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Effect of synbiotic supplementation on fecal calprotectin levels and *lactic acid bacteria, Bifidobacteria, Escherichia coli* and *Salmonella* DNA in patients with cervical cancer

Luis Humberto de Loera-Rodríguez[1], Genaro Gabriel Ortiz[1], Paloma Rivero-Moragrega[1], Irma Ernestina Velázquez-Brizuela[1], Juan F. Santoscoy[1], Ana Rosa Rincón-Sánchez[2], Claudia Charles-Niño[2], José A. Cruz-Serrano[3], Alfredo de J. Celis de la Rosa[4], Fermín P. Pacheco-Moises[5] and Refugio Medrano-González[6]

[1]Laboratory of Development, Aging and Neurodegenerative Diseases. Neurosciences Division. Western Biomedical Research Centre. Mexican Social Security Institute. Guadalajara, Jalisco. Mexico. [2]Health Sciences Center. University of Guadalajara. East Guadalajara, Jalisco. México. [3]Kurago Biotech SA de CV. Zapopan, Jalisco. México. [4]Department of Public Health. University Center for Health Sciences. Guadalajara, Jalisco. México. [5]Department of Chemistry, Exact Sciences and Engineering Center. University of Guadalajara. Guadalajara, Jalisco. México. [6]Jalisco-Institute of Cancerology SSA. Guadalajara, Jalisco. México

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Correspondence: Genaro Gabriel Ortiz. Laboratory of Development, Aging and Neurodegenerative Diseases. Neurosciences Division. Western Biomedical Research Centre. Mexican Social Security Institute. Sierra Mojada, 800. 44340 Guadalajara, Jalisco. México

e-mail: genarogabriel@yahoo.com

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ABSTRACT

Background: patients with cervical cancer (CC) receiving chemotherapy and radiotherapy have several gastrointestinal adverse effects.

Objective: to evaluate the effect of dietary synbiotic supplementation on fecal calprotectin (FCP), bacterial DNA levels, and gastrointestinal adverse effects in patients with CC.

Methods: clinical, controlled, randomized, double-blind trial. Patients consumed synbiotics or placebo three times a day for seven weeks. FCP was assessed by Elisa method. DNA from probiotic and pathogenic bacteria were determined by quantitative real-time polymerase chain reaction. Diarrheal evacuations were evaluated with the Bristol stool form scale and nausea and vomiting were measured using the scale of the National Institute of Cancerology of the United States.

Results: after a seven-week treatment, FCP concentration was lower in the synbiotic group compared to the control group ($p < 0.001$). Stool consistency in the placebo and synbiotic groups was similar at baseline. A significant improvement in stool consistency was obtained in both groups at the end of the intervention ($p < 0.001$). The concentrations and total proportions of the probiotic and pathogenic bacteria were similar in both groups. Nausea significantly diminished in both groups ($p < 0.001$) at the end of the trial. Furthermore, the synbiotic group had a statistically significant decrease in the frequency and intensity of vomiting when compared to the control group ($p < 0.001$).

Conclusions: the synbiotic treatment decreases significantly the FCP levels and the frequency and intensity of vomiting in patients with CC.

Key words: Fecal calprotectin. Cervical cancer. Synbiotic. qPCR.

RESUMEN

Introducción: los pacientes con cáncer cervical (CC) tratados con quimioterapia y radioterapia tienen efectos gastrointestinales adversos (EGA).

Objetivo: evaluar el efecto de la suplementación dietética con simbióticos en la calprotectina fecal (FCP), el DNA bacteriano y los EGA en pacientes con CC.

Métodos: se realizó un ensayo clínico, aleatorizado y doble ciego. Los pacientes ingirieron simbióticos o placebo tres veces al día durante siete semanas. La FCP se evaluó mediante el método de ELISA. El ADN bacteriano se cuantificó mediante PCR en tiempo real. Las evacuaciones se evaluaron con la escala de Bristol y las náuseas y los vómitos se cuantificaron utilizando la escala del Instituto Nacional de Cancerología (USA).

Resultados: después de siete semanas de tratamiento, la concentración de FCP fue menor en el grupo tratado con simbióticos en comparación al grupo control ($p < 0,001$). La consistencia de las heces en los grupos tratados con placebo y simbióticos fue similar al inicio del estudio. Se obtuvo una mejora significativa en la consistencia de las heces en ambos grupos al final de la intervención ($p < 0,001$). Los niveles de las bacterias probióticas y patogénicas fueron similares en ambos grupos. Los casos de náuseas disminuyeron en ambos grupos ($p < 0,001$) y el grupo tratado con simbióticos tuvo una disminución significativa en la frecuencia e intensidad de los vómitos en comparación al grupo control ($p < 0,001$).

Conclusiones: el tratamiento simbiótico disminuye significativamente los niveles de FCP y la frecuencia e intensidad del vómito en pacientes con CC.

Palabras clave: Calprotectina fecal. Cáncer cervical. Simbióticos. qPCR.

INTRODUCTION

The International Agency for Research on Cancer of the World Health Organization (WHO) estimates that the incidence and the worldwide mortality of cancer are increasing. About ten million new cases of cancer are reported annually (1). Seventy-eight percent of the cases of CC occur in developing countries in which the use of preservatives is infrequent and early diagnostic testing, in general, is not available for the population (2). In México, CC is the second most common cause of malignant tumors in women ≥ 25 years old and the highest incidence rate is present in women older than 45 years, specifically in those between 60 and 64 years of age (incidence rate of 15.5), followed by those between 45

and 49 years of age (incidence rate of 12.6); in all cases, the rates are calculated for every 100,000 women more than ten years of age (3).

CC initiates as a cellular dysplasia of the cervix epithelium and has been found to be highly associated to the human papilloma virus (HPV), which is able to cause genetic transformation in the epithelial cells of the cervix, through the expression of proteins E6 and E7 which interact with p53 and RB proteins. Thus, affecting the cell cycle (4).

CC is classified histologically as: epidermoid carcinoma, adenocarcinoma, and adenosquamous carcinoma. There are several classifications of the clinical staging. The most commonly used is the classification established by the International Federation of Gynecology and Obstetrics (FIGO) (5). The treatment of CC depends on the stage of the disease and includes surgery, chemotherapy and radiation. However, chemotherapy and radiotherapy have serious side effects. For instance, 5-15% of patients treated with radiation will develop enteritis, which persists months or even years after therapy is completed (7).

About 5% of the total protein contents of leukocytes and 30-60% of their cytosolic protein is calprotectin (CP). In infectious and inflammatory processes, plasma CP levels increase 5 to 40 fold above normal values (6-8). The FCP is a marker strongly associated with colorectal inflammation; when fecal CP/plasma CP ratio is higher than 6, it has a 100% sensitivity and 94% specificity in the diagnosis of enteritis (6-8). For this reason, FCP level has been chosen in this study as a measurement of the enteric inflammatory status.

Synbiotics are products that contain both prebiotics and probiotics (*Bacteroides, Eubacterium, Lactobacilli* and *Bifidobacteria*), which are known to exert various health benefits, such as immunomodulation, reduction of glucose and cholesterol, inactivation of carcinogens, anti-inflammatory and maintenance of intestinal integrity (9,11). Prebiotics, such as inulin and oligofructose, are substances that stimulate the growth of non-pathogenic bacteria such as *Lactobacilli* and *Bifidobacteria*, which inhibit the growth of pathogenic bacteria, thus decreasing inflammation. Oral supplementation with synbiotics has been shown to reduce the inflammatory process in intestinal diseases such as interspecific chronic ulcerative colitis, Crohn's disease and radiation enteritis (12-14). In

addition, prebiotics such as oligofructose-enriched inulin are associated with an early reduction in FCP (14,15). Therefore, the aim of this work is to assess the effect of dietary synbiotic supplementation on FCP, bacterial DNA levels, and gastrointestinal adverse effects in patients with CC who were treated with chemotherapy and radiotherapy at the Jalisco Institute of Cancerology (IJC).

MATERIAL AND METHODS

Study design

This study was a randomized, double-blind, controlled trial in patients with the diagnosis of CC, histologically classified as adenocarcinoma or epidermoid, in stage II and III of FIGO, who were currently in treatment with chemotherapy and radiotherapy at the Jalisco Institute of Cancerology. The size of the sample was obtained with the calculation of the formula when it is expected to find a difference in averages and the standard deviation is known, given a 95% reliability and a power of 80%, using as a variable the fecal calprotectin (16). All participants were fully informed of the study procedures and provided written informed consent. This study was performed according to the updated Declaration of Helsinki, the General Health Law on Research for Health and the Guidelines for Good Clinical Practices in Research for Health 2012. All procedures were approved by the Committee of Ethics, Teaching and Research of the Jalisco Institute of Cancerology (Registry number 008/2011). Identification numbers were assigned to assure patient confidentiality. Patients were randomly assigned in a 1:1 ratio to receive synbiotic treatment or placebo, with a computer-generated randomization sequence. To ensure masking between the synbiotic and placebo, gels were identical in appearance, packaging, and labeling. The synbiotic used contained: 1 x 10^7 colony-forming unit (CFU)/g biogel of *Lactobacillus acidophilus* NCFM, *Bifidobacterium lactis* Bi-07 1 x 10^6 CFU/g biogel, and blue agave inulin.

These patients were divided into two groups: 35 patients received the synbiotic supplement and 35 received placebo. Both groups received 20 g gels; patients had to ingest one gel 30 minutes before breakfast, one gel 30 minutes before lunch and one gel

30 minutes before dinner, for seven weeks. The diet of each patient was monitored every 15 days to ensure that no diet modifications were done throughout the intervention. This was supervised by the nutritionist's team at the IJC.

Patients were randomly assigned in a 1:1 ratio to receive oral synbiotics or placebo, with a computer-generated randomization sequence (blocks of 2-4). To ensure masking between the synbiotics and the placebo, gels were identical in appearance, packaging and labeling.

Sample collection

Fecal samples (> 10 g) were collected from each participant in a sterile container at 0, 4 and 7 weeks. Fecal samples were immediately frozen at -70 °C until use.

Determination of fecal calprotectin

Fecal calprotectin was quantified using the enzyme-linked immunosorbent assay, with a highly specific monoclonal antibody for calprotectin, known as the rapid test of calprotectin Quantum Blue®. The assays were performed according to the manufacturer's instructions. The signal intensities of the samples were measured quantitatively by the Quantum Blue® reader from BÜHLMANN (9).

DNA extraction and real-time PCR

The proportions of bacteria of interest in stool samples were determined using the ZR Fecal DNA MiniPrep™ kit (catalog no. D6010, Zymo research) according to the manufacturer's instructions. For the amplification of the specific genomes of the bacteria of interest, the oligonucleotides described elsewhere were used (17,18). The oligonucleotides were synthesized by the company Invitrogen and working solutions were prepared at 2.5 pmol/μl, as indicated previously (17,18).

Determination of gastrointestinal side effects

The instrument validated to measure the characteristics of diarrheal evacuations in patients was the Bristol stool form scale (BSFS) and nausea and vomiting was measured

with the INC (scale of the National Institute of Cancerology of the United States). The BSFS is a diagnostic medical tool designed to classify the form of human feces ranging from the hardest (type 1) to the softest (type 7). Types 1 and 2 represent hard stools and a slow transit (constipation). Types 3, 4 and 5 are usually considered to be the most "normal" stool form. Types 6 and 7 are considered as abnormally loose/liquid stools (and in conjunction with other symptoms, indicative of diarrhea) (19).

Adhesion to treatment

Participants reported daily consumption of the supplement in a consumption posting sheet. The percentage adherence for each subject was determined by the following formula: (number of gels consumed) / (number of gels returned to the physician) × 100. We considered it as an optimal adherence if the percentage was higher than 80%.

Statistical analysis

The statistical analysis was performed with the Microsoft Excel system (Windows 8), and with the Statistical Package for Social Sciences (SPSS) in its version 20.0. Results are presented in graphs and comparative tables for each of the variables using descriptive statistics, univariate and multivariate contrasts, parametric statistics (Student's t-test and ANOVA), non-parametric statistics (Friedman, Wilcoxon, Mann-Whitney and Kolmogorov-Smirnov tests). With regard to the sociodemographic characteristics of the patients, such as marital status, number of sexual partners, place of origin and occupation, they were also performed and analyzed with both programs (Excel and SPSS), making the descriptive statistics with their respective case numbers, as well as the total percentages for both groups with the respective p value.

RESULTS

Sociodemographic and clinical data

At the beginning of the study, a total of 165 medical records of patients with CC at the JIC were reviewed. Ninety-seven patients met the inclusion criteria for the study. Seventeen

patients were excluded from the study: five patients were taking dietary supplements, five died, and seven refused to sign the informed consent, leaving a total of 80 patients. During the study, five patients in each group were removed from the protocol due to changes in treatment, poor adherence to the supplement consumption (< 80%) and presentation of side effects other than nausea and diarrhea.

Patients in both groups had similar sociodemographic (Table I) and clinical (Table II) characteristics at baseline. The mean age of both groups of treatment was similar ($p = 0.441$). No significant differences were found in the marital status ($p = 0.586$), occupation ($p = 0.586$), clinical staging ($p = 0.502$) and histological variant ($p = 0.374$) between both groups (Table II).

Outcomes measurements

A significant increase in the level of FCP was observed at week 7 in the placebo group ($p < 0.001$) at the end of the intervention. In contrast, a significant decrease in the level of fecal CP was detected in the synbiotic group at week 7 ($p < 0.001$) (Fig. 1).

Stool consistency in the placebo and synbiotic groups was similar at baseline. A significant improvement in stool consistency was obtained in both groups at the end of the intervention ($p < 0.001$). However, there is no statistical significance between both groups after a seven-week treatment (Fig. 2).

The levels of nausea in patients with cervical cancer treated with placebo and synbiotics were compared before, during and after the intervention and they were statistically similar. The levels of nausea the patients presented during the intervention significantly diminished in both groups; a $p < 0.001$ was obtained using the Friedman and Wilcoxon tests (Fig. 3).

We report the number and percentage of cases according to the degree of vomiting in patients with CC treated with placebo or synbiotics. In the control group, vomiting results for grade 2 in 75% of patients before treatment, and this effect was subsequently present in only 10% of the patients. For grade 1, corresponding to the presence of a vomiting in 24 hours, there was an increase of 25% of the patients to 90%, which showed improvement

in relation to this side effect. In the other group treated with synbiotics, for vomiting grade 2, 63.9% of the patients presented it before treatment, and after the intervention only in 5.6% of the patients, for grade 1 vomiting occurs in 36.1% of the patients before treatment, for 94% of the patients after the intervention. With the Chi-square test analysis, the comparison of the degree of vomiting between treatments at each of the evaluated moments was significant only during the treatments ($p = 0.025$); the Friedman test and the Wilcoxon test were significant for both treatments ($p < 0.001$) (Fig. 4).

The results of the qPCR in this study did not show significant changes in relation to the concentrations and total proportions of the probiotic bacteria: *Acid lactic* and *Bifidobacteria versus* the pathogenic bacteria, *E. coli* and *Salmonella,* both in the placebo group and the synbiotic group (Fig. 5).

DISCUSSION

The average age of the patients enrolled in this study was 49 years and was similar to the results published previously for Mexico (4) and the statistics published by the WHO, in which the highest prevalence was found between the ages of 35-50 (WHO 2016). Sociodemographic data showed that 71% of the patients enrolled in this study have a low educational level and 85% do not have a job. These data coincide with studies reporting that a low socioeconomic level is a risk factor for developing CC. Regarding the number of sexual partners of the patients, this study reports that two patients were nubile, 48.5% had one sexual partner, 40% had two to five sexual partners, and 8.5% had more than five sexual partners. These data are in consonance with those previously reported, suggesting that having multiple sexual partners is a risk factor for CC (4).

In this study, FCP levels at the end of the clinical assay were found to increase significantly in the placebo group. In contrast, a significant decrease of calprotectin levels was found in the synbiotics group ($p < 0.001$) (Fig. 3). Fecal calprotectin is a non-invasive marker for the evaluation of the excretion of macrophages in the intestinal lumen and is strongly associated with colorectal inflammation with 100% sensitivity and 94% specificity (22).

The FCP values were used to determine response to treatment and to evaluate asymptomatic patients, as well as to predict relapses of inflammatory bowel disease, taking into consideration that the reference values for healthy individuals are < 50 μg/kg of wet feces. Calprotectin levels ranging 100-500 μg/kg of wet feces are indicative of colorectal cancer, and 200-20,000 μg/kg of wet feces are characteristic of chronic inflammatory bowel disease (23).

The anti-inflammatory effect of the synbiotic treatment is demonstrated by the decrease in the concentration of FCP. In fact, it has been proposed that calprotectin level is the surrogate marker for treatment outcome in relapsing inflammatory bowel disease.

There is increased awareness of the role of the microbiota in maintaining health, since gut microbes are capable of producing a vast range of essential molecules for the human metabolism. For instance, many enzymes produced by microbes influence digestion and health. Therefore, it has been suggested the consumption of synbiotics, lactic ferments of probiotics associated with prebiotic substances such as vitamins and fructo-oligosaccharides, to strengthen the immune system (12,20,21). Probiotic bacteria are effective when they demonstrate: a) effective cell adhesion, which impedes the adhesion of pathogenic bacteria; b) resisting the medium and multiplying; c) being safe, non-invasive, and non-cancerous; and d) living together with the saprophyte microbiota (11,12,20).

The mechanisms of action of probiotics in the human organism is to competitively exclude the enteric pathogens, promote the synthesis of cytokines, adhere to the surface of intestinal cells, produce toxic metabolites such as hydrogen peroxide, neutralize dietary carcinogens, inhibit bacterial growth through the production of lactic acid and bacteriocins, reestablish the saprophytic bacterial microbiota after the antibiotic therapy, and stimulate trophic enterocyte (12,20). It is advisable to incorporate synbiotic foods into the diet in order to strengthen the immune system and inhibit the development of cancer. The synbiotics act as inhibitors of certain oncogenes, and as modulators of the immunological response to combat the infectious agents that have colonized the gastrointestinal tract (20). The groups of pathogenic bacteria with the greatest

antagonism against probiotic bacteria are *E. coli* and *Salmonella* species, a situation that was considered in this study to determine and quantify the interaction of these bacterial groups against the probiotic bacteria such as *Lactobacilli* and *Bifidobacteria* (12,20).

In a previous study using an endotoxic shock model, it was found that synbiotic treatment has beneficial effects. For instance, synbiotic treatment improved the survival rate of lipopolysaccharide-treated rats, ameliorated the clinical symptomatology, reduced the production of serum proinflammatory cytokines (TNF-α, IL-6, IL-1β), and preserved the mitochondrial membrane fluidity and ATPase activity (24). In a recent, randomized, double-blind, placebo-controlled clinical trial conducted in two public hospitals, it was found that the administration of a synbiotic gel is a safe and simple way to get a significant reduction in prevalence and monthly episodes of vomit, heartburn, and stomachache, as well as a significant decrease in gastrointestinal symptoms severity in hemodialysis patients (25). Furthermore, short-term synbiotic treatment in patients with end-stage renal disease can lead to the increase of *Bifidobacterium* counts, maintaining the intestinal microbial balance (26).

Our data support the potential prophylactic role of synbiotics for the treatment of some gastrointestinal side effects of radiotherapy and chemotherapy in patients with CC. However, it is necessary to do longer studies to investigate if the treatment with synbiotics improves the levels of probiotic bacteria in these patients.

There were some limitations in this study: one was the sample size to evaluate the microbiological outcomes and, regarding patient care, it lacks long term follow-up evaluations. It is important to mention that we are currently starting a new project with a microarray assay where we will directly analyze the microbiota before, during and after the treatment with these synbiotics.

CONCLUSION

In this study, we found the possibility of reducing some side effects of chemotherapy and radiotherapy in CC patients with the supplementation of synbiotics. Synbiotic treatment reduces the level of fecal CP, as well as the frequency and intensity of vomiting.

CONFLICT OF INTEREST

The synbiotic supplement gel and the placebo gel were kindly provided free of charge by Kurago Biotech SA de CV.

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Table I. Sociodemographic characteristics of patients with cervical cancer treated with placebo or synbiotics

Characteristics	Synbiotic		Placebo		Total		p-value
	n	%	n	%	n	%	
Civil status							
Single	9	25.7%	1	2.9%	10	14.3%	0.586
Free union	4	11.4%	3	8.6%	7	10.0%	
Married	11	31.4%	18	51.4%	29	41.4%	
Separated	4	11.4%	5	14.3%	9	12.9%	
Widow	7	20.0%	8	22.9%	15	21.4%	
Total	35	100.0%	35	100.0%	70	100.0%	
Number of sexual partners							
0	0	0.0%	3	8.6%	3	4.3%	0.444
1	19	54.3%	21	60.0%	40	57.1%	
2-3	12	34.3%	7	20.0%	19	27.1%	
4-5	3	8.6%	2	5.7%	5	7.1%	
> 5	1	2.9%	2	5.7%	3	4.3%	
Total	35	100.0%	35	100.0%	70	100.0%	
Birthplace							
Guadalajara metropolitan area	9	25.7%	5	14.3%	14	20.0%	0.544
Interior of the State	12	34.3%	15	42.9%	27	38.6%	
Other States	14	40.0%	15	42.9%	29	41.4%	
Total	35	100.0%	35	100.0%	70	100.0%	
Occupation							
Home	31	88.6%	31	88.6%	62	88.6%	0.586
Worker/merchant	3	8.6%	4	11.4%	7	10.0%	
Professional	1	2.9%	0	0.0%	1	1.4%	

Total	35	100.0%	35	100.0%	70	100.0%

Table II. Baseline clinical characteristics of patients with cervical cancer treated with placebo or synbiotics

	Placebo n = 35	Synbiotic n = 35	p-value
Age, years	48.6 ± 14.7	51.2 ± 14.7	0.441
FIGO stage	II (77.1%), III (22.8%)	II (71.4%), III (28.6%)	0.502
Histological type	Adenocarcinoma (20%) Epidermoid (80%)	Adenocarcinoma (17.1%) Epidermoid (82.9%)	0.374
Diabetes mellitus	17.5%	11.1%	0.429
Hypertension	27.5%	16.7%	0.258
Anemia	50.0%	63.8%	0.445
Leukocytosis	22.5%	22.2%	0.977
Hyperglycemia	30.0%	33.4%	0.874
Hypercholesterolemia	20.0%	25.0%	0.267

Data are expressed in number, percentage or mean and standard deviation. Student's t-test, Chi-square test or the Fisher's exact test were used.

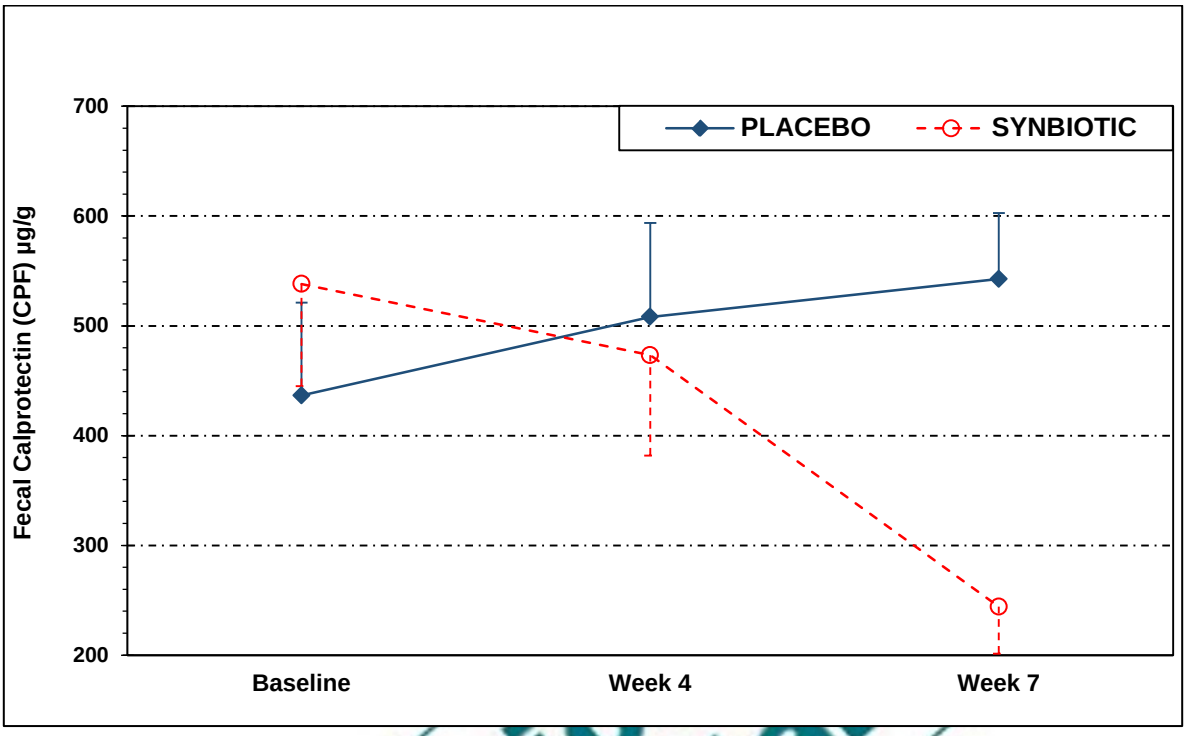

Fig. 1. Fecal calprotectin levels in synbiotic and placebo groups.

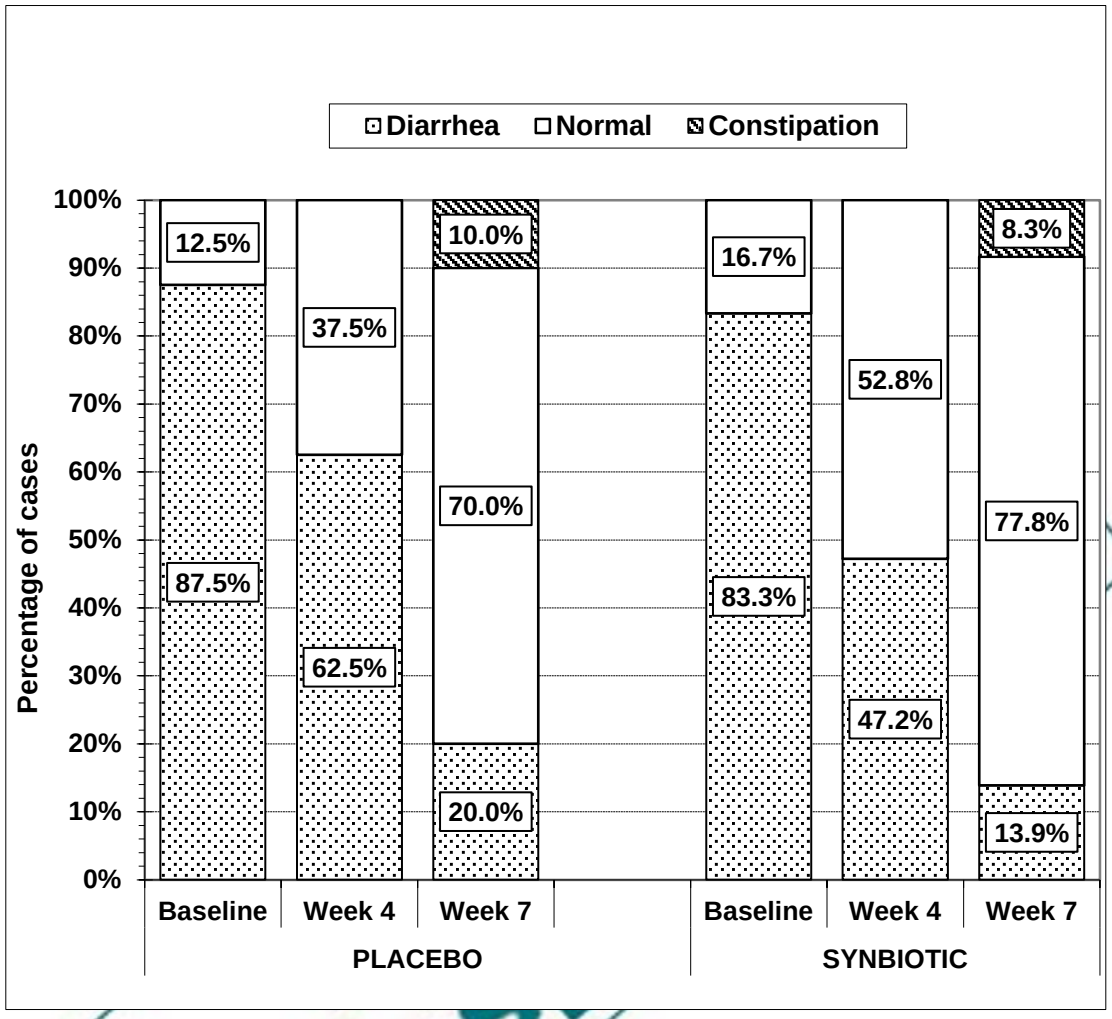

Fig. 2. Percentage of cases according to the categories of the Bristol stool form scale in patients with uterine cervical cancer treated with placebo or synbiotics.

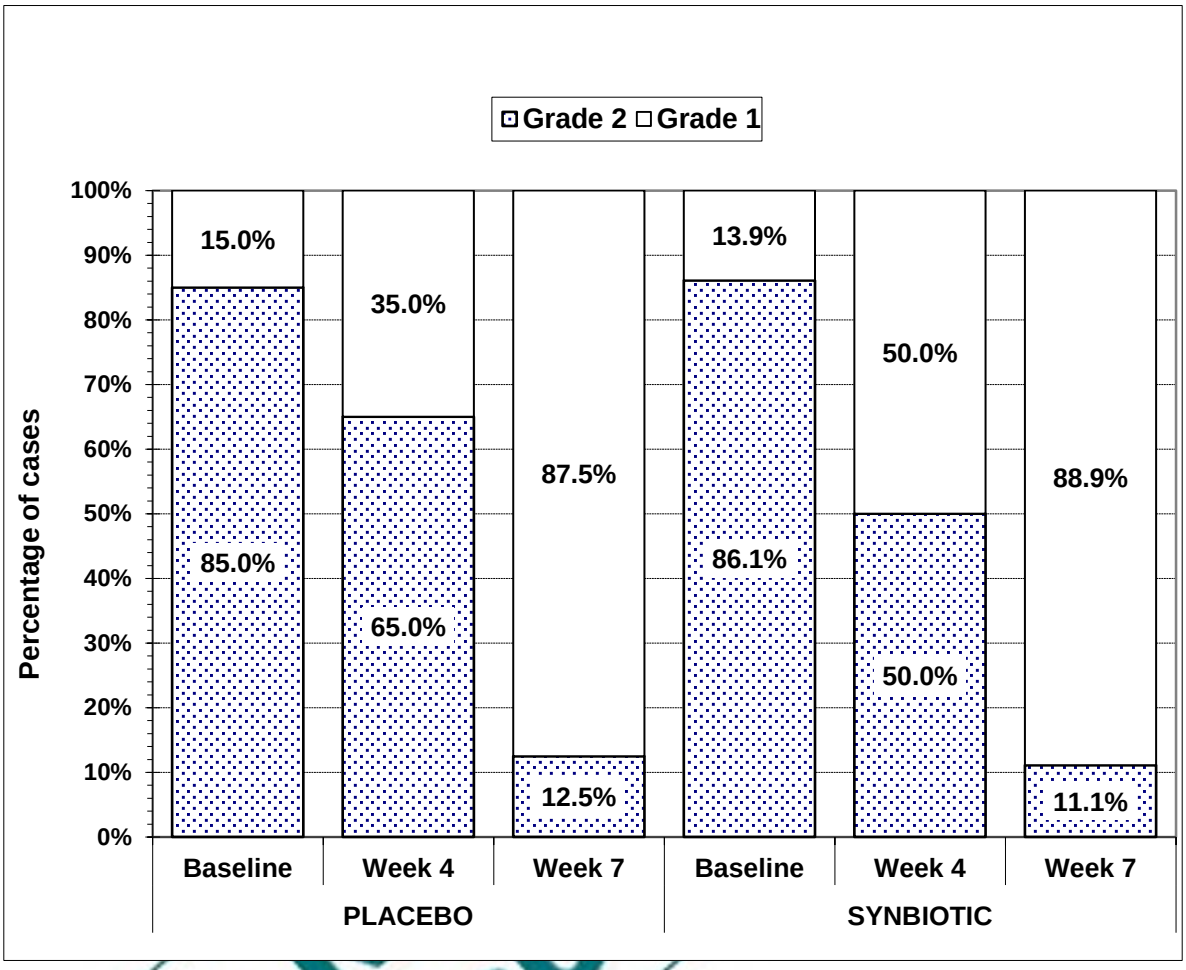

Fig. 3. Percentage of cases according to the scale of nausea in patients with uterine cervical cancer treated with placebo or synbiotics.

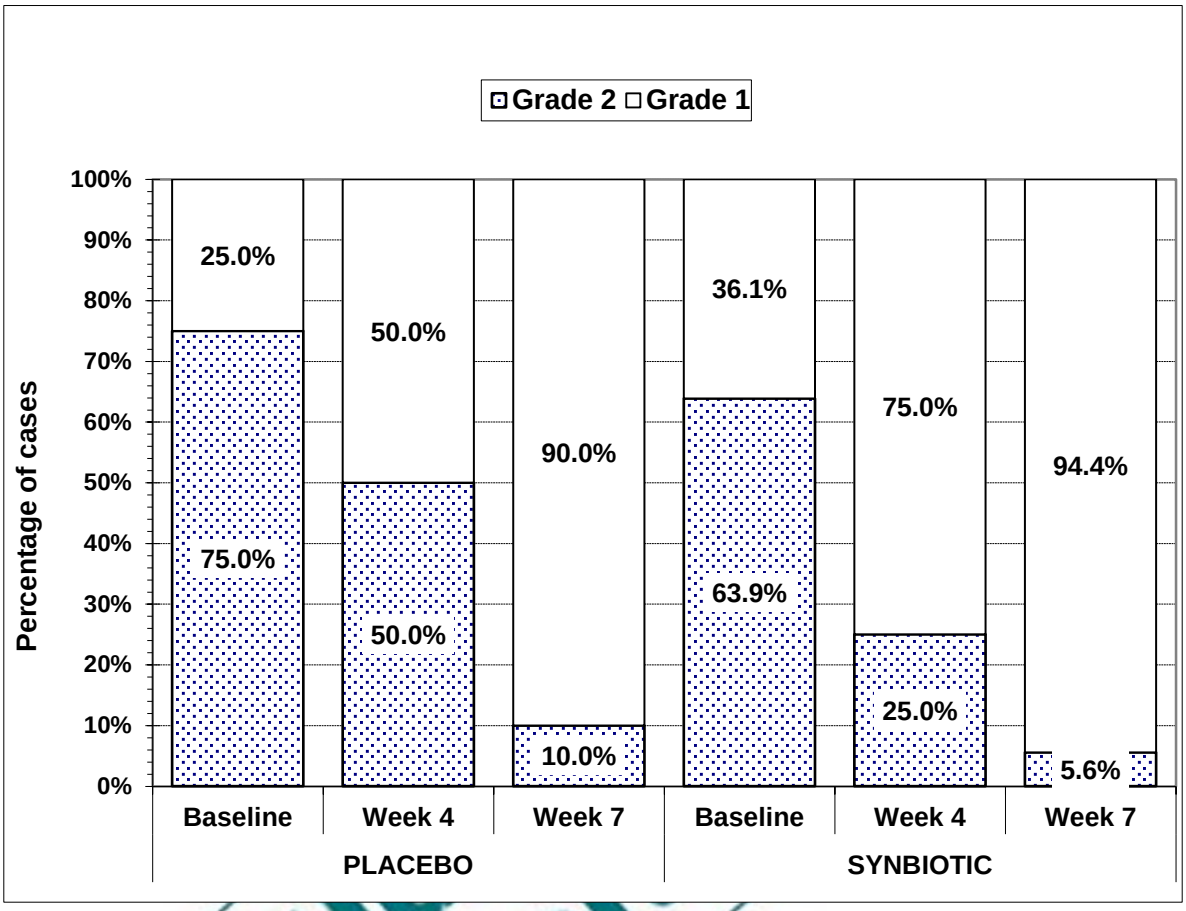

Fig. 4. Percentage of cases according to the scale of vomiting in patients with uterine cervical cancer treated with placebo or synbiotics.

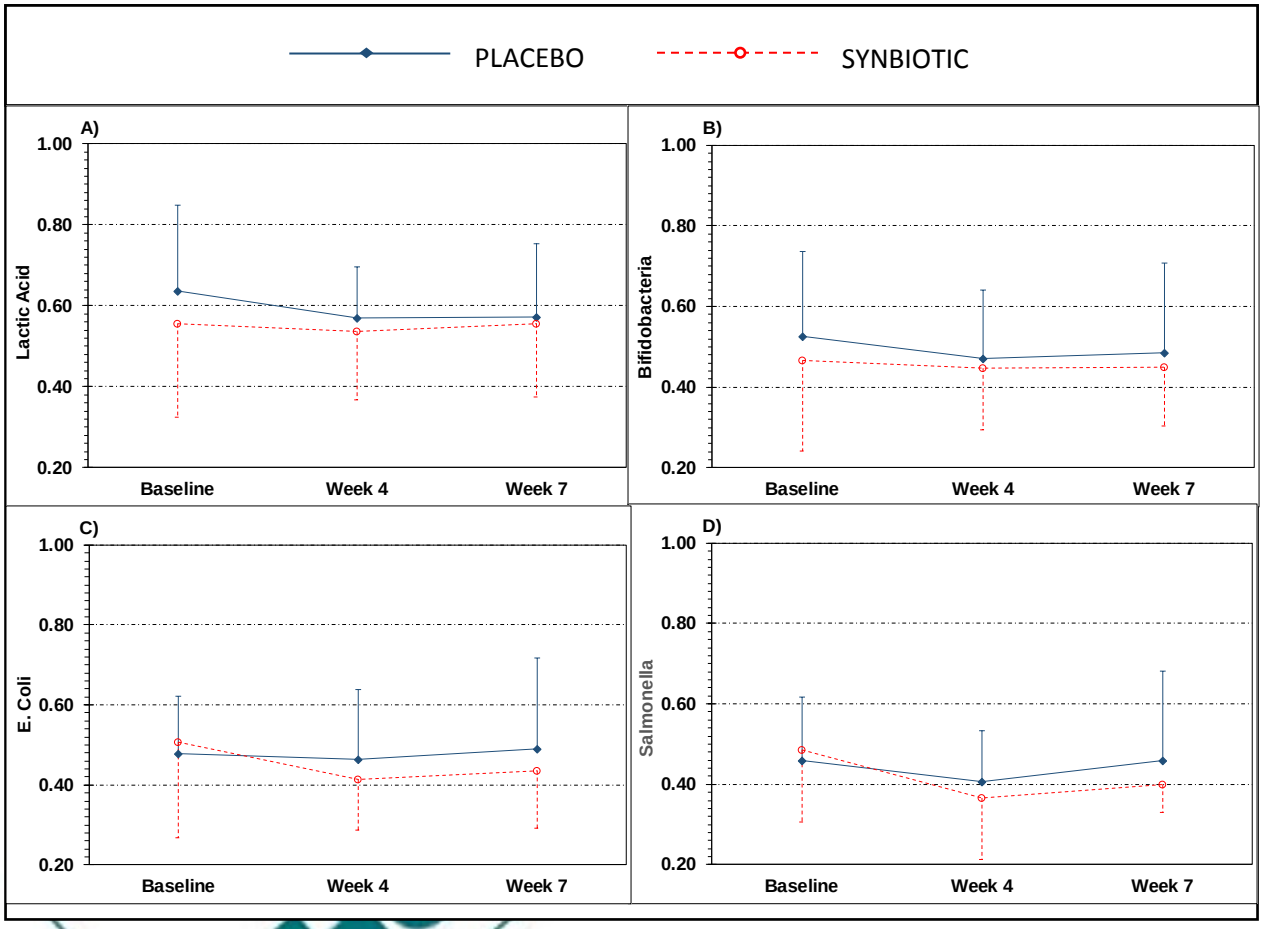

Fig. 5. Proportion of *Lactic acid bacteria* (A), *Bifidobacteria* (B), *E. coli* (C), and *Salmonella* (D) in samples from patients with uterine cervical cancer treated with placebo or synbiotics.