

Probiotic Effect of Lebanese Darfiyeh Cheese: A Randomized Case Control Prospective Study in the Elderly

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Received 12 July 2015; accepted 4 September 2015; published 7 September 2015

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Abstract

A randomized case control prospective method was adopted in this study in order to assess the health benefit associated with the Darfiyeh cheese, a traditional Lebanese raw goat milk cheese ripened in goat skin. 21 participants were recruited and divided into 2 groups: test group ($n = 10$) and control group ($n = 11$). Venous blood and stools were collected at day 14, 21 and day 0 and 21 respectively. Flow cytometry was performed on blood sample. Fecal samples were plated on Plate Count Agar (PCA) and de Man, Rogosa and Sharpe Agar (MRS) agar in order to monitor any alteration in the gastrointestinal tract (GIT). At day 24, a significant increase by 27.67% ($p = 0.043$) in the bacterial count was seen on the MRS agar for the test group in comparison to no change in the total bacterial count. At day 14 a significant drop ($p = 0.026$) in CD3+/CD4+ population in the test group (35.67 vs 42.87), and a non-significant drop ($p = 0.818$) in CD8+ (24.76 vs 28.28) is seen when compared to control group. At day 24, no significant change ($p = 0.20$) in the CD3+/CD4+ population in comparison between both groups and no change in CD8+ or Geo means in both groups. In conclusion, an alteration in the GIT microflora could be hinted at by the lactobacilli bacterium, which was not reflected on the total bacterial count.

Keywords

Lactococcus lactis Subsp. *Lactis*, *Lactobacillus plantarum*, Darfiyeh Cheese, Probiotic Effect, Elderly

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1. Introduction

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) working group described that probiotics must have live microorganisms and that they should confer a measured physiological health benefit, as well as possessing well-being effects in the human host [1]. Probiotics are widely used as food additives because they have the ability of fermentation, which can affect the taste of food [2]-[4]. In addition, many probiotic strains such as Lactic Acid Bacteria (LAB) are used in studies as vaccine vehicles, as microbial interference treatments or as adjuvant for antibiotics [1].

LAB are physiologically present in the human digestive tract [5]. They have evolved symbiotically with the digestive tract [1] [6]. These bacterial strains are able to ferment nutrients into lactic acid or other byproducts [1] [5] [6]. LAB also have the ability to lower the pH in the GIT and female genital tract, which is a healthy and beneficial phenomenon since low pH implement an antibacterial effect [7] [8].

Raw goat milk, which is best known for its indigenous microflora is highly linked to probiotic effects in the human body [9]. The microflora mostly consisting of LAB allows this specific type of milk to have beneficial and valuable health impact on the consumer. Raw goat milk has served for many decades in the manufacturing of Darfiyeh, a cheese ripened in goat skin [10]. A highly developed and diverse ecosystem is found in this cheese: bacteria, molds and yeasts [11]. The bacteria present in this cheese are *Streptococcus thermophilus*, *Enterococcus faecium*, *Enterococcus faecalis*, *Lactobacillus plantarum*, *Staphylococcus haemolyticus*, *Escherichia coli* and *Clostridium* spp.

It has been shown that *Lactococcus lactis* subsp. *lactis* does not possess any invasive capacity, thus it has shown no bacterial translocation after the admission of 109 CFU of viable *Lactococcus lactis* subsp. *lactis* in human at the mesenteric lymph node sites [6].

Lactobacillus plantarum does not possess any infective capacity and is not able to cross the intestinal barrier, and therefore does not have the capacity to cause bacteremia [6] [12] [13]. In addition, *Lactobacillus plantarum* has been shown to possess an antibacterial activity as resultant of secretion of bacteriocins against common pathogens [14]. Moreover, this bacterium has the ability to enhance the systematic immunity by increasing the number of B lymphocytes, natural killer cells and the activation of CD4+ and CD8+ cells [15] [16]. In accordance to the FAO and the WHO guidelines, probiotic organisms, which are used in food, must have the ability to stay alive in the gastric juices and they should be resistant to the exposure to the bile in the human gut. In addition, they must have the ability to flourish and colonize the digestive tract of humans [17].

Among the possible mechanisms of probiotic therapy is promotion of a nonimmunologic gut defense barrier, which includes the normalization of increased intestinal permeability and altered gut microflora. Another possible mechanism of probiotic therapy is improvement of the intestine's immunologic barrier [18]. LAB have been shown to enhance the proliferation of Th and Treg cells. More specifically, *Lactobacillus plantarum* enhances the proliferation of CD3 and CD4, which are markers for Th and Treg cells [19] [20]. Few studies assessing the potential health benefit of each of the two-bacterium *Lactococcus lactis* sub species *lactis* and *Lactobacillus plantarum* have been done [16] [21]. No study was done combining both bacteria and testing their effects on human health. To evaluate the role of the two-bacterium, a randomized case control prospective method was adopted in the study in order to assess their health benefit associated with the intake of Darfiyeh in Lebanese elderly.

2. Methods

2.1. Darfiyeh Cheese Samples

Experimental cheeses were processed at small cheese scale farming in North Lebanon Mountains, and ripened in goatskins [10]. After 21 days of ripening, the goatskin was opened through the neck. Representative cheeses were selected randomly and taken out for microbiological analyses. Fractions of whole cheese samples (20 g interior and surface) were homogenized in 180 mL of sterile 2% (w/v) tri-sodium citrate dehydrate solution serially diluted (10^{-1} - 10^{-7}) in a sterile physiological solution and plated onto different media (Salmonella-Shigella Agar (SS), MacConkey Agar, and Mannitol Salt Agar (MSA)). The absence of growth on the aforementioned media reflects in order the absence of *Salmonella* spp., *Shigella* spp., potential pathogenic gram-negative bacteria, and *Staphylococcus*.

2.2. Subjects and Trial Design

This trial was conducted, in winter 2013, in subjects older than 64, institutionalized in the Foyer Saint George in Beirut, where they benefit from a pre-planned diet and a twenty-four hours medical supervision. Exclusion criteria were 1) suffering from lactose intolerance, 2) suffering from metastatic cancer, 3) documented intolerance to milk or dairy products, 3) use of antibiotics, or nutritional supplements in the previous month, 4) intake of any dairy product (yoghurt, etc.) all over the trial period and 5) BMI <16.

21 elderly subjects were randomized. Subjects were divided into 2 groups based on their randomized number (odd number formed the test group ($n = 9$), even number formed the control group ($n = 12$)).

The test group was given 20 g of cheese as described by Mane *et al.* [21]. The cheese was administered every 2 days all over the period of 24 days. The study design is shown in **Figure 1**.

The Body Mass Index (BMI) of each participant was calculated in order to monitor his diet status, which was used as an external variable. A stool culture (dependent method) was also performed in order to monitor the basal colonies forming units (CFU) of each of the two studied strains in the feces of each participant. MRS and plate count agars are used.

2.3. Flow Cytometry

The flow cytometry technique was adopted to quantify the components of the cellular immunity. The following lymphocytes were quantified: Th, Tc and Treg, using specific markers (**Table 1**). The flow cytometry methodology was performed using two different protocols: one for extra cellular markers (CD3, CD4, CD8, CD19) and a second one for intracellular markers (FoxP3) [22].

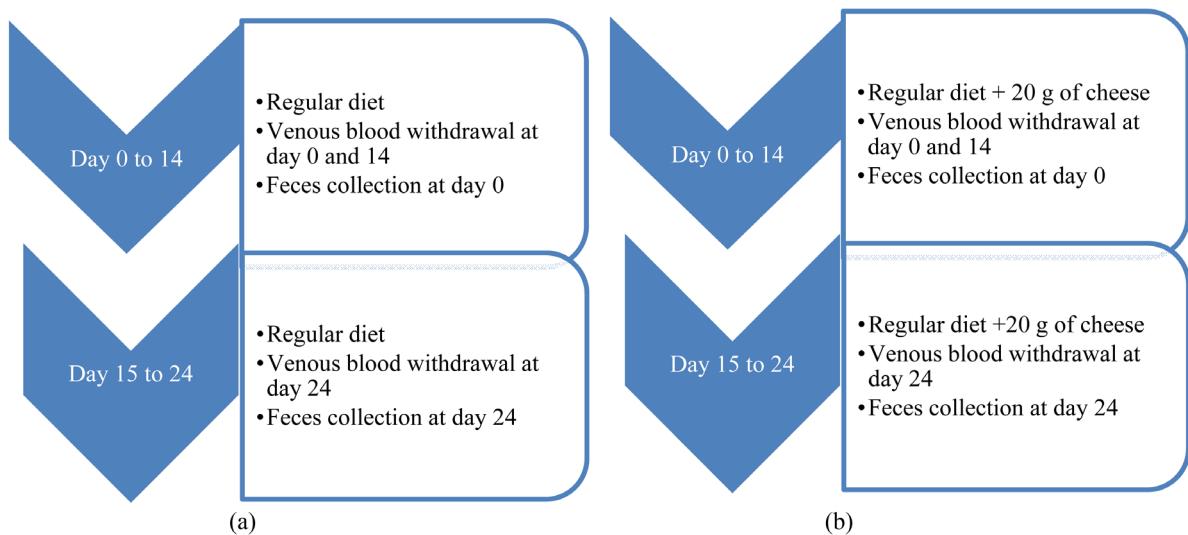


Figure 1. Study design scheme for control (a) ($n = 12$) and test (b) ($n = 9$) groups, during the course of the trial 9 out of 21 participants left the trial. 5 out of 9 left the test group.

Table 1. Association between Th, Tc, Treg cells with their CD markers.

Marker	Th	Tc	Treg
CD3	+	+	+
CD4	+	-	+
CD8	-	+	-
CD25	-	-	+
FoxP3 (intracellular)	-	-	+

2.4. Bacterial Count

Samples of feces (20 mg) were collected and put under sterile conditions in 10 mL of Bactotryptone. A series of dilution was performed and 1mL of each dilution was plated on LAB plate count agar and MRS agar. The plates were kept for 3 days at 30 degree Celsius. Colonies were counted and the results were plotted in parallel for each group.

2.5. Ethical Considerations

This study was given the approval of the Institutional Review Board (IRB) and the Ethical Committee of the University of Balamand and the Saint George Hospital University Medical Center. All subjects gave their informed consent to participate in the study.

2.6. Statistical Analysis

Simple descriptive statistics to describe our studied population was used. Means, standard deviations and medians were used for quantitative data; proportions were used for qualitative data. Mann-Whitney test was used for comparison among the two groups when appropriate; Wilcoxon signed ranked test was also used for the analysis when it was appropriate. All statistical analyses were performed using the SPSS software for windows. p values below 0.05 were considered as significant.

3. Results

At day 0 and 24, the sample age of the participants was carefully calculated and double checked on their medical file. The average age of the test group is 86 years while the average age of the control group is 85 years. Using the medical records of the participants their gender was identified. 35% of the participants are male and 65% are female (data not shown). In addition to their age and gender the weight and height of each participants was also checked in the medical record. The BMI of each participants was carefully calculated using the following formula: weight (kg)/height² (m²). Any participant with a BMI less than 16 was excluded from the study at day 0. The average BMI of all the participants at days 0 and 24 is shown in **Table 2**.

Vomiting, constipation and diarrhea were also monitored before, during and at the end of the trial (data not shown). These data were used to evaluate any physiological effects, which affect the bowel. Data were collected at days 0 and 24. These data refer to any episode of diarrhea or vomiting during a period of 5 days before the date of the data collection. The results show that 5 out of 12 participants showed constipation during and at the end of the trial. 2 out of those 5 belong to the test group. Regarding diarrhea, 1 out of the 12 participants showed to have more than one episode of diarrhea during the past week from the data collection date. This participant belongs to the control group. One participant showed to have one episode of vomiting during the past week from the data collection date. The latter belongs to the control group.

Bacterial count was done on MRS and PCA agars at days 0 and 24. The number of stool samples collected at days 0 and 24, was 14 and 11 samples respectively. The CFU count was made by applying the following formula: $50 \times 20 (\text{mg}) \times 10^{\text{number of dilution}}$. The mean CFU for the MRS agar showed 356×10^6 and 371×10^6 for days 0 and 24 for the control group. The mean CFU for the MRS agar showed 112×10^6 and 143×10^6 for days 0 and 24 for the test group. The mean CFU for the PCA agar showed 1444×10^6 and 1530×10^6 for days 0 and 24 for the control group. The mean CFU for the PCA agar showed 4075×10^6 and 4231×10^6 for days 0 and 24 for the test group (**Table 2**).

Different dyes were used in order to quantify the CDs marker. Results are shown in **Figure 2**. The statistical interpretation of the CD3+/CD4+ population of cells, the CD8+ cells and the CD3+/CD4+ X and Y Geo mean of both groups is shown in **Table 3**.

Table 2. BMI distribution and bacterial count (expressed in $\text{CFU} \times 10^6$) for control test and control groups at days 0 and 24.

	Test group		Control group	
	Day 0	Day 24	Day 0	Day 24
Average BMI	25.3	26.05	26.3	26.8
CFU [*] ($\times 10^6$)-PCA	4075	4231	1444	1530
CFU ($\times 10^6$)-MRS agar	112	143	356	371

Table 3. Flow cytometry data (mean \pm standard deviation) at days 14% and 24% and % change between days 14 and 24.

	Mean \pm SD at day 14	Mean \pm SD at day 24	% change between days 14 and 24
Control group CD3/CD4	42.87 \pm 7.67	36.08 \pm 10.10	-8.60
Control group Others CD3	22.01 \pm 12.17	32.77 \pm 3.96	19.64
Test group CD3/CD4	35.67 \pm 3.61	44.07 \pm 4.73	10.53
Test group Others CD3	30.14 \pm 11.10	25.38 \pm 12.05	-8.57
Control group CD8	28.28 \pm 6.04	30.14 \pm 4.75	3.18
Test group CD8	24.76 \pm 8.70	28.31 \pm 10.32	6.69
Control group CD3/CD4 X Geo	21.11 \pm 0.21	31.45 \pm 1.28	19.67
Control group CD3/CD4 Y Geo	427.37 \pm 33.51	438.75 \pm 87.32	1.31
Test group CD3/CD4 X Geo	20.17 \pm 1.73	29.73 \pm 3.40	19.16
Test group CD3/CD4 Y Geo	418.2 \pm 43.94	434.45 \pm 45.26	1.91
Control group CD8 Y Geo	63.03 \pm 5.78	65.59 \pm 3.05	1.99
Test group CD8 Y Geo	59.5 \pm 3.22	63.36 \pm 2.16	3.14

Note a mean of CD3+/CD4+ of 42.87 and 36.08 at day 14 and 24 for the control group. The mean of CD3+/CD4+ showed 35.67 and 44.07 at day 14 and 24 for the test group. The mean of CD8+ showed 32.71 and 28.35 at day 14 and 24 for the test group. The mean of CD8+ showed 31.96 and 30.14 at day 14 and 24 for the control group. CD3/CD4 X and Y Geo means were also noted.

4. Discussion

It has been documented that probiotic strains are able to upkeep a healthy digestive system and functioning [23]. The Darfiyeh cheese contained two well-documented probiotic strains: *Lactococcus lactis* subsp. *lactis* and *Lactobacillus plantarum* [5] [24]. Participants were under dairy product-free diet. The latter was used to control and avoid overlapping in the results. This overlapping might be due to the fact that dairy products can contain the two bacteria of interest. This overlapping could also result from orange juice, corn flakes and chocolate [24]. These products were never been administered to the participants.

Darfiyeh cheese contains a high concentration of salt (up to 0.5 M), and therefore has a salty taste. This fact had raised the burden of an increased blood pressure. Therefore, the weight of the cheese was accurately monitored to ensure that only 20 grams were administered. The blood pressure was checked regularly. No sign of increase in the blood pressure was noted. No side effects related to the administration of the cheese were observed during the trial period. The percentage of participants with diarrhea at day 24 remained the same in comparison with day 0 in both groups (data not shown). The percentage of constipation and vomiting was also constant in between day 0 and day 24 in both groups. No change more than 5% was noted in the BMI of each participant (**Table 2**).

The data showed in **Table 2** suggests a 4.21% increase in the bacterial count for the control group on MRS agar. This count increased dramatically by 27.67% for the test group on the MRS agar considered to be statistically significant. This difference suggests a baseline increase of the lactobacilli found in the stools for the test group who consumed the Darfiyeh during the past 24 days. This difference is not observed in the control group. On PCA agar 5.95% ($p = 0.177$) and 3.82% ($p = 0.2$) are the level of increase shown on this agar for the control and test group respectively. This increase cannot be considered of significant interest and could not indicate any change in the total count of aerobes in the microflora of the GIT. These counts could suggest that the total count for bacteria in both groups was not affected but the specific count of lactobacilli shows a difference between the two groups [24] [25]. This could be explained by the fact that the lactobacilli found in the cheese was able to reach the GIT and alter the microflora of the GIT of the participants in the test group [5] [18]. Moreover, *Lactococcus lactis* subsp. *lactis* was identified on PCA without being selective for the *Lactococcus lactis* subsp *lactis* [3]. This has created a burden in analyzing the effect of the cheese on the microflora of the GIT [17] [18].

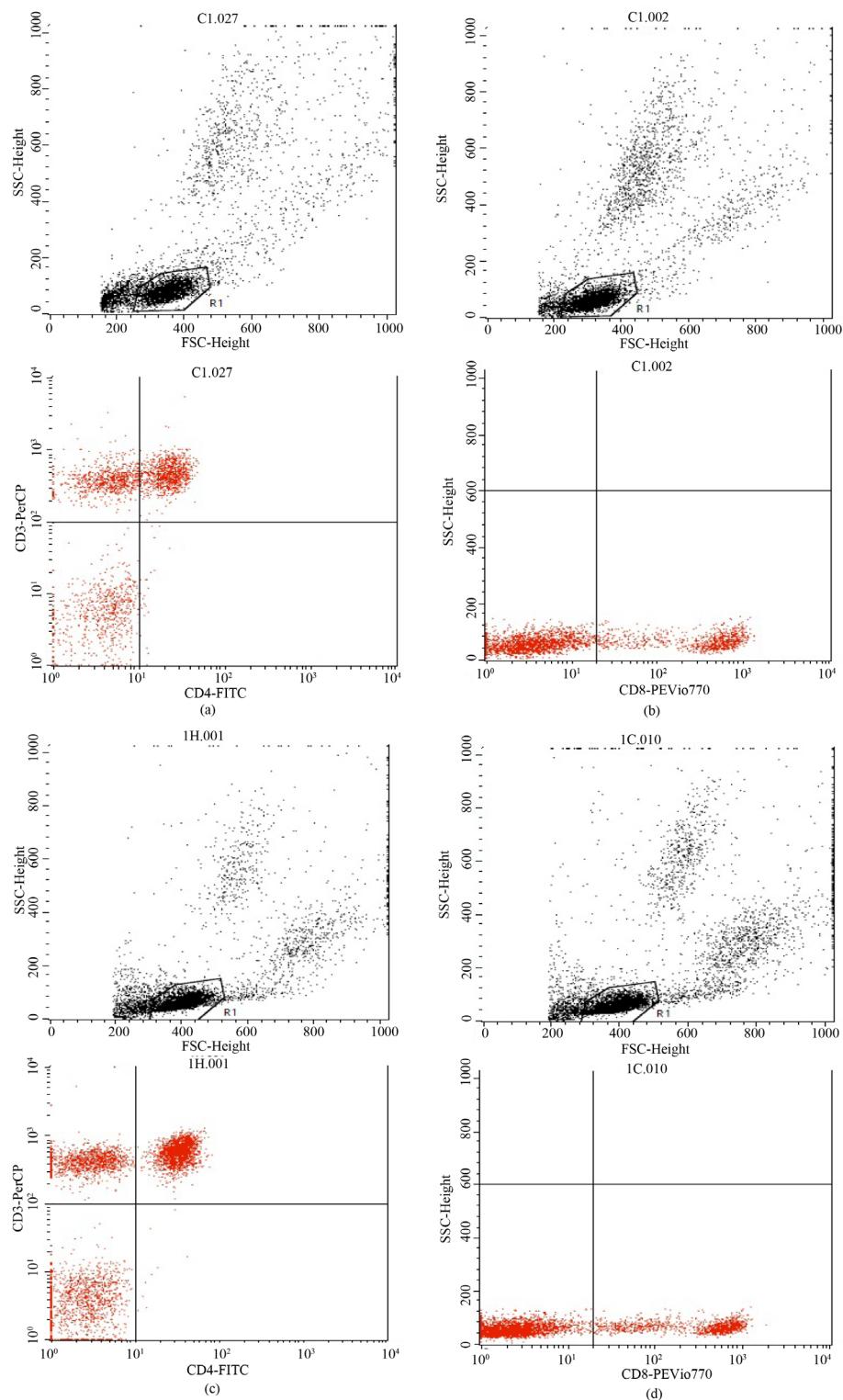


Figure 2. (a) Lymphocyte gate for CD3+/CD4+ cells at day 14: %CD3+ cells are located in the upper left (UL) and upper right (UR) quadrants. %CD4+ cells are located in the UR quadrant; (b) Lymphocyte gate for %CD8+ cells at day 14. %CD8+ cells are located in the lower right (LR) quadrant; (c) Lymphocyte gate for CD3+/CD4+ cells at day 24. %CD3+ cells are located in the upper left (UL) and upper right (UR) quadrants. %CD4+ cells are located in the UR quadrant; (d) Lymphocyte gate for %CD8+ cells at day 24. %CD8+ cells are located in the lower right (LR) quadrant.

In general, inflammation and immunity modifications are critical to advance nearly every complex condition in the human body, thus the need to use “tools” which are able to modulate immunity either by up regulating or by down regulating the immune response [23]. *Lactobacillus plantarum* and *Lactococcus lactis* subsp *lactis* are good “tools”, classified as probiotics, able to create immunomodulation in the GIT. This fact has been shown in this study by a decrease in CD3+/CD4+ population in the test group in comparison to the control group. This decrease is considered to be statistically significant at day 14 ($p = 0.026$). The drop in CD8+ is not statistically significant ($p = 0.818$). Regarding the Geo Means they were statistically not significant; drop in all test groups at day 14 ($p = 0.009$). However, when added all together, it looks like the test group show a significant decrease in Th cells and its level of CD3+ and CD4+, and no significant change was noted regarding the CD8+ cells expression. This could be attributed to a stimulation of the Th cells leading to a cellular response which did not involve the lymphocyte T cytotoxic and could have involved other type of immunity which was not quantified in this study [26]-[28].

At day 24, there is an increase in the CD3+/CD4+ population ($p = 0.2$) in the test group in comparison to the control group (44.07 vs 36.08). No change in CD8+ or Geo means of all (p -values of 0.8181 and 1 respectively). This could be explained by a potential investment of the cells stimulated up to day 14 [28] [29]. This stimulation could have been invested in the stimulation of a humoral immunity, which was not quantified in this study. This humoral immunity could involve the production of interleukin (IL 1-IL10) and the secretion of immunoglobulin (IgG, IgM, IgA) [28] [30]. In other studies, *Lactobacillus plantarum* and *Lactococcus lactis* subsp *lactis* were able to stimulate the cellular and humoral immunity. This has been observed in this study by a down regulation then an up-regulation of the Th cells. In addition, a drop by 8.6% ($p = 0.029$) is seen in the population of CD3+/CD4+ reflecting a drop in the Th cells in the control between day 14 and day 24. A 10.53% increase in the population of Th cells for the test group was noted ($p = 0.029$). This up regulation could suggest a sensitization of the Th cells created by the intake of the Darfiyeh cheese. Regarding the Tc cells, a modest up regulation for the test group is seen in comparison with the control group, suggesting the absence or modest effect on the intake of the cheese on the sensitization process of these cells. An over sensitization of the Th cells could aggravate Th-mediated autoimmune disease such as rheumatoid arthritis. Thus there is a need to quantify the cellular immunity and the production of IL1 and IL10 in future studies [28]. Note that to attribute the down and up regulation in the population of CD3+/CD4+ cells to the *Lactobacillus plantarum* and *Lactococcus lactis* subsp *lactis* solely is not accurate. Thus the need to identify the potential molecules which could be responsible of this stimulation and use them in an in vitro context (cell culture) with non-sensitize lymphocyte (naïve lymphocytes). This sensitization has to be monitored in order to identify a potential sensitization and lymphocytes proliferation which could happen after the stimulation with the potential molecules.

The results suggest that the ratio of the two bacterial strains present in the cheese could modulate an alteration in microflora of the GIT. However this is not enough to play a role in boosting the immune system of elderly people as stated in previous studies [3] [5] [24] [25]. This finding could be linked to the consumption of the Darfiyeh during the trial period leading to the conclusion that the bacterial strains were able to cross and pass all over the GIT [23].

5. Conclusion

Our study was able to investigate an up and down regulation of Th cells which could be related to the probiotic effect of the two bacterial strains. In addition, during the course of the study, we were able to observe an alteration in the microflora of the GIT but no changes were observed regarding the total bacterial count in the feces. This study has set the basics and the standards for future studies. These basics could be summarized by the baseline, which is created by the consumption of the normal Darfiyeh; in addition to the optimization of the methodology used in order to generate results. The small sample size was a limitation in this study. Note that dealing with elderly people was a real advantage and problem at the same time. The advantage was obvious in the quantification of results and making the subject follow the strict diet. The problem was illustrated by their difficult behavior and mis judgment on daily facts which could affect others opinion. A prospective study dealing with elderly people having different immune reactions is recommended to be made on larger scale, among different age groups of Lebanese elderly.

Acknowledgements

The authors acknowledge and thank the Foyer Saint George in Beirut for data collection.

Funding

The study was funded by the University of Balamand.

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