PHARMACIA

Pharmacia 70(4): 1411–1422
DOI 10.3897/pharmacia.70.e111822

Research Article

# Combination of ketogenic diet and probiotics inhibits breast cancer in mice by immune system modulation and reduction of Insulin growth factor-1

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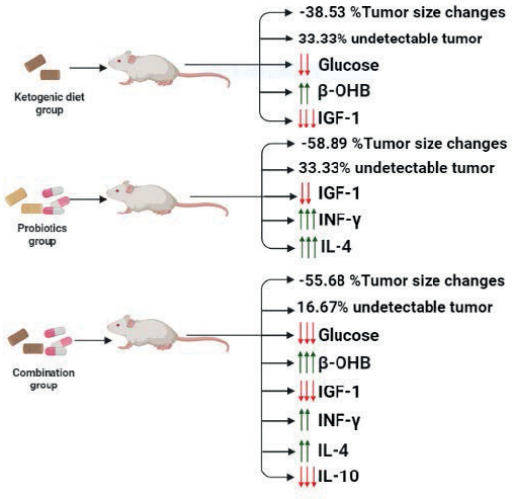
**Received** 30 August 2023 ♦ **Accepted** 11 November 2023 ♦ **Published** 30 November 2023

**Citation:** Kamal AKS, Talib WH (2023) Combination of ketogenic diet and probiotics inhibits breast cancer in mice by immune system modulation and reduction of Insulin growth factor-1. Pharmacia 70(4): 1411–1422. https://doi.org/10.3897/pharmacia.70.e111822

## Abstract

Breast cancer (BC) is the most prevalent malignancy in women and the first tumor type in the world. Ketogenic diets (KD), which are high in fat, low in carbohydrates, and sufficient in protein, can be used alone or as adjuvants with cancer drug medication as cancer therapy or prevention methods. Probiotics are nonpathogenic microorganisms or groups of bacteria that live in the gut and nourish the host body. In this work, we tested a new KD-probiotic combination against breast cancer implanted in mice. Several combination of probiotics ($1\times10^9$ CFU/0.5ml) and KD (14.1 kcal/2g) reduced tumor size and enhanced cure rate. KD and combination therapy groups increased beta-hydroxybutyrate (β-OHB) while decrease blood glucose, and IGF-1.IFN-γ, IL-2, IL-4, and IL-10 serum levels were measured to assess immune reaction to various therapies. Combination and probiotics therapy raised IFN-γ, and IL-4 levels, however IL-10 level did decrease in all treated group with highest decrease in combination group. In the safety profile, probiotics, ketogenic diet, and their combination were safe. Overall, the combination of a ketogenic diet and probiotics has the potential to be utilized in the future development of anti-cancer nutrition to augment conventional therapies.

**Graphical abstract.** Summary of the effect of Ketogenic diet, Probiotics and its combination on EMP-6 breast cancer cell and *in vivo*:



**Keywords**

antitumor, combination therapy, breast cancer, high fat diet

## Introduction

Breast cancer (BC) is the most prevalent malignancy in women and the first tumor type in the world (organization). Breast cancer is metastatic cancer that may often spread to distant organs such as the bone, liver, lung, and brain (Kotecki et al. 2018; Bale et al. 2019; Shao and Varamini 2022). Mutations and aberrant oncogenes play critical roles in tumorigenesis, amplification, and progression. Recent studies have understand the molecular mechanisms of numerous genetic factors in human breast cancer, such as phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), P53, nuclear factor Kappa-light-chain-enhancer of activated B cells (NF-kB), breast cancer-associated gene 1 and 2 (BRCA1 and BRCA2), Human epidermal growth factor receptor 2 (HER2), Epidermal Growth Factor Receptor (EGFR), cyclooxygenase-2 (COX-2), and c-myelocytomatosis oncogene (c-Myc) (Sun et al. 2017; Waks and Winer 2019; Messaoudi et al. 2022). Several risk factors might raise the chance of breast cancer, age is the most prominent risk factor for breast cancer, after gender (Sun et al. 2017; Momenimovahed and Salehiniya 2019). Clinical and epidemiological studies that have controversial results showed that prolactin, insulin-like growth factor (IGF-1), diabetic women, obesity, family history of breast cancer, BRCA2 gene mutation, smoking, exposed to radiation, and oral contraceptives used longer than 10 years have all been incriminated as risk factors for breast cancer (Momenimovahed and Salehiniya 2019; Rajaneesh 2021). On the other hand, physical exercise, breastfeeding, diet, and surgical removal of the ovaries can all reduce the risk of breast cancer (Sun et al. 2017; Rajaneesh 2021).

Nutrition is a salient component of breast cancer treatment, recovery, and quality of life. As a result of cancer and its treatment side effects, such as nausea and vomiting, many cancer patients are at risk for poor nutritional status. Nutrition education is critical due to increasingly diverse populations of the United States and many other Western countries, such as Canada, Australia, and European countries, as well as the rising costs of cancer treatment (Rakhmanovna 2022; Regeer et al. 2022).

Several lines of the study suggest the assumption of low-toxicity therapeutic techniques based on specialized diets like ketogenic diets (KD), which comprise high fat, moderate to low protein, and extremely low carbohydrate, forcing the body to use fat instead of glucose to produce adenosine triphosphate (ATP) (Nordli et al. 2020; Goldberg et al. 2021). In general, the fat-to-carbohydrate-plus-protein ratio is 3:1 or 4:1 by weight, resulting in an energy distribution of roughly 8% protein, 2% carbohydrate, and 90% fat in a diet (D'Andrea Meira et al. 2019; Goldberg et al. 2021; Sukkar and Muscaritoli 2021). Multiple lines of research suggest the use of KD as cancer treatment or prevention method, either alone or in combination with medicines. They have been used as a dietary treatment for epilepsy, recent findings revealed that KD reduces the incidence of diabetes and neurological deterioration (D'Andrea Meira et al. 2019; Vinciguerra et al. 2020). Furthermore, KD has the potential to be an effective complementary or alternative therapeutic strategy by suppressing tumor growth factors in a wide range of malignant tumors, including breast cancer, glioblastoma, prostate, colon, pancreatic, and lung cancer (Klement et al. 2018; Mitchell et al. 2019; Khodabakhshi et al. 2020). Mechanistically, KD exhibits anti-angiogenic, anti-inflammatory, and apoptosis-promoting properties in cancer cells, elevating ketone bodies and decreasing glucose required to promote the Warburg effect (Seyfried et al. 2017; Seyfried et al. 2020; Zeppieri 2021). Experimentally, weight loss associated with cancer patients consuming KDs makes them more likely to respond to chemotherapy or radiation therapy.

Probiotics are nonpathogenic microorganisms or groups of bacteria that live in the gut and nourish the host body (Pandey et al. 2015; Rocha-Ramírez et al. 2021). A good source of fermentation bacteria are found in many fruits and vegetables (e.g., cabbage, olives, sauerkraut, kimchi, and cucumbers), fermented milk products (e.g., yogurt, kefir, and cheese), and meat products (Salamis and Sausages) (Ozen and Dinleyici 2015; Zielińska and Kołożyn-Krajewska 2018). Two bacterial phyla are present in the human intestines: gram-positive Firmicutes (Bacillus species, *Lactobacillus* species, and *Clostridium* species) and gram-negative Bacteroidetes (Zielińska and Kołożyn-Krajewska 2018). Several experimental studies demonstrated the impact of probiotics on different type of cancer (Shirazi et al. 2020). In homeostasis, aging, and cancer, there are clearly organ/tissue-specific changes in the composition of the related microbiomes with both overlapping and dissimilar species and abundance to that of the colon (Ding and Schloss 2014; Byrd et al. 2021). In accordance with the majority of existing evidence, breast cancer is associated with bacterial dysbiosis in both the gut microenvironment and breast tissue (Xuan et al. 2014; Shirazi et al. 2020). According to current theories, changes in the composition and functions of various breast and gut bacterial may contribute to the development and progression of BC via a variety of pathways. Furthermore, the microbiome has a crucial role in determining the success of both conventional chemotherapies and immunotherapeutic treatments, as well as the development of cancer (Badgeley et al. 2021). It also has the potential to affect the bioavailability of medications (Laborda-Illanes et al. 2020; Badgeley et al. 2021). Another important role for

the gut microbiota is the regulation of steroid-hormone metabolism, particularly estrogen metabolism, which is a component that plays an important role as a risk factor for breast cancer development, particularly in postmenopausal women. It has been proven that a subgroup of bacteria inside the gastrointestinal system regulates estrogen metabolism and the balance of circulating and excreted hormone levels (Flores et al. 2012; Kwa et al. 2016).

A combination of ketogenic diet with probiotics has not been evaluated in the literature to treat breast cancer. Accordingly, this study was designed to test the therapeutic potential of a new combination consisting of ketogenic diet and probiotics against breast cancer implanted in mice.

# Experimental

## Cell line and culture conditions

One mouse mammary cell line was used in this study, the parent (EMT6/P) cell line was purchased from the European Collection of Authenticated Cell Cultures (ECACC; Salisbury, United Kingdom). The cell line was cultured using a minimum essential medium supplemented with 10% fetal calf serum, 1% L-glutamine, 0.1% gentamycin, 1% penicillin-streptomycin solution, and 0.5 mL of 0.1% Non-Essential Amino acids 100×. Perfect cell culture conditions were provided for cell growth using complete tissue culture media (MEM).Cell was incubated at 37 °C, 5% carbon dioxide, and 95% humidity.

## Probiotic preparation

Commercially available probiotics (Jamieson) used in this study were suspended in a phosphate buffer solution (PBS) to obtain the desired bacterial cell concentration of $1\times10^9$ CFU/0.5 ml, and supplied by oral gavage route (Tiptiri-Kourpeti et al. 2016).

## Ketocal preparation

Commercially available ketogenic diet "Ketocal" used in this study. Each mouse was delivered daily 14.1 kcal/2g (Zhang et al. 2020). KetoCal was mixed with water in a (2:1) ratio and supplied to the animals orally (ad libitum) every day (Talib 2020).

## In vivo experiment

### *Animals*

This study was conducted according to standard ethical guidelines. The Research and Ethical Committee approved all the experimental protocols at the Faculty of Pharmacy, Applied Science Private University. This study was conducted using 24 healthy female Balb/C mice, ranging between 21–25 grams of weight and 6–8 weeks of age. Mice were supplied by the animal house in the Applied Science Private University, Amman, Jordan. All protocols of animal experiments were validated by the Research and Ethical Committee of Applied Science University with Standard ethical guidelines. Mice were accommodated in well ventilated rooms, at room temperature 25 °C and 50–60% humidity, as well as alternating cycles of dark and light every 12 hours, in order to accomplish all required environmental conditions. They were raised in cages equipped with wooden shavings for bedding, a special water bottle, and food.

### *Tumor inoculation*

We started to prepare the inoculation of mice with EMT6/P (parent breast cancer cell line). Briefly, EMT-6/P in MEM medium was prepared for the whole 24 mice. Tumor inoculation was achieved by injecting a tumor induction dose of 1000000 cells (in 0.1 mL) subcutaneously in the abdominal area of each female Balb/C mouse. Injected cancer cells were left to grow and form new tumors.

### *Treatments*

Twenty-four mice were used in this treatment, mice were divided into four groups (n = 6/group). Control group, Ketogenic diet group, Probiotics group, and combination of Probiotics and Ketogenic diet group Table 1. The treatments of Probiotics, started one weeks before tumor inoculation and 10 days after tumor inoculation (Tiptiri-Kourpeti et al. 2016). The treatment of Ketogenic diet started 10 days after tumor inoculation and continued until finish of experiment (Zhang et al. 2020). The treatment lasted for 25 days. During treatment, blood samples, and mouse weight were taken at five time-points over the tumor inoculation on days 10, 13, 17, 20, and 23 Fig. 1. Ten days after inoculation, tumor volumes were measured using a digital caliper to define the length and width. In this context, Tumor volumes and tumor progression were calculated using the following equations, respectively (Faustino-Rocha et al. 2015).

$$V = \frac{(L \times W^2)}{2}$$

Where: V, L, and W are the volume, length, and width of the tumor, respectively.

$$\%\,tumor\ change = \frac{F-I}{I} \times 100\%$$

F, I represent the final and initial tumor volumes, respectively.

Finally, after 25 days from the beginning of the experiment, mice were sacrificed by cervical dislocation, and

**Table 1.** In vivo experimental treatment schedule (No = number).

| No. of groups | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| No. of mice | 6 mice | 6 mice | 6 mice | 6 mice |
| Treatment | Unrestricted diet (SD) | Ketogenic diet (KD) | Probiotics | Combination of Probiotics and Ketogenic diet |
| Daily dose | 12 kcal/5g | 14.1 kcal/2g | $1\times10^9$ CFU/0.5 ml | ($1\times10^9$ CFU/0.5ml) of Probiotics and (14.1 kcal/2g) of Ketogenic diet |

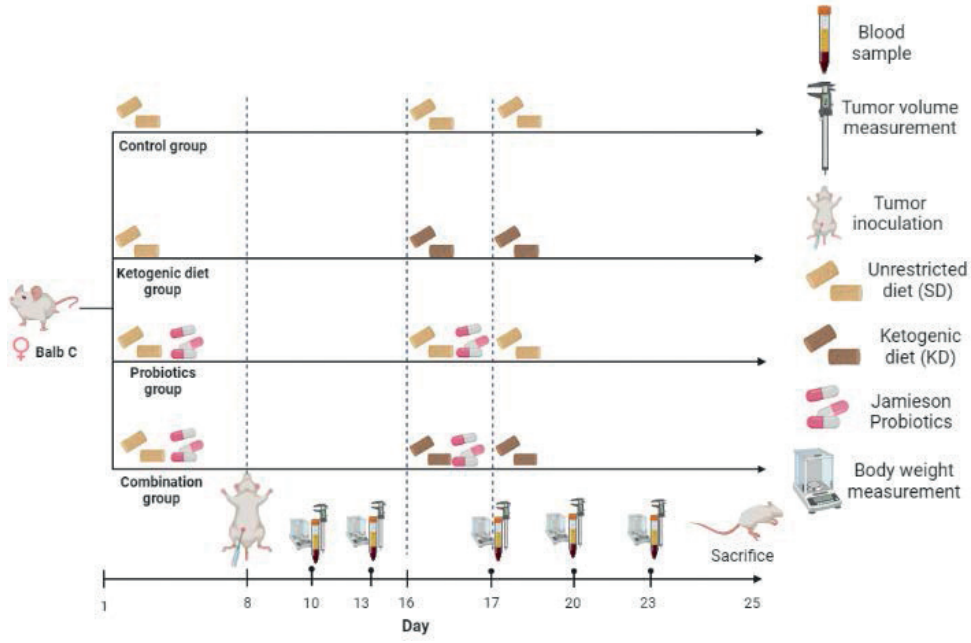



**Figure 1.** Experimental timeline.

dissected tumors were weighed and stored in 10% formalin to preserve their morphology.

## Blood analysis

### *Evaluation glucose and beta-hydroxybutyrate (β-HB) levels in treated mice*

Blood levels of glucose and β-HB were assessed on days: 10, 13, 17, 20, and 23, and compared with normal-untreated mice bearing no tumor. Blood glucose levels were measured using the Accu-Chek blood glucose monitoring system (Roche, Basel, Switzerland). β-HB Assay Kit (Sigma, United States) was used to measure the levels of β-HB in the serum (Talib 2020).

### *Measuring serum IGF-1*

Mouse IGF-1 ELISA kits (Invitrogen, United States) had been used to assess IGF-1 levels. IGF-1 ELISA kits were investigated to negative control (untreated mice with tumor), and different treatment groups.

### *Detection of IFN-γ, IL-2, IL-4, and IL-10 serum levels*

The effect of each treatment on the immune response of tumor-bearing mice was determined using Mouse Th1/Th2 ELISA kit (Thermo Fisher Scientific, Toronto, Canada). Blood samples were collected from mice subjected to different treatments and serum samples were prepared. Interferon (IFN)-γ, interleukin (IL)-2, IL-4 and IL-10 were detected using kit instructions (Talib 2017).

### *Lipid profile (Total Cholesterol, TG, HDL, and LDL)*

Serum lipid profile had been evaluated by measuring total cholesterol, TG, LDL, and HDL using Beckman Coulter AU480 (Beckman Coulter, Inc, USA) for negative control (untreated mice with tumor), positive control (normal mice without tumor), and different treatment groups.

## Assessment of liver and kidney functions in mice

Serum levels of alanine Transaminase (ALT), aspartate Transaminase (AST), and creatinine were measured in negative control (untreated mice with tumor), positive control (normal mice without tumor), and different treatment groups using Beckman Coulter AU480 (Beckman Coulter, Inc, USA) to assess any relative nephrotoxicity and liver toxicity.

## Statistical analysis

Data were presented using the mean ± SEM (Standard Error of Mean). The statistical significance among the groups were determined using SPSS (Statistical Package for the Social Science, Chicago, Illinois, version 22) by one-way analysis of variance (ANOVA; post hoc test: Tukey), and Kruskal-Wallis test. Differences between groups were considered significant when the p-value was less than 0.05 ($p < 0.05$).

# Results and discussion

## Antitumor effects of probiotics, ketogenic diet ant their combination on EMT6/P cells implanted in mice

Generally, treatment of tumor bearing mice with a combination of probiotics ($1\times10^9$ CFU/0.5 ml) and ketogenic diet (14.1 kcal/2g) caused significant ($p < 0.05$) decrease

in tumor size with a percentage change in tumor size of (-55.68%) compared with an increase of (106.82%) in the negative control group. A slightly lower percentage change (-38.53%) was observed in the group treatment with Ketogenic diet group (14.1 kcal/2g). Interestingly, probiotic group ($1\times10^9$ CFU/0.5 ml) resulted in the highest reduction in tumor size (-58.89%).

Table 2 the same cure percentage (33.33%) were obtained in groups treated with probiotics and ketogenic diet. However, (16.67%) undetectable tumor was reported for mice treated with combination therapy Fig. 2. Slight difference in average tumor weight were recorded for ketogenic diet and probiotics groups with average tumor weight (0.257 and 0.182 g) respectively. However, these values were insignificant ($p>0.05$) and far below that of the negative control group (0.445 g). Interestingly, the lowest average tumor weight was reported in the combination group (0.069 g). Moreover, the percentage change in body weight of mice at different treatment was measured.

Table 2 probiotics and combination groups exhibited reduction in body weight with percentage change around (-6%) and (-1%) respectively compared with the negative control group that showed an increase of (2.5432%) in body weight.

We used mice bearing EMT6/P tumor cells to examine the effect of each treatment in vivo. We should note that the beginning tumor sizes of each group were different from other. Variable tumor sizes within each group were observed at the end of the study in all groups as indicated by high standard deviation values. Such difference is mainly due to the difference in response toward each treatment. Although all mice were inoculated using the same concentration of EMT6/P cells. We found that ketogenic diet treatment showed significantly reduction in tumor size. Several studies showed that the effect of the ketogenic diet against implanted breast tumors can vary according to the duration of treatment or formula concentration of fat, carbohydrate, and protein. For example, in a

**Table 2.** Effect of different treatments on tumor size, cure percentage, tumor and body weight. Where ($mm^3$) is a cubic millimeter, (g) is gram, (av.) is an average, and (n) is a number.

| Groups (n = 6) | Av. initial tumor size ($mm^3$) ± SEM | Av. final tumor size ($mm^3$) ± SEM | % Change in tumor size | % cure | Av. tumor weight (g) | % Change in body weight |
|---|---|---|---|---|---|---|
| Control | 279.50 ±30.66 | 578.04 ± 118.76 | 106.82 | 0 | 0.445 | 2.543 |
| Ketogenic diet | 292.87 ± 30.34 | 180.02 ± 63.50 | -38.53 | 33.33 | 0.257 | 0 |
| Probiotics | 422.51 ± 56.79 | 173.69± 56.66 | -58.89 | 33.33 | 0.182 | -6.238 |
| Combination | 401.13 ± 31.09 | 177.76 ± 40.50 | -55.68 | 16.67 | 0.069 | -1.275 |

% Change in tumor size = (final size – initial size) / initial size × 100
% Cure= (number of cure mice *100)/total number of mice
% Change in weight = (final weight – initial weight) / initial weight × 100.

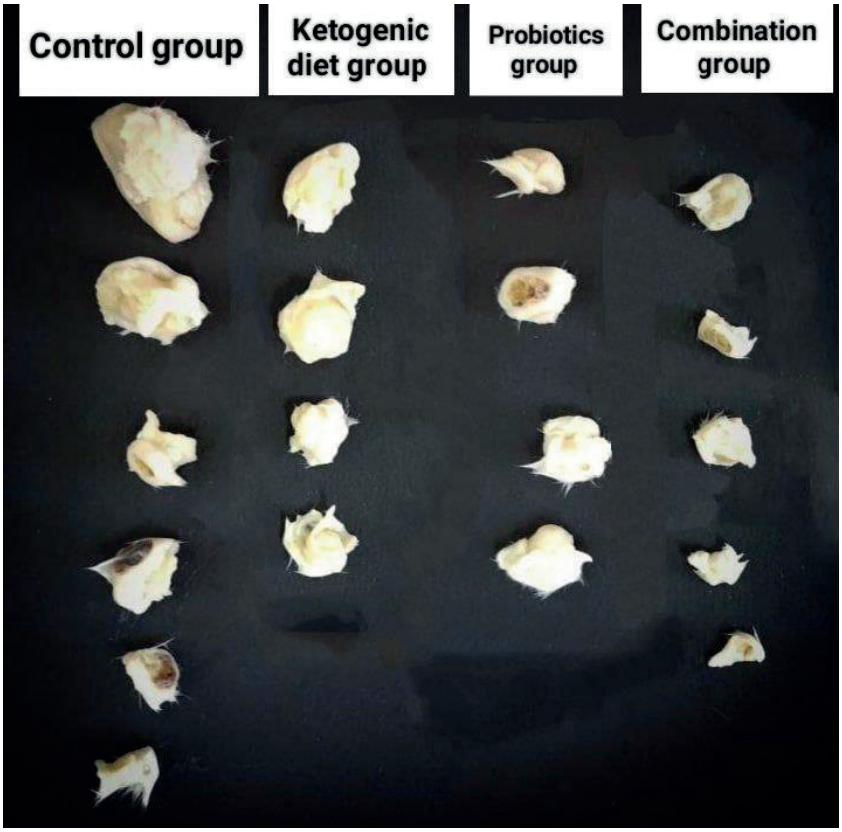



**Figure 2.** Effect of different treatments on tumor size and cure percentage. N = 6 for each group.

previous study, KD (comprised of 6% calories from CHO, 19% from protein, and 55% from FAT) was provided to breast cancer patients for 90 consecutive days concurrent with the first 12 weeks of chemotherapy. KD led to a significant decrease in stage and tumor size compared to the control group. The tumor size in the KD group showed a significant reduction compared to the baseline; the reduction in tumor size was 27 mm in the intervention group compared to 6 mm in the control group (Khodabakhshi et al. 2021). In another study, Four-week-old female FVB/N-Tg (MMTV-PyVT) 634 Mul/J mice, where (100%) develop breast tumors spontaneously during their lifetime. Then, ketogenic diet (KD) (carbohydrate/fat/protein with 0.1/89.9/10.0 percentage respectively) was orally administered. The ketogenic diet remarkably reduced tumor size in the KD group than that of standard diet (SD) mice (Zou et al. 2020) . These results are consistent with the results obtained in our study which shows the ability of KD to cause tumor regression.

In our study, we also examined the effect of probiotics on tumor growth in vivo, we found that probiotics treatment showed the highest reduction in tumor size. Our findings are in agreement with previous studies which reported the ability of probiotics to reduce tumor size in mice breast cancer cells (Maroof et al. 2012; Azizi et al. 2021).

In a different study, probiotics were taken for limited time for mice with cancer. Significant reductions in body weight were observed in treated group (He et al. 2020; Kita et al. 2020). While ketogenic diet group showed no change in body weight. KetoCal (KC) is a nutritionally complete, commercially available 4:1 (fat: carbohydrate + protein) ketogenic formula that is an effective non-pharmacologic treatment for the management of refractory pediatric epilepsy, Abdelwahab et al used C57BL/6 mice with malignant glioma implanted feeding KD (KetoCal) for two weeks. Animals fed KD had no change in body weight compared with other groups (Abdelwahab et al. 2012). Although, compared with single treatments, the combination treatment showed the strongest effect on tumor weight. The significant reduction in tumor size, and weight were correlated to a significant reduction in body weight.

## Effect of the treatments on glucose and b-Hydroxybutyrate levels

The subsequent analysis of glucose levels showed that treatments with combination therapy (ketogenic diet and probiotics) had the lowest level of glucose ($p < 0.001$). Also, ketogenic diet treated group had lower glucose levels than the control group, noteworthy the reduction was significant Fig. 3. On the other hand, ketogenic diet and combination groups showed the highest β-hydroxybutyrate compared with other treatments, nevertheless, the increase was insignificant Fig. 4.

The use of ketogenic diet inhibits breast cancer in mice by reducing blood glucose. Also, the use of this diet resulted in an increase in blood levels of β-hydroxybutyrate which is known of its antitumor effects (Woolf et al. 2016).

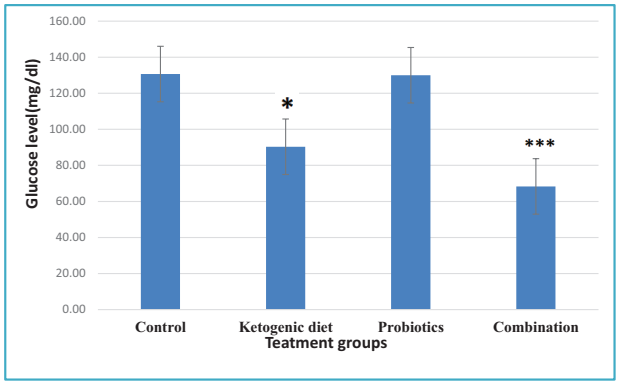



**Figure 3.** Serum levels of glucose for different treatment groups. Lowest glucose level was observed in group taking combination therapy. Results are expressed as means ± SEM (n = 3).*P < 0.05, *** P < 0.001. (Treatment groups compared with the control group).

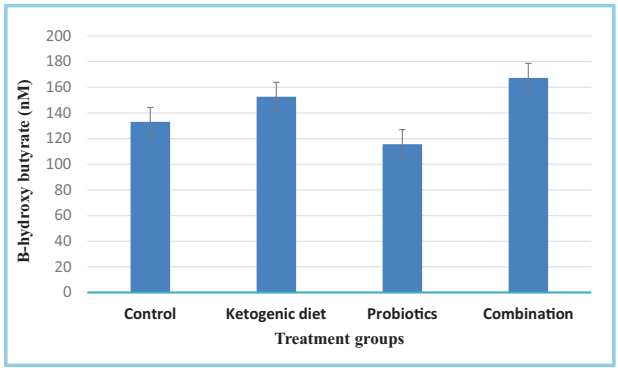



**Figure 4.** Serum levels β-hydroxybutyrate for different treatments. Highest β- hydroxybutyrate levels was observed in group taking combination therapy. Results are expressed as means ± SEM (n = 3).*P < 0.05, *** P < 0.001. (Treatment groups compared with the control group). Test was performed in duplicate.

Our results are consistent with previous findings that showed similar effect of ketogenic diet against breast cancer in different in vivo and human studies (Khodabakhshi et al. 2021). A variety of findings have led to suggestion that probiotics, which are described as living bacteria that, when supplied in suitable proportions, impart a health benefit on the host, and may have potential advantages in maintaining good glucose metabolism. Moroti and co-workers (Moroti et al. 2012) reported that consumption of probiotics and fructo-oligosaccharides reduced fasting glycaemia in elderly subjects with type 2 diabetes mellitus, whereas consumption of a placebo product did not. Probiotics may also regulate glucose by targeting insulin action; Andreasen and co-workers (Andreasen et al. 2010) reported an improvement in insulin sensitivity in diabetic and non-diabetic volunteers following consumption of probiotics. Several animal research, in addition to human studies, have given encouraging findings on the effectiveness of probiotics in the maintenance of healthy fasting and postprandial blood glucose levels, as well as associated outcomes (Abdelazez et al. 2018; Al Kattar et

al. 2020). Although, metabolic products of the gut microbiota could be important in the control of blood glucose (Cabello-Olmo et al. 2021).

## Effect of different treatments in IGF-1

Remarkably, Treatment of Blab/C mice with ketogenic diet group (14.1 kcal/2g) caused decrease in the level of IGF-1 (14.815 ng/ml) compared with the negative control group (59.795 ng/ml). On the flip side, the levels of IGF-1 were (37.170 ng/ml) for probiotics group, and (59.795 ng/ml) for the negative control group. Regarding to the mice treated with combination ($1\times10^9$ CFU/0.5 ml of probiotics, 14.1 kcal/2g of ketogenic diet), Reduction of IGF-1 levels to (16.069 ng/ml) was significant comparing with control group Fig. 5.

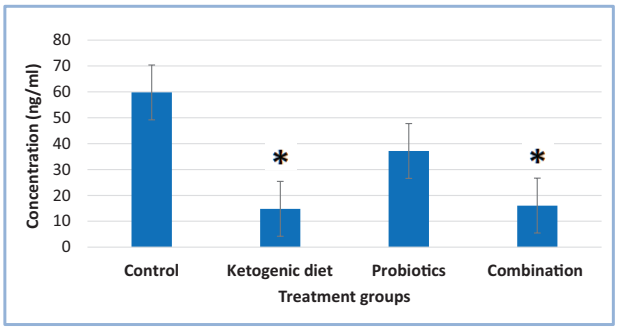



**Figure 5.** Serum levels of IGF-1 for different treatments. The lowest IGF-1 level was detected in ketogenic diet group. Results are expressed as means ± SEM (n = 3).*P < 0.05, *** P < 0.001. (Treatment groups compared with the control group). Test was performed in duplicate.

The ability of a ketogenic diet to reduce serum IGF-1 levels could be a target mechanism for using it as a cancer adjuvant therapy. Increased IGF-1 levels are associated with an increased risk of several cancers such as breast, lung, colorectal, and prostate cancer (Shanmugalingam et al. 2016). IGF-1 can induce cancer cell proliferation by activating Ras/MAPK and PI3K/Akt pathways (Krishnamurthy and Kurzrock 2018). Increased activation of these pathways will stimulate cancer cell growth initiation, and progression (Shanmugalingam et al. 2016). We found that there were differences between the treatment groups in serum IGF-1 compared to the control group. Based on our study results, the ketogenic diet for 25 days was decreasing serum IGF-1 levels in mice. This result is in line with another study that showed that the ketogenic diet for eight weeks could reduce plasma IGF-1 levels in mice (Widiatmaja et al. 2022). Furthermore, in a study of prostate cancer xenografts, the ketogenic diet was shown to cause lowered insulin and IGF-1, decreased phosphorylation of AKT, and slowed tumor growth (Kalaany and Sabatini 2009). Besides that, oral administration of glucose solution combined with postoperative probiotics on inflammation, and intestinal barrier function in patients after colorectal cancer surgery significantly decreased in IGF-1 compared with a group who received glucose alone (Xu et al. 2019).

## Effect of ketogenic diet and/or probiotics on the immune system

To explore the effect of different treatments on the immune response in animals, the serum levels of IFN-γ, IL-2, IL-4 and IL-10 were detected Fig. 6. The level of IFN-γ increased in probiotics group (21.01 pg/ml), and combination group (16.917 pg/ml) treated animals compared to that in the control group (4.182 pg/ml), this increase was significant. The level of IL-2 was almost stable and did not change significantly across the ketogenic diet, and combination groups compared with the control group. However, a significant increase in IL-2 was observed in probiotics group compared with control group. In contrast, the level of IL-4 increased in the combination group (1.077 pg/ml), and significantly in probiotics group (2.690 pg/ml) treated animals compared to the control group (0.182 pg/ml). IL-10 level did decrease in all treated group with highest decrease in combination group (8.854 pg/ml) compared to that in control group (15.210 pg/ml).

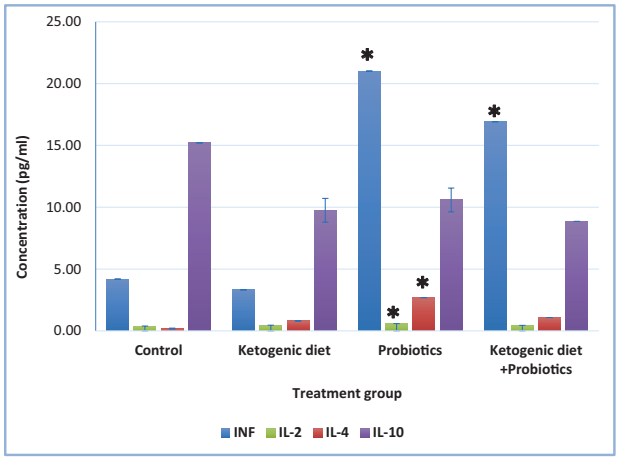



**Figure 6.** Effect of different treatments on serum levels of INF-γ, IL-2, IL-4, and IL-10. Concentration of serum cytokines (pg/ml) in different treated mice. The highest level of INF-γ, IL-4, IL-2, and lowest IL-10 were detected in probiotics group. The lowest level of IL-10 was detected in combination group. Results are expressed as means ± SEM (n = 3).*P < 0.05, *** P < 0.001. (Treatment groups compared with the control group). Test was performed in duplicate.

In cancer xenografts, the anti-proliferative action of IFN-γ, probably due to enhanced cell death by up-regulation of some caspases and anti-angiogenic activity, have been found (Sidky and Borden 1987; Bouker et al. 2005). Further actions of IFN-γ are the following: (a) increased expression of major histocompatibility complex (MHC) class I and class II molecules; (b) activation of monocytes and macrophages able to induce a cytokine cascade (IL-6, TNF-α and IL-8), and a successive complete activation of various subsets of T or B cells; (c) differentiation

of CD4+ to Th1 cells and inhibition of Th2 proliferation (Thomson and Lotze 2003). Oral administration of *L. casei* significantly increased the production of IFN-γ and NK cytotoxicity in spleen cells culture of test mice (Dallal et al. 2012). In the present study probiotics induced a high level of INF-γ, while the ketogenic diet did not make any change. The probiotics group caused systemic activation in the immune system resulting in an increase in INF-γ, IL-2, and IL-4 levels. This enhanced immune activation, and other anticancer mechanisms to get the high cure percentage obtained in this study. Another study indicated that oral administration of *L. acidophilus* ($2\times10^8$ CFU) for 15 consecutive days was able to alter the cytokine production in tumor-bearing mice into a Th1 protective pattern, favorable to anti-tumor immunity, with a Significant increase in splenocyte production of IFN-γ, IL-4 (Maroof et al. 2012). Both IFN-γ and IL-4 synergize to enhance MHC class II expression on the surface of melanoma and breast cancer cells (Obiri et al. 1994). Furthermore, previous work demonstrated the ability of IL-4 to inhibit growth and induce apoptosis in human breast cancer cells (Obiri et al. 1994; Gooch et al. 1998; Blais et al. 1996). Similarly, IFN-γ alone or in combination with IL-4 might elicit an antitumor immune response against breast cancer cells after probiotics and combination treatments in our experiment.

IL-2 induces the proliferation of activated T cells and the differentiation of cytotoxic T lymphocytes (CTL); it also has effects on other immune cells including NK cells, B cells, monocyte/macrophages, and neutrophils (Waldmann 2018). Results from a pilot phase I study in metastatic HER2+ breast cancer indicated that IL-2 combined with trastuzumab was well tolerated and provided clinical benefit (Repka et al. 2003). In contrast to our study, in probiotics treated animals, the serum level of IL-2 cytokine was significantly increased. IL-10 is a pleiotropic cytokine that exhibits both tumor-promoting and inhibitory properties. IL-10 is expressed in patients with breast cancer and has been associated with poor prognosis (Ishigami et al. 2019). In an in vivo study, BALB/c mice were injected with 4T1 cancer cells and treated orally with Kefir water for 28 days. Kefir contains various kinds of microbial flora, including the most commonly studied species with probiotic potential such as *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactococcus lactis* subsp *lactis* (Kakisu et al. 2011; Zamberi et al. 2016). The levels of immunomodulation-related cytokines (IFN-γ and IL-2) were significantly increased in the Kefir water-treated group. Furthermore, Kefir water also inhibited the level of IL-10 cytokine, which is associated with poor prognosis (Zamberi et al. 2016). Finally, the combination of probiotics and ketogenic diet resulted in a decrease in IL-10 level with an increase in IFN-γ, IL-2, and IL-4 levels. Some cytokines (IL-1, IL-6, and IL-11) stimulate, while others (IL-12, IL-18, IFNs) inhibit breast cancer proliferation and/or invasion. Similarly, high circulating levels of some cytokines seem to be favorable (IL-2) while other are unfavorable (IL-1b, IL-6, IL-8, IL-10, IL-18) prognostic indicators. So far IL-2, IFN-α/β, occasionally IFN-γ, IL-6, and IL-12 have been the cytokines used for the anti-tumor treatment of advanced breast cancer either to induce or increase hormone sensitivity and/or to stimulate cellular immunity (Waldmann 2018; Berraondo et al. 2019). Our results are consistent with these findings and probiotics are the main stimulator of the immune system in our combination as low change of these cytokines levels were detected in the ketogenic diet as a single treatment.

## Effect of treatment therapy in lipid profile

Measured serum lipid distribution for different treatments using Beckman Coulter AU480. Serum lipid levels of the normal mice without any tumors were measured and used as a reference for normal levels. The changes in serum triglyceride levels were lower in all groups compared to healthy mice (125 mg/dl) Fig. 7. Total cholesterol levels were elevated in all groups compared to healthy group, although cholesterol levels in the combination group (125 mg/dl) was higher to those in the control group (92 mg/dl) Fig. 7. High-density lipoprotein (HDL) levels were higher in treatment groups than in healthy group Fig. 7. The shifts in low-density lipoprotein (LDL) levels were diminish in combination group (5 mg/dl), and ketogenic diet group (10 mg/dl) compared to control group (13 mg/dl). Whereas probiotics group (15 mg/dl) exhibited LDL level higher than the level measured in the control group Fig. 7. The treated group exhibited insignificant differences in serum TG ($p > 0.950$), total cholesterol ($p > 0.998$), HDL ($p > 0.999$), and LDL ($p > 0.968$) levels compared to normal untreated mice.

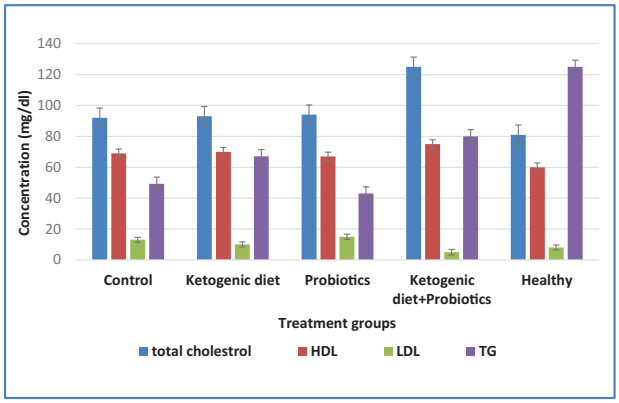



**Figure 7.** The effect of different treatments on serum levels of triglyceride (TG), total cholesterol, high density lipoprotein (HDL), and low-density lipoprotein (LDL). The measured levels (mg/dl). Results are expressed as means ± SEM (n = 3). Test was performed in duplicate.

We found that there were no significant differences between the groups in total cholesterol, TG, HDL, and LDL. This study's results showed that both combination, and ketogenic groups had in insignificant reduced serum LDL concentrations compared to the control group. Numerous clinical trials on cancer patients or obese patients reported that blood LDL or/and triglyceride concentration decreased after ketogenic diet intervention for a while

(Schmidt et al. 2011). As in our samples, there were also a reduction in TG in the treatment groups. In previous studies examining KD in in vivo or cancer patients, serum cholesterol has also been evaluated, but few have observed significant changes in its level (Meidenbauer et al. 2014; Lien and Vander Heiden 2019; Khodabakhshi et al. 2020). Ranji et al, was detect the Effects of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* probiotics on serum lipid levels in comparison with the azoxymethane (AOM), and health groups in a mouse model of colon cancer. Oral consumption of *L. acidophilus* and *B. bifidum* probiotics decreased the TG, cholesterol, and increase HDL (Heydari et al. 2019). In our study, the cholesterol and HDL levels top-up in treatment groups, and the levels of change were not statistically different among groups.

## Assessment of liver function and kidney function

The potential of developing toxicity associated with different treatments was investigated by measuring the serum levels of AST, and ALT liver enzymes, as well as creatinine. Serum levels were also measured for normal mice with no tumors (as a reference for normal liver and kidney function). The treated group exhibited insignificant differences in serum ALT ($p > 0.997$), and AST levels ($p > 0.9909$) compared to normal untreated mice. No significant change in the AST level was observed across untreated animals (229 U/l), healthy normal animals (242 U/l), and animals treated with a combination of ketogenic diet (14.1 kcal/2g), and probiotics ($1\times10^9$ CFU/0.5ml) (171 U/l) Fig. 9. Although the AST level in probiotics (300 U/l), and ketogenic diet (254 U/l) treated groups were higher than that in the normal group, the increase in the reading was statistically insignificant. The ALT level in the combination treated group (44 U/l) was lower than in the normal group (86 U/l), however, this low level was not statistically significant. Moreover, in all other treated groups no significant changes in ALT levels were detected Fig. 9. Finally, in all different treatments, the creatinine levels did not significantly change compared with those in the normal group ($p > 0.988$) Fig. 8. Therefore, AST, ALT and creatinine levels were considered to be normal compared

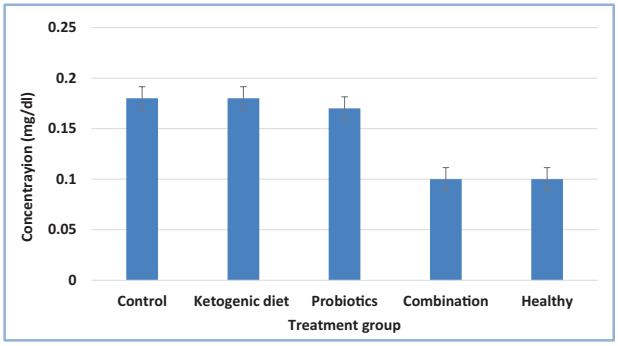



**Figure 8.** The effect of different treatments on serum levels of creatinine. The measured level (mg/dl) of creatinine in animals with different treatment groups. Results are expressed as means ± SEM (n = 3). Test was performed in duplicate.

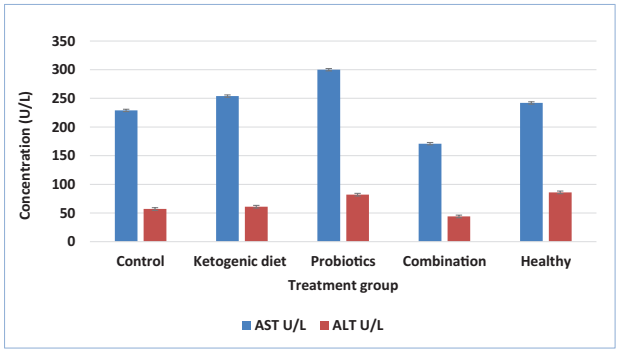



**Figure 9.** The effect of different treatments on serum levels of aspartate transaminase (AST), and alanine transaminase (ALT). The measured levels (U/l) of AST and ALT creatinine in animals with different treatment groups. Results are expressed as means ± SEM (n = 3). Test was performed in duplicate.

to their levels in the normal group. As a result, liver and kidney function tests showed normal values after using this combination which indicates its safety.

# Conclusion

This study shows that the ketogenic diet and probiotics are important ways to treat breast cancer in animal model system. Gut microbiota and human health have already been linked in a clear way by the growing field of microbiome research. Microbial driven processes affect inflammation, promote metabolite exposures, impact gene expression through transcription, translation, epigenetic modifications, and modulate immunological signaling. The ketogenic diet is a common cancer therapy. In fact, the ketogenic diet has been tested in a variety of cancer animal models. The results of a ketogenic diet on its own vary depending on the kind of cancer, however the combination of a ketogenic diet with chemotherapy or radiation seems encouraging all pathways involved in the genesis, and pathophysiology of cancer. The evidence supporting the involvement of nutrition in cancer risk, development, and prognosis has a complex link. However, looking at the relationship between diet and cancer with the lens of microbes may give us new insights. This research found that combining a ketogenic diet with probiotics caused regression of breast cancer in mice. The combination of a ketogenic diet and probiotics has the potential to be utilized in the future development of anti-cancer nutrition. Understanding which microbial metabolites of dietary components provide the most anti-cancer benefits could aid in the development of new preventive or therapeutic strategies by optimizing exposure across individuals, either through microbiota manipulation or direct administration of bioactive microbiota-derived metabolites, also known as postbiotics. However, significant concerns about the relationships between food, microbiota, and cancer persist, posing obstacles to the development of preventive or therapeutic nutritional treatments for particular cancer types. At the most fundamental level, it is necessary to understand which components of microbial consortiums interact with certain dietary components to

form compounds with anti-cancer properties, and then to demonstrate their frequency throughout diverse human populations and across the lifetime. Determining the biological mechanisms by which the ketogenic diet and microbial metabolites regulate cancer-related pathways is also necessary for establishing bioavailability, effective intake levels, and identifying possible interactions with chemotherapies treatment. Developing human studies that stratify test populations based on their microbiota composition and ability to produce targeted metabolites, while continuing to investigate cellular-level interactions, are the next steps required to define and refine the microbiota as a hub between nutrition and cancer.

Acronyms, abbreviations, symbols. Breast cancer (BC), Ketogenic diets (KD), Beta-hydroxybutyrate (β-OHB), Phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), Breast cancer-associated gene 1 and 2 (BRCA1 and BRCA2), Human epidermal growth factor receptor 2 (HER2), Epidermal Growth Factor Receptor (EGFR), Cyclooxygenase-2 (COX-2), c-myelocytomatosis oncogene (c-Myc), Insulin-like growth factor (IGF-1), Adenosine triphosphate (ATP), Phosphate buffer solution (PBS), Unrestricted diet (SD), Interferon (IFN)-γ , Interleukin (IL), Alanine Transaminase (ALT), Aspartate Transaminase (AST), KetoCal (KC), High-density lipoprotein (HDL), low-density lipoprotein (LDL), Azoxymethane (AOM)

## Acknowledgements

The authors are grateful to the Applied Science Private University, Amman, Jordan, for the full financial support granted to this research project.

This study was conducted according to standard ethical guidelines. The Research and Ethical Committee approved all the experimental protocols at the Faculty of Pharmacy, Applied Science Private University (approval number: 2022-PHA-38).

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

This research project was fully financially supported by Applied Science Private University, Amman, Jordan.

AKSK and WHT both conceived and designed the study. AKSK carried out the study, interpreted the data, and drafted the manuscript. WHT supervised the study. Both authors read and approved the final manuscript to be published.

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