

ORIGINAL PAPER

MICROBIOLOGICAL, CHEMICAL AND SENSORY ANALYZES OF PRODUCED PROBIOTIC YOGHURTS ADDED CLOVE AND PROPOLIS

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ARTICLE INFO	ABSTRACT
<p>Keywords Probiotic yoghurt, probiotic bacteria, propolis, clove</p>	<p>Clove (<i>Syzygium aromaticum</i>) is an aromatic bud has antioxidant, antifungal, antiviral, antimicrobial, antidiabetic, anti-inflammatory, anti-thrombotic, anesthetic, analgesic and insect repellent effects. Propolis is a sticky resinous material with same properties like clove. Therefore, the aim of this study was to produce probiotic yoghurts added propolis (0.03%) and clove in different concentrations (0.1%, 0.3% and 1.0%). Five different probiotic yoghurts were produced, and analyzed for their microbiological, chemical and sensory properties. Microbiological analyzes of probiotic yoghurts were carried out according to IDF and ISO standards. Their chemical and sensory analysis were performed according to Turkish Standards Data were</p>

analyzed by SPSS. Propolis showed antibacterial effect except *S. thermophilus* while clove improve the development of bacteria especially Bifidobacterium. The titratable acidity of all groups was increased while their pH value was decreased. Propolis showed antibacterial effect except *S. thermophilus* while clove improved the development of bacteria especially Bifidobacterium. Clove and propolis together changed the microbiological, chemical and sensory properties of probiotic yoghurts while probiotic yoghurt with propolis got the lowest score according to sensory properties.

INTRODUCTION

The importance of functional foods having an impact on the well-being of the human health is increasing by the consumer (1). According to the definition of the Medical Institute of the US National Academy of Sciences, functional foods are “foods that contain potentially healthy products, including modified foods or food ingredients, that can provide health benefit beyond the nutrients it contains” (2). They help reduce the risk of certain diseases and improve optimal health conditions that have a positive effect on health (1).

Functional foods are usually used to reduce the harmful effects of plants, fruits, leaves, crustaceans and industrial products and to treat diseases (3). These foods also have a positive effect on general health by reducing the risk of diseases such as the risk of cancer, cardiovascular disease, regulating the gastrointestinal tract, reducing menopausal symptoms, maintaining the health of the urinary tract and lowering blood pressure (1,4). It has recently been known worldwide due to these beneficial effects on the health of functional food (5). Probiotics are functional foods because of containing useful biological active compounds (2), and among the functional foods, probiotics are the most popular (4). It was known that probiotics are viable microorganisms ensuring health benefits to the host when administered in sufficient quantities (6). They affect the function of the commensal bacterium and host defenses. The effects of probiotics are provided by various mechanisms such as limiting bacterial translocation, suppressing LPS translocation and reducing visceral sensitivity by affecting immunological responses (7). Probiotics can stimulate immunomodulatory cells which enhance immune function and affect immunological response in the host (7,8). Probiotics can antagonize pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Shigella sonnei*, *Shigella flexneri*, *Campylobacter jejuni* and *Salmonella typhimurium* through the production of antimicrobial and antibacterial compounds (9). They promote lactic acid producing microflora which reduces the pH of the intestines (8). Probiotic bacteria improve the balance of intestinal microbiota and human health by supporting mucosal defenses. Probiotics are useful in the prevention and treatment of diarrhea, reduction of lactose intolerance and supporting immune

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system functions. They are also recommended for the treatment of atopic dermatitis, necrotizing enterocolitis, pseudomembranous colitis, chronic liver disease, allergic diseases and food allergies (10). Human take probiotics from sources such as fermented milk, yogurt, baby food, energy drink, confectionery and chewing gum. Fermented foods as a probiotic product are more available in the market and more demanded (5). The medical activity of probiotic food products are depending on the number of active cells, the total number of live cells per mL/L or per gram of the food (4). Probiotic foods must be contain at least 1.0×10^6 cfu/g probiotic microorganism (11).

Yogurt as the main source of probiotics among the fermented products, has an important role in supporting and improving health. The most favorable effects of yogurt on health are the decreasing lactose intolerance, promoting weight management, strengthening the immune system and preventing from some diseases (12). When probiotic cultures are added into normal yogurt, the functional properties and contribution to health of food increase. Therefore, regular consumption of probiotic yogurt is recommended for a healthy lifestyle (13). Studies have shown that probiotic yogurt can help to reduce the risk factors for cardiovascular disease in type 2 diabetics by lowering total and LDL cholesterol concentrations (14).

Propolis is collected by the bees from plant extracts which contains at least 200 chemical constituents (15). It is a sticky resinous material with a properties of antibacterial, antifungal, antiviral, cytotoxic, antioxidant, antiinflammatory, immunomodulator effects (16). A study reported that Egyptian propolis (10 and 25%) mixed yogurt may reduce the serum cholesterol and glucose levels in rats (15). Clove (*Syzygium aromaticum*) is an aromatic bud obtained from a tree of the *Myrtaceae* family (17). It has been used to treat or prevent many diseases (18). Its properties have been reported in studies such as antioxidant, antifungal, antiviral, antimicrobial, antidiabetic, anti-inflammatory, anti-thrombotic, anesthetic, analgesic and insect repellent (19). Recently, it was reported that clove has positive effect on bacterial growth in a dose dependent manner (20). There is no research on the effect of propolis and clove on yogurt bacteria together.

To our knowledge, this is the first study aimed to develop the probiotic yogurt enriched with clove and propolis at different concentrations together. Therefore, the produced yogurts were investigated for their microbiological, chemical and sensory properties.

MATERIALS AND METHODS

Materials

The starter culture for preparation of probiotic yoghurt used in this study was obtained commercially from Maysa Gıda, Adana, Turkey. Pasteurized milk was obtained from the supermarket (Turkey). According to manufacturer's instructions, each 10 mL of water based propolis solution used in the study contains 300 mg Brazilian propolis and was provided by the

Sepe Natural Organic Products (Turkey). The peptone used in microbiological analysis is commercially supplied in powder form from Merck (Turkey). Distilled water was produced in our department laboratory in Bezmialem Vakıf University. MRS, MRS-CC, M17 and TOS-MUP agars were obtained commercially from Merck (Turkey). MRS, MRS-CC, M17, TOS-MUP agars were used for these bacteria respectively, *L. bulgaricus* subsp. *delbrueckii*, *L. acidophilus*, *Sc. thermophilus* and *B. animalis* subsp. *lactis*.

Clove and its content analysis

The chemical compounds of clove powder were analyzed by a gas chromatograph (GC) equipped with a flame ionization detector (FID) to determine the total peak percentage of the solvent (Phytotherapy Center, Bezmialem Vakıf University, Istanbul, Turkey). The total chemical mass was calculated by multiplying the mass of each extract with the percentage representing the total peak area of the aroma compounds (21). The conditions of utilized GC-MS system were described earlier (22).

Chemical analysis of pasteurized milk

The commercially obtained milk was analysed for the fat ratio. Therefore, a 10 mL sulfuric acid was placed in the butyrometer and 11 mL milk was slowly added onto it. One mL of amyl alcohol (Merck, Turkey) was added the mixture in butyrometer and was closed with stopper. The homogenized mixture was centrifuged in the Gerber centrifuge (Funke Gerber Germany, T = 65 °C, 1350 rpm) for 5 min. Then, the ratio of fat in milk was evaluated in the mixture (23).

The protein ratio in milk was evaluated according to Turkish Standards (2002). Phenolphthalein (1%) (Merck, Turkey) and saturated potassium oxalate (Merck, Turkey) were added into 50 mL of pasteurized milk sample and the mixture was titrated with NaOH (Merck, Turkey) until the having a slightly pink colour. After the titration, formaldehyde (Merck, Turkey) was added in this mixture and it was titrated again until the having slightly pink colour. The used NaOH amount was calculated.

The fat free dry matter of milk was also analysed according to Turkish Standards (24). The petri dishes waiting for 1 hour were removed from the desiccator, cooled and weighed in a precision weighing. A 5 mL of pasteurized milk was placed on petri dishes and weighed again for the precise weighing. The weighed petri dishes were placed in the water bath and taken to the drying oven after about 30 minutes. After 2-3 hours, the petri dishes were immediately taken out of the desiccator, cooled and weighed on a precision weighing (Ohaus Co., USA).

According to Turkish Standards (24), pH and titratable acidity of pasteurized milk were carried out. The pH of milk was measured using a pH meter according to manufacturer's instructions (Mettler-Toledo, Switzerland). For the determination of titratable acidity in milk each 25 mL of pasteurized milk samples were placed in a beaker. One mL of phenolphthalein solution (1%)

(Merck, Turkey) was added in these samples. The solution was titrated with a sodium hydroxide solution until a pink colour was removed.

Preparation of experimental probiotic yoghurt groups

Pasteurized milk (500 mL) was heated to 42 °C. A 50 mL of this milk was added to the starter culture. The clove in different concentrations (0.1%, 0.3% and 1.0%), and propolis at a concentration of 0.03% were added and mixed into the 50 mL pasteurized milk. This mixture was transferred into 450 mL of milk waiting in 42 °C oven. After 6 hours of incubation, yoghurt was removed from the oven. Yoghurts were cooled to room temperature. Yoghurt ripening was carried out in refrigerator at + 4 °C for 24 hours. The preparation of experimental probiotic yoghurt groups (n = 5) were repeated three times and then analyzed as described below. All experimental groups were presented in the Figure 1 (a-e).

Microbiological analysis of probiotic yoghurt groups

The pepton water was prepared by adding 0.1% pepton powder and sterile distilled water. The mixture was sterilized by an autoclave (Nuva Materials Manufacturing Industry and Trade Co., Turkey). Probiotic yoghurt samples were diluted with 0.1% pepton water. A 90 mL of pepton water was added to a sample of 10 g of probiotic yoghurt placed in a sterile stomacher bag. This mixture was homogenized on the Stomacher (VWR, Italy). A 1 mL of homogenate was taken into the first tube (10^{-2}) for preparing dilution series (10^{-2} to 10^{-6}). A 100 microliters from each dilution were inoculated into MRS, MRS-CC, TOS-MUP and M17 agars (25). According to IDF and ISO standards, MRS, MRS-CC, TOS-MUP agars were incubated in an incubator (BINDER, Germany) for 72 hours at 37 °C in anaerobic condition, and M17 agars were incubated for 24 hours at 45 °C in aerobic condition. Agar containing only 25-250 colonies were evaluated using a colony counter (Interscience, France). Bacteria were calculated as colony-forming unit (cfu)/g yoghurt.

Chemical analysis of probiotic yoghurt groups

The fat ratio of experimental yoghurt groups was analysed according to Turkish Standards (26). The yoghurt sample was weighed 50 g in a beaker. A 5 mL ammonia (Merck %28-30) was added into this yoghurt, and mixed. An 11 mL of mix and 10 mL sulfuric acid (Merck %90, d = 1.82) were placed in the butyrometer. A 1 mL N-amyl alcohol (Merck %100, d = 0.815) were added into butyrometers, and they were centrifuged in the Gerber centrifuge (Funke Gerber Germany, T=65 °C, 1350 rpm) for 5 min. After the butyrometers were removed from the centrifuge, the results were analyzed. The results were read from the butyrometer, and the percentage fat mass was calculated.

Fat free dry matter analysis of experimental yoghurt groups was done according to Turkish Standards (26). Some sea sand was added to the petri dishes and dried in the oven. These petri dishes were cooled in desiccator. Then they were weighed on a precision weighing scale. Five

grams of homogenized yoghurt sample were added to these petri dishes. They were dried in the oven at 103 ± 2 °C for 2 h. After cooled petri dishes in desiccator, it was weighed again in order to calculate the percentage amount (m/m).

The quantitative analysis of protein of experimental yoghurts were performed using 50 g of yoghurt sample and adding 50 mL of distilled water. And then 0.5 mL of phenolphthalein (2%) (Merck, Turkey) and saturated potassium oxalate (Merck, Turkey) were added and mixed. After 2 minutes, it was slightly titrated to a pink colour with 0.1N NaOH (Merck, Turkey). Then 10 mL of formaldehyde (Merck, Turkey) were added and mixed. After incubation 1 minute, the slightly pink colour was again titrated to the pink colour. The titrated amount of 0.1 N NaOH were used in order to calculate the amount of protein in yoghurts (27).

The pH determination of each yoghurt sample was carried out using a pH meter (Mettler-Toledo, Switzerland) (27). The determination of titratable acidity of experimental yoghurt groups was done weighing 10 gram yoghurt and adding 10 mL of distilled water to a beaker. After adding of 0.5 mL of phenolphthalein solution (1%) was titrated with 0.1 M NaOH until being pink for about 30 seconds (26).

Sensory analysis of probiotic yoghurt groups

Sensory analysis of probiotic yoghurts were performed blind by our study team (8 researchers). Panelists evaluated yoghurt samples (n = 15) at the same time and independent from each other. According to Turkish Standards (26), each yoghurt should be assessed based on 4 characteristics in terms of appearance, consistency, smell and taste. Each yoghurt should be get at least 4 points from each characteristic and at least 16 points in total.

Statistical analysis

A total of 15 probiotic yoghurt samples were analyzed using SPSS 16.0 statistical package program. One-way ANOVA test was used to compare the microbiological and chemical analysis results of the experimental groups with the control group and determine the statistical difference ($P < 0.05$). The data obtained from sensory analysis was calculated as the total score for each group.

RESULTS

As reported in the our previous study, the essential oils of powdered clove was found from high to low ratio respectively: Eugenol (62.152%), Acetyeugenol (17.543%), Caryophyllene (17.049%), Humulene (1.847%), Alfa coapene (0.613%), Cadinene (0.371%) and others (0.426%) (Günes-Bayir and Bilgin 2019a).

The number of each bacterium was calculated, the results were compared among the experimental yoghurt groups in Figures 2 (a-d). The number of *B. animalis* subsp. *lactis* increased in yoghurt groups with clove and propolis depending on clove concentration (Fig.

2a.). These colonies were found to be reduced in the probiotic yoghurt group with propolis. However, these results were not found statistically significant. They found to be similar results for *L. acidophilus* and *L. bulgaricus* subsp. *delbrueckii* (Figures 2b and 2c). Propolis has a significantly repressive effect for these bacteria in probiotic yoghurts ($p < 0.05$). Clove showed an increasing effect on the number of them in probiotic yoghurt with propolis. The number of these bacteria was lower in probiotic yoghurt with propolis and clove than in normal probiotic yoghurt (control group). As clove concentration increased, the number of *L. acidophilus* decreased and the number of *L. bulgaricus* subsp. *delbrueckii* did not change. The results of microbiological analysis on *Sc. thermophilus* were presented in Fig. 2d. When the control group and probiotic yoghurt with propolis were compared, the increase in the number of *Sc. thermophilus* was not found to be significant. Clove and propolis have a reducing effect for *Sc. thermophilus* in probiotic yoghurt ($p < 0.05$). In addition, clove concentration shown a decreasing effect on this bacteria in probiotic yoghurt with propolis but not significantly.

Chemical analysis were performed on fat, fat-free dry matter, protein, titratable acidity and pH properties of yoghurts (Table 1). Propolis reduced the amount of fat in probiotic yoghurt. Fat was found to be low in probiotic yoghurt with clove and propolis compared to probiotic yoghurt. Fat rates of experimental yoghurts rise in clove concentrations increased. The additives in probiotic yoghurt slightly increased the fat-free dry matter. For this reason, it was found that the content of fat-free matter in the added probiotic yoghurts was higher than probiotic yoghurt ($p < 0.05$). The additives added to yoghurts do not have a high protein content. There was no significant difference in protein amount for all probiotic yoghurts. Clove and propolis have an increasing effect on acidity in probiotic yoghurts ($p < 0.05$). There was no significant change in pH values.

As a result of sensory analysis, the control group had the highest value (160 points) in total. Probiotic yoghurt with clove and propolis (1%) had the highest scores as 148 points. The yoghurt with propolis had the lowest value in sensory analysis (127 points). The results of sensory analysis are as indicated in Table 2.

DISCUSSION

Fermented dairy products, such as yoghurt, are very popular food for live probiotic cells. In recent years, the popularity of functional dairy products containing probiotics has increased significantly due to various therapeutic benefits documented and improving awareness of health (1). Present study was aimed to improve the effect of beneficial bacteria on probiotic yoghurt by adding clove and propolis at different concentrations. The number of all examined bacteria are effected by the substances concentrations added in the yoghurt groups. In studies, clove has been reported to have antibacterial properties (19). The antibacterial properties of cloves were observed in probiotic yoghurt with clove and propolis. Some spices such as pepper extract and

garlic extract have been reported to have an inhibitory effect on *L. acidophilus* (28). This result is in agreement with the present study which is conducted by clove and propolis.

It was reported that some plant extracts demonstrated the high inhibitory activity against the number of Bifidobacterium and Lactobacillus (28). In our study, propolis decreased the number of bifidobacteria while the used clove powder increased the bacterial number depending on its concentration. An inhibitory effect of some extracts on *L. bulgaricus* subsp. *delbrueckii* was demonstrated early (29). In the present study, propolis generally has inhibition effect on probiotic bacteria of yoghurts. We can say that the propolis has antibacterial properties. In this case, taking the propolis together with probiotic foods instead of single intake suggests that it is more useful for bacterial flora. It is controversial how the propolis effect on the bacterial flora in the human body when used a single. Also it must be investigate spices containing bioactive substances such as clove and cinnamon may have positive effects. On the other hand, a study has shown that some essential oils increased the number of *Sc. thermophilus* (29). In contrast to this study, the number of *Sc. thermophilus* was decreased depending on increased clove concentration while the propolis increased the bacterial number in yoghurt.

Yoghurt is a protein-rich fermented food. It is easier to digest than milk, as a fermented food. Yoghurt groups included different concentrations propolis and clove were evaluated in terms of oil, dry fat, pH, protein and titratable acidity by chemical analysis. In the yoghurt with saffron, fat content decreased compared to normal yoghurt (30). It was observed that propolis and cloves in different concentrations added to the yoghurt caused a decrease in the amount of fat in our study. However, amount of fat is increased by rising clove concentration. The fat content found as $3.2 \pm 0.1\%$ in yoghurt groups produced with milk containing 3% fat. Propolis caused to increase amount of fat dry matter. Adding clove powder supported this positive effect on fat dry matter. Yoghurts with propolis and cloves contained $8.52 \pm 0.28\%$ fat-free dry matter. This increase may depend on the rate of evaporation during the incubation. According to one study, there was not much difference in fat-free dry matter ratios in yoghurt with three different essential oils (31). In a study, protein content almost unchanged in yoghurt when added components (30). Because the additives do not have high protein value, the amount of protein in yoghurt groups was found to be same (3.54%) in our results. Yoghurt increased the titratable acidity with the addition of essential (31). Clove density generally increase acidity in yoghurt and the acidity value was on average 1.03%, it is the same as this study. Propolis reduced the amount of pH in probiotic yoghurt and clove showed similar effect. However, increasing concentrations of clove prevent this decline. When saffron as a spice were added to the yoghurts, it was found that the pH value decreased (30). As a result, the addition of propolis and cloves at different concentrations increased some microbial bacteria of the product and did not have a significant effect on the chemical properties. Also, there was no significant sensory effect.

CONCLUSIONS

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In this study, probiotic yoghurts were prepared with propolis (0.03%) and clove in three different concentrations as 0.1%, 0.3% and 1%. These additives were examined about their effects on probiotic yoghurt's microbiological, chemical and sensory properties. In microbiological analysis, it was found that generally propolis has an antibacterial effect on bacteria colonies except *Sc. thermophilus*. It was shown that, clove reduce the suppressive effect of propolis on bacteria colonies and supports the number of *B. animalis* subsp. *lactis*. In general, clove effect on the microbiological qualities of probiotic yoghurts positively. When the probiotic yoghurts were analyzed for their chemical properties, there was not much negative results. Propolis and clove had similar effects on probiotic yoghurts for example the fat ratios of probiotic yoghurts were reduced by propolis and clove supplementation. The additives increased the fat-free dry matter in probiotic yoghurt. There was no change on protein amount. Titratable acidity was increased while the pH was decreased in all probiotic yoghurts. Propolis showed negative activity on sensory properties of probiotic yoghurt but clove was more preferable about it.

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Table 1. Chemical analysis of experimental probiotic yogurt groups including fat, fat-free dry matter, protein and titratable acidity in percentages, and pH values are demonstrated as mean ± SD. SD: Standard deviation.

	CONTROL (NORMAL)	PROPOLIS (0.03%)	CLOVE (0.1%)AND PROPOLIS (0.03%)	CLOVE (0.3%)AND PROPOLIS (0.03%)	CLOVE (1%)AND PROPOLIS (0.03%)
FAT MATTER (M/V %)	3.3±0.01	3.2±0.01	3.2±0.06	3.2±0.01	3.3±0.01
FAT FREE DRY MATTER (M/M %)	8.15±0.01	8.18±0.02	8.24±0.04	8.34±0.04	8.59±0.04
PROTEIN (M/M)	3.54±0.02	3.54±0.01	3.54±0.03	3.54±0.03	3.54±0.02
TITRATABLE ACIDITY (M/M %)	0.91±0.06	1.02±0.02	1.04±0.01	1.01±0.02	1.03±0.01
PH	3.98±0.09	3.91±0.01	3.85±0.02	3.92±0.02	3.91±0.03
APPEARANCE	40	31.7	30.3	39.3	39.3
TEXTURE	40	32	36.7	35.7	33.7
ODOUR	40	31.3	36.7	35.3	31.7
TASTE	40	32.3	36	35.7	33
TOTAL	160	127	140	146	148

Table 2. The sensory analysis of each probiotic yogurt group are presented according to Turkish Standards (TS 1330).

FIGURES and FIGURE LEGENDS

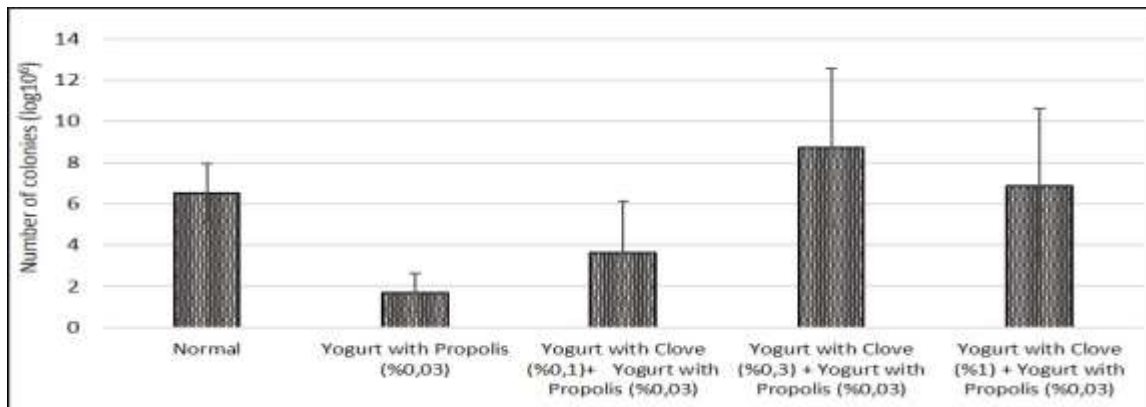
Figure 1.

Figure 1. All experimental probiotic yoghurt groups were presented as **a)** yoghurt without propolis and or clove (control group); **b)** yoghurt with propolis (%0.03); **c)** yoghurt with propolis (%0.03) and clove (%0.1); **d)** yoghurt with propolis (%0.03) and clove (%0.3); **e)** yoghurt with propolis (%0.03) and clove (%1.0), respectively

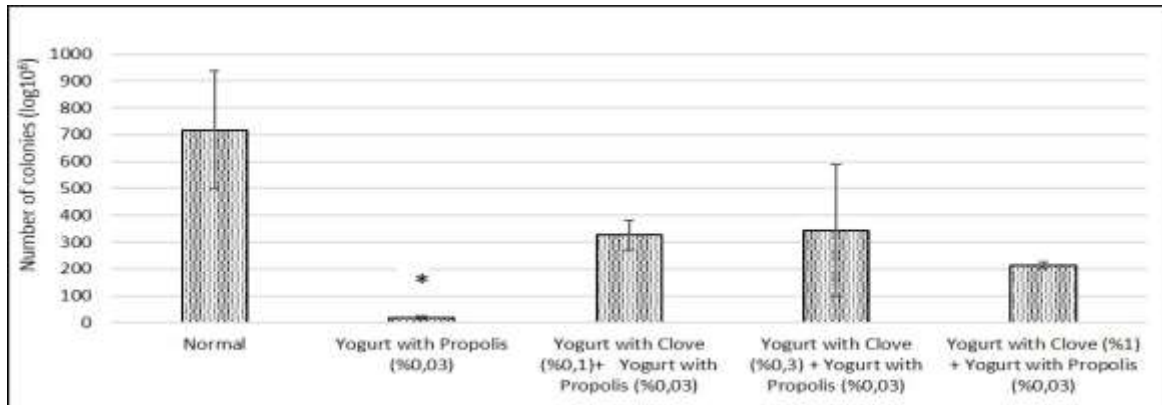


Figure 2.

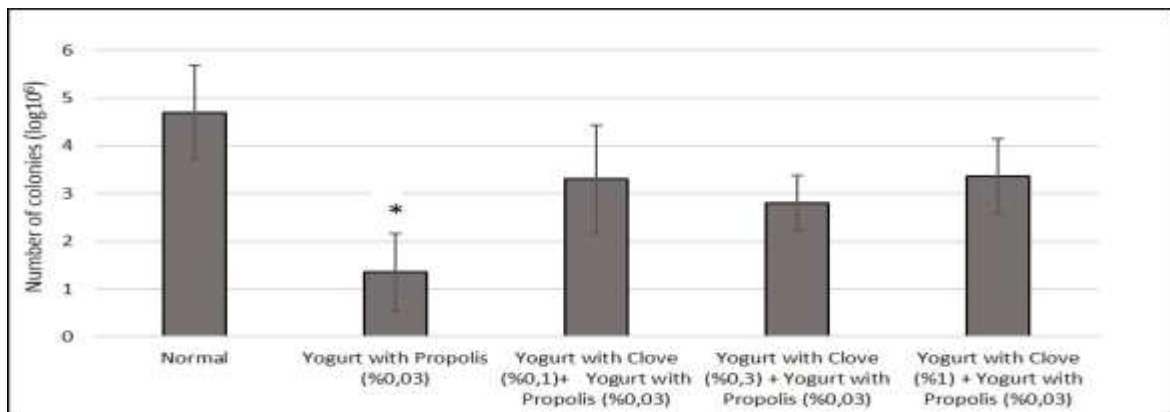
a)



b)



c)



d)

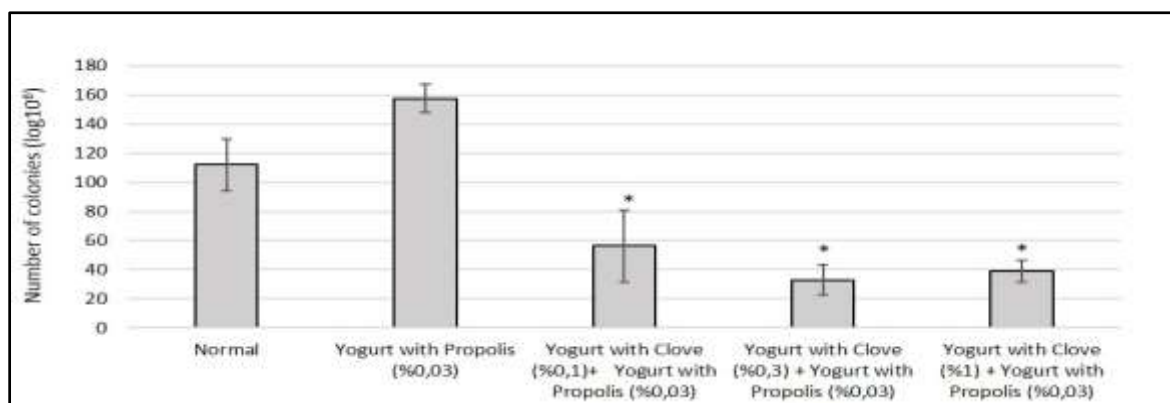


Figure 2. The bacteria counts in probiotic yoghurt groups are determined as colony forming unit. **a)** *Bifidobacterium animalis* subsp. *lactis*, **b)** *Lactobacillus acidophilus* **c)** *Lactobacillus bulgaricus* subsp. *delbrueckii* and **d)** *Streptococcus thermophilus*. * Differences between control group and other groups are statistically significant (p<0.05).