobacteria and alga C. vulgaris powder. For testing the effect of treated yogurt on rats liver functional enzymes, the treatments were feeding on standard diet (C-), high-fat diet HFD only as a positive control (C+), HFD + yogurt (T1), HFD + Lactobacillus treated yoghurt (T2), HFD + Bifidobacteria treated yoghurt (T3), HFD + C. vulgaris treated Yogurt (T4), HFD + Lactobacillus+ C. vulgaris treated Yogurt (T5), HFD + Bifidobacteria/C. vulgarisYogurt (T6), and HFD + Lactobacillus/Bifidobacteria/C. vulgaris treated Yogurt (T7). The results showed that there was a significant decrease in the level of AST, ALT and ALP enzymes in all treatments dosed with yogurt added to the probiotic without or with C. vulgaris, compared to the positive control treatment (high-fat diet only), which recorded the highest enzymes levels. All the functional liver enzymes were significantly decreased in rats dosed with yogurt treated with probiotics bacteria and C. vulgaris (T5, T6, T7) compared to the positive and the negative control treatments. It was noticed that the level of ALT and ALP enzymes decreased and returned to the normal state due to the probiotics bacteria and C. vulgaris compared to the positive control, which recorded the highest increase in the level of both ALT and ALP.

Keywords: yogurt, probiotic, C. vulgaris

1. Introduction

Chlorella vulgaris is a type of blue-green algae that is a source of beneficial nutrients such as carotenoids, for example - β-carotene, lutein, and zeaxanthin, as well as pigments, proteins, polyunsaturated fatty acids, and vitamins (K), E, B12, (A, [11]. A probiotic or probiotic is an oral nutritional supplement or food product that contains a sufficient number of viable microorganisms that have the ability to alter the intestinal flora and bring about beneficial healthy changes in the host when taken in adequate amounts [2], AL Hamid [1] indicated that the process of mixing probiotics with prebiotics in symbiotic Consisting primarily of prebiotics and probiotics, it is defined as a type of dietary intervention approach to target the gut microbiota that is now gaining increasing attention. [10]foods has beneficial effects on consumer health by introducing indigestible food with beneficial microorganisms into the gastrointestinal tract that ferment it and produce a suitable environment and beneficial compounds. And bring about positive effects by improving the balance of the intestinal flora in the digestive system of the host. The effectiveness of the probiotics is enhanced by incorporating the prebiotics with Chlorella vulgaris in the food matrix to produce compounds that stimulate the growth and function of the probiotics. The biomass of microalgae generally contains 8-17% carbohydrates, and they represent the main derivative products that have the main role in stimulating the probiotics, which are the polysaccharides. These polysaccharides have been shown to selectively stimulate the growth and activity of bacteria primarily from the genera Lactobacillus and Bifidobacterium, which contributes to improving the health of the host [11,4]. Mixing C. vulgaris with probiotic strains in nutritional supplements or functional foods will allow the creation of a new segment of health-promoting food products. Therefore, the study objectives were culturing Chlorella vulgaris, and using its extract to stimulate yogurt starter and Bifidobacteria + Lactobacillus growth of probiotic bacteria and testing the effect of treated yogurt with Chlorella vulgaris and probiotics bacteria on rat's liver functional enzymes.

2. Materials and Methods

Culture and development of algae

A pure isolate of C. vulgaris was obtained from the microbiology laboratory-College of Sciences at the University of Kufa. The alga was developed using

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BG11 medium, as 5 liters of BG11 medium were taken and 150 ml of C. vulgaris algae were added and placed in a glass beaker on the MAGNETIG STIRRER device for mixing and preventing clumping at the bottom and keep the alga in the middle for 25-30 days to full growth at 25 C under constant light.The algae were separated after the end of the culture period, using filter paper of 15 cm diameter and the Pump Vacuum Stage device. Then it was dried at room temperature for 24 hours [13-14].

Starters and probiotic bacteria

A frozen yogurt starter containing Lactobacillus delbrueckii Subsp bulgaricus and thermophilus Streptococcus (Italian company SACCO) was used in a ratio of 1:1.

Lactobacillus probiotic bacteria and BB-12 were obtained from Al-Ameen Center for Advanced Research and Biotechnology. The bacteria were used after the starter was activated for three times on MAS Broth medium. They were incubated at 42°C and at 37°C for the yogurt starter and the probiotic, respectively, until the working culture Daily starter was reached [3].

Processing the functional yogurt

Yogurt treatments containing probiotic bacteria were prepared with C. vulgaris powder added to it, at a concentration of (0.6%) after reconstitution using 100 ml of liquid milk with 7 treatments and three replicates for each treatment. Whole milk powder was used and milk samples were heat treated at 85 °C for 15 minutes. After cooling the milk to the fermentation temperature, the milk was divided into 7 groups to conduct the treatments. The treated groups were only whole milk with 1ml of yogurt starter (G-), whole milk with 1ml of yogurt starter to be treated with 1 ml of Lactobacillus (A1), or 1 ml Bifidobacteria (A2), 1ml Chlorella vulgaris (A3), or 1ml Bifidobacteria +1ml C. vulgaris (A4), or 1ml Lactobacillus +1ml chlorella vulgaris (A5), or 1ml of each Lactobacillus/ Bifidobacteria/C. vulgaris (A6).

Samples of each treatment were mixed well using a food processor to ensure even mixing. Sampels were packed in 150 ml plastic containers under sterile conditions and incubated at 40 °C until the pH reached 5.4 ± 0.02. At the end of the fermentation process, the samples were cooled and kept in the refrigerator at 5±1 °C for 30 days. Then, the numbers of microorganisms were determined and calculated after 24 hours of incubation (primary counting) and after seven days (secondary counting) after modification [6].

Estimation of bacteria numbers

The total number of starter bacteria was estimated according to the method described [7]. Dilutions of graduated yogurt samples were prepared using a solution of 0.1% sterile peptone water, and the pouring method was used on MRS Agar media. The dishes were incubated under anaerobic conditions at a temperature of 37°C for 24 hours, and the number of growing colonies was counted using a colony counting device.

Estimation of the numbers of *Lactobacillus* bacteria

The numbers of Lactobacillus bacteria were calculated during fermentation periods (1-7) days, and the decantation method was used to count the bacteria. This was done by taking 1 ml of yogurt and adding it to 9 ml of peptone water. After homogenization, 1 ml of the mixture was transferred to make a series of dilutions until the dilution was reached. Transfer 1 ml of the appropriate dilution to a sterile petri dish.Then an amount of MRS Agar medium was added, then the plate was moved horizontally in all directions until the contents were homogeneous, and left to solidify under sterile conditions and placed in an anaerobic incubation cylinder. 30-300 with appropriate dilution using a colony counting device [8]

Estimation of the number of Bif bacteria. Lactis BB-12

The method described by [8] was used to estimate the number of Bif-12 bacteria. lactis BB, as 1 ml of decimal dilutions prepared from bacterial cultures were transferred to sterilized Petri dishes, then MRS agar was poured on them in homogeneous quantities with horizontal stirring to various sides and the dishes were left to solidify. Then, they were incubated at 37°C for 48 hours under anaerobic conditions. After the end of the incubation period, the dishes in which the number of colonies ranged between 30-300 were counted with the appropriate dilution using a colony counting device.

Nutritional experiment

In this experiment, 54 adult male rats (Rattus norvegicus) with weights between (100 ± 130) gm, at the age of 4 weeks, obtained from the animal house / University of Tikrit, were used in this experiment. The rats were then randomly divided into 9 groups, with 6 rats per group, placed in plastic cages with a metal clip under controlled conditions of temperature (25 ± 2) C °, humidity (40-50%), and lighting 12:12 hours light/darkness. Rats were left for 7 days to adapt to free access to food and water. The condition of the rats was monitored throughout the experiment period to keep them healthy until the end of the experiment.

The experimental rats were divided in to 9 groups according to the treatments, where the treatments included feeding rats on standard diet (Table1) as a negative control (C-), high-fat diet HFD only as a positive control (C+) (Table1), HFD/1ml plain yogurt (T1), HFD/Lactobacillus treated yoghurt (T2), HFD/Bifidobacteria treated yoghurt (T3), HFD/C. vulgaris treated Yogurt (T4), HFD/Lactobacillus+ C. vulgaris treated Yogurt (T5), HFD/Bifidobacteria+C. vulgaris treated yogurt (T6), and HFD/Lactobacillus+Bifidobacteria+C. vulgaris treated yogurt (T7).

Estimation of liver functional enzymes (AST, ALT, and ALP) activity

The colorimetric method developed by the Swiss company AGAPPE was followed in estimating the activity of the two liver enzymes, Aspartate Amino Transferase (AST) and Alanine Amino Transferase (ALT). 1ooo microliter of the reagent and 100 microliters of the sample were mixed well, and the mixture was incubated for 1 minute at 37 °C, after which the absorbance was measured at 340 nm during 3 minutes. The activity of the two enzymes (AST) and (ALT) was calculated in international unit / liter (IU / L). According to the following law:

Enzyme activity (unit / liter) = absorption rate / number of minutes x 1745

Estimation of alkaline phosphatase ALP enzyme level

$$\text{para-nitro phenyl phosphate} + H2 \xrightarrow{ALP} \text{para-nitro phenyl} + \text{inorganic phosphate ALP} = \text{Alkaline phosphatase}$$

The same method was used following the Swiss company (AGAPPE) instructions. The basic principle is the reaction of para-nitro phenyl phosphate with water in the presence of the enzyme alkaline phosphatase. It will produce para-nitro phenyl and inorganic phosphate with the aid of the enzyme alkaline phosphatase:

Table2. Ingredients and their proportions (g/100 g) in the standard diet and high-fat diet (HFD) used to feed the rats used in the experiment

Ingredients (g/100g)	Standard diet	High-fat diet
	%	%
carbohydrate	25	25
Protein	65	65
Antioxidants	2	2
Fats	4	4
Pure Cholesterol	-	3
Animal fats	-	3
Powder milk	-	3

The experiment lasted for 5 weeks, including one week, the experimental animal adoption period [1]. After that, the experiment was terminated, data and measurements were taken, and observations were recorded.

Statistical analysis

The statistical analysis program 12.1. GenStat, 2009 (GenStat V) was used to analyze the data at probability level of 5%. Differences among treatments means were determined according to Duncan's multiple range tests ($P \leq 0.05$).

3. Results and Discussion

Effect of Chlorella vulgaris on the growth of probiotic bacteria

We note in Table (3), we find that the addition of algae to the yogurt led to significant increases in the total number of bacteria, where measured by coefficients (G- and A1), (A2) and the two periods of bacterial counting (first and final), which were recorded during the first counting period (7.41, 7.42, 7.42). and 7.32 CFU, respectively, and it was recorded in the final count (7.64, 7.74 and 7.78) CFU, respectively. We also find that there is a significant increase in the mixed addition of *Lactobacillus* and *Bifidobacteria* with *C.vulgaris* algae in the number of total bacteria in the first and final counts, compared to the coefficients of adding algae alone, A3, or with one of the two types of bacteria, A4, A5, and A6, which were recorded during the first count (7.82, 8.54, 8.42 and 8.57) CFU, respectively, and in the final count it recorded (8.85, 8.95, 8.52 and 9.25) CFU, respectively. The results also showed that there were no significant differences in the total number of bacteria in the groups added to the probiotic A1 and A2 bacteria, and the control treatment, G-, for the two counting periods. This shows the superiority of all treatments to which *C. vulgaris* was added, compared with the negative control treatment-G, which recorded 7.64 CFU. Where treatment A6 excelled over the rest of the treatments as it gave 9.25 CFU.

Table3. Logarithm of the bacterial count of probiotic microorganisms in the different treatments at the beginning and end of fermentation (CFU).

Treatments	First count	Final count
G-	7.42 a	7.64 a
A1	7.41 a	7.74 a
A2	7.32a	7.78 a
A3	7.82 a	8.85 bc
A4	8.54 b	8.95 b
A5	8.42 b	8.52 b
A6		9.25c

* Different letters within the same column indicate a significant difference among the means according to Duncan's multiple range tests (P≤0.05); treatments are: only whole milk with 1ml of yogurt starter (G-), whole milk with 1ml of yogurt starter to be treated with 1 ml of Lactobacillus (A1), or 1 ml Bifidobacteria (A2), 1ml Chlorella vulgaris (A3), or 1ml Bifidobacteria +1ml C. vulgaris (A4), or 1ml Lactobacillus +1ml chlorella vulgaris (A5), or 1ml of each Lactobacillus/ Bifidobacteria/C. vulgaris (A6)

The difference in the number of probiotic bacteria may be due to the fact that the dried biomass of microalgae generally contains 8-17% carbohydrates, which represent the main products derived from photosynthesis and carbon fixation metabolism. These oligosaccharides have been shown to selectively stimulate the growth and activity of bacteria primarily from the genera Lactobacillus and Bifidobacterium, in stimulating the growth of probiotic bacteria and thereby contributing to the improvement of host health [9,10]. However, the chemical profile and metabolic pathways of carbohydrates differ between species and the composition and properties of the cell wall also depend on the growth stage and the moss cultivation conditions [11,12]. In particular the polysaccharide profile of C. vulgaris cell wall contains rhamnose (45-54%) in combination with lactose (14-26%), xylose, mannose, arabinose and glucose. The most important complex carbohydrate is β-1,3-glucan, a branched polysaccharide comprising β-D-glucose units and is well fermentable in the colon. β-1,3-glucan is a water-soluble polysaccharide that has a positive effect on the activities of digestion and immune regulation [13,14].

After adding Bifidobacteria to yogurt, a significant effect on prebiotics were evaluated based on cell count. Where comparisons were made with the control treatment at a significant level (0.05), and similar conclusions were obtained in the previous study that evaluated the effects of prebiotic growth or positive Chlorella vulgaris [11].Behestipour et al [6] reported that the addition of 0.5 or 1.0% (w/v) of C. vulgaris and Arthrospira platensis in yogurt fortified with probiotics for Lactobacillus acidophilus and Bifidobacterium animalis subsp. fermentation compared to a controlled yoghurt.An improved viability of Bifidobacterium longum and Lbc was supported by a study by Cantú-Bernal [15]. plantarum in dairy products with the addition of Chlorella sorokianiana (in addition, the results indicated an antiviral effect of cells against rotavirus after treatment with probiotics and microalgae, The positive effects of microalgae on probiotic survival may be related to the fact that it supports lactic acid bacteria and probiotic bacteria, provides nutrient-rich and stimulating substances, and encourages their growth and activity. These include exopolysaccharides, adenine, hypoxanthine, free amino acids, and essential vitamins and minerals [8].

Effect of oral dosing using yogurt supplemented with probiotics and alga *Chlorella vulgaris* on rat's liver functional enzymes

The results (Table 4) showed that there was a significant decrease in the level of AST enzyme in all treatments dosed with yogurt added to the probiotic without or with seaweed, compared with the positive control treatment (high-fat diet only), which recorded the highest level of 231.3. Where the two treatments 4T and T7 recorded the least value levels among all the treatments, 171 u/l and 174.8u/l, respectively. The level of ALT enzyme increased in the positive control C+ treatment fed on a high-fat diet compared to the negative control treatment C- standard diet. A dose of yogurt with probiotic bacteria T2 and T3 recorded a decrease in the level of ALT enzyme compared to the C + positive control treatment.

Table4.Effect of different treatments of oral dosing using yogurt supplemented with probiotics and alga *Chlorella vulgaris* on rat's liver functional enzymes (30 DPT)

Treatments	AST(U/L)	ALT(U/L)	ALP(U/L)
C-	167.6 a	20.22 a	383.1 a
C+	231.3 b	42.55 b	572.5 b
T1	178.2 a	23.01 a	408.7 a
T2	164.4 a	23.7 a	410.8 a
T3	165 a	23.65 a	409.2 a
T4	171 a	22.97 a	376.4 a
T5	149.8 a	14.97 a	366.6 a
T6	151.4 a	19.05 a	a350.2
T7	174.8 a	15.79 a	a357.6

*values are means of 6 replications, means followed by different letter(s) are significantly different according to Duncan's multiple rage tests (P≤0.05). Treatments are: feeding rats on standard diet as a negative control (C-), high-fat diet HFD only as a positive control (C+), HFD/1ml yogurt (T1), HFD/Lactobacillus treated yoghurt (T2), HFD/Bifidobacteria treated yoghurt (T3), HFD/C. vulgaris treated Yogurt (T4), HFD/Lactobacillus+ C. vulgaris treated Yogurt (T5), HFD/Bifidobacteria+C. vulgaris treated yogurt (T6), and HFD/Lactobacillus+Bifidobacteria+C. vulgaris treated yogurt (T7).

A significant decrease in the level of ALT enzyme in the treatments dosed with yogurt with probiotic bacteria and algae (T5, T6, T7) compared to the positive control treatment.

From the results, it was noticed that the level of ALT and ALP enzymes decreased and returned to the normal state compared to the positive control, which recorded the highest increase in the level of both ALT and ALP compared to the negative control, while the T5 treatment recorded the highest decrease in the level of both enzymes compared to all treatments. The estimates of liver enzymes are considered one of the most important indicators of liver and other organs health. The decrease recorded in the concentrations of enzymes during the experiment indicates the positive effects shown by the yogurt treated with probiotic bacteria, and the yogurt treated with probiotic bacteria and algae. The results are consistent with the results of a previous study [11] in which rats fed diets containing 0.5% (w/v) fermented colostrum supplemented with 1.0% (w/v) Chlorella vulgaris powder showed a significant decrease in ALT and ALT levels. AST levels, compared to the control group levels.

Similar results were found in the study of Al-Hamdani (2019) [8] in the occurrence of a decrease in liver enzymes due to the effect of the types of biocatalysts, and the enzymes maintained normal levels. It was also found that the use of probiotics bacteria Le paracasei

TD3 in doses of male rats (Wistar rats) with hypercholesterolemia led to a decrease in the levels of liver enzymes in the blood serum of treated animals [16]. The results of the current study did not differ from the results of the study by Ghazal (2020) [13] in recording a decrease in the levels of liver enzymes ALT and AST in the blood serum of mice that were fed with synergistic cheese.

4. Conclusion

Based on the findings of this study it can be concluded that the addition of *C. vulgaris* powder had positive effects in stimulating the growth of the probiotics bacteria (*Bifidobacteria* + *Lactobacillus*) and increased the number of cells CFU over the untreated control. also gave better results compared to the treatment of yogurt with bacteria in the absence of *C. vulgaris*. Positive effects were also detected on liver functional enzymes AST, ALT, and ALP which were clearly decreased in rats dosed with yogurt supplemented with probiotics bacteria, especially in combination with the alga *C. vulgaris*.

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