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Use of *Lactobacillus* and *Bifidobacterium* Gut Microbiota Counts as an Indicator of Cancer Presence and Chemotherapy Effect

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Abstract

Globally, cancer has been estimated to cause about 13% of all deaths in the world, especially in developing countries. Cancer cells are abnormal cells that disrupt the process of cell division, and there are more than 100 types of cancer. The diagnosis of cancer is still the most important factor in the success of cancer treatment. Treatment strategies of cancer may include radiation, chemotherapy, and surgery. This study was conducted to estimate microbiota counts in cancer patients, finding a method for detection and treatment follow up. *Lactobacillus* and *Bifidobacterium* counts were determined by qPCR depending on the standard curve from known bacterial counts obtained from probiotics. The main results of this study showed that the *Lactobacillus* count significantly increased in newly diagnosed cancer patients, while the count of *Bifidobacterium* decreased compared to the control. Also, chemotherapy led to a decrease in both bacteria counts, which may suggest supporting chemotherapy with probiotics.

Keywords: cancer, microbiota, *Lactobacillus*, *Bifidobacterium*, absolute qPCR

Резюме

В световен мащаб се счита, че ракът причинява около 13% от всички смъртни случаи, особено в развиващите се страни. Раковите клетки са анормални клетки, които нарушават процеса на клетъчно делене. Съществуват повече от 100 вида рак. Диагнозата рак все още е най-важният фактор за успеха на лечението на рак. Стратегиите за лечение на рак могат да включват радиация, химиотерапия и хирургия. Това проучване е проведено за оценка на микробиота при пациенти с рак, за да се намерен метод за откриване и проследяване на лечението. Броят на лактобацилите и бифидобактериите е определено чрез qPCR в сравнение със стандартна крива от известния брой бактерии, установен в пробиотици. Основните резултати от това проучване показват, че броят на *Lactobacillus* значително се увеличава при новодиагностицирани пациенти с рак, докато броят на *Bifidobacterium* намалява в сравнение с контролата. Също така, химиотерапията доведе до намаляване на броя на бактериите, което може да предполага поддръжка на химиотерапия с пробиотици.

Introduction

The cancer disease is characterized by the random division and conversion of atypical cells and it is a major cause of mortality and morbidity, with about 600,000 of most cancer deaths recorded in the United States alone, and 1.7 million newly identified cases (Hanahan and Coussens 2012). The complicated interactions between cells surviving genetic damage and their macro- and micro-environments are referred to as cancers of tissues (Siegel *et al.,* 2016). Further to the severe suffering inflicted, most cancer cases impose a significant financial burden, whereby $125 billion is spent per year in the United States, exceeding health care costs (Mariotto *et al.,* 2011).

Intestinal microbiota is concurrent with the human host to influence the host's physiology and digestion in various capacities. Undoubtedly, the host and microbiota are in agreement with each other. Specifically, the metabolic movement of gut microorganisms was proposed to work as an assistant, a real organ (Dibaise *et al.,* 2012). *Lactobacillus*, a genus of anaerobic or small-scale aerophilic, gram-positive heterogeneous lactic acid-producing

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microorganism is widespread in the intestinal microbiota of healthy adults. Neonates receive lactobacilli at birth by exposure to vaginal lactobacilli (Salami *et al.,* 2012). Acidic and succinic acids are also produced, however, in little amounts. Lactic acid during glucose fermentation is the major metabolic and final product of lactobacilli (Slover *et al.,* 2008). It has also been reported that lactobacilli help the body to resist illnesses such as inflammatory bowel disease (IBD), celiac disease (Lieske *et al.,* 2005; Schmidt *et al.,* 2010), type 1 diabetes (T1D) (Petrovsky, 2010; Roesch *et al.,* 2009), and multiple sclerosis (MS) (Salami *et al.,* 2012). *Bifidobacterium* was initially isolated from a healthy breastfed infant in 1899 by the method of Tissier at the Pasteur Institute, France. They are anaerobic gram-positive, non-spore-forming pleomorphic bacilli, that were initially named *Bacillus bifidus* communis (Ishibashi *et al.,* 1997; Bevilacqua *et al.,* 2012). It has been proven that bifidobacteria have various health benefits, which include the elimination of procarcinogens, immunomodulation, diarrhea, prevention of infections of the intestinal tract, vitamin production, production of antimicrobials against severe intestinal bacteria, fortify the mucosal epithelium barrier against the invasion of pathogenic bacteria, and alleviation of constipation (Wasilewska and Bielecka, 2003).

This study was conducted to find a correlation between cancer and microbiota counts, especially *Lactobacillus* and *Bifidobacterium*, by absolute qPCR.

Materials and Methods

Collection of samples

In this experimental study, 50 stool samples were collected and distributed as follows: 25 samples from cancer patients, and 25 samples from apparently healthy persons. The stool samples were collected from individuals between ages 9-67 years during the period from July 2017 to May 2018. Cancer patients were diagnosed with cancer at the Ramadi teaching hospital.

Extracting DNA from stool samples

Total DNA was extracted from stool samples of patients and controls by AccuPrep Stool DNA Extraction Kit from Bioneer Cat. number (K-3036), as described by the instruction manual. The extracted samples were checked for purity and concentration using NanoDrop.

Primers

The primers for detection of normal *Lactobacillus* and *Bifidobacterium* flora (Table1) were designed according to the sequence of specific genes obtained from NCBI (https://www.ncbi.nlm.nih.gov/) by the online tool Primer3^{+} (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/).

Standard curves construction for bacteria

The standard curve method is generally established on the Ct values of each, an input set of known DNA concentrations or a dilution series of a reference DNA sample. The standard curves were designed for *Lactobacillus and Bifidobacterium* bacteria through DNA extracted from probiotics capsules obtained from Protexin Pharmaceuticals. Each capsule contained 200 million bacterial cells. A 10-fold serial dilution of the extracted DNA, extending from 1×10^{5} to 1×10^{9}, was utilized to develop the standard curves for both *Lactobacillus* and *Bifidobacterium*. The values of CT in dilutions were estimated by absolute qPCR with the *Lactobacillus* and *Bifidobacterium* to generate the standard curves for both bacterial species. The initial template logarithm of their copy numbers was plotted against the CT values. A standard curve was created through plotted points linear regression of each bacteria.

Estimation of bacterial numbers

The bacterial numbers for both *Lactobacillus* and *Bifidobacterium* for patients and control stool samples were determined by qPCR, depending on the standard curve using a ready-to-use premix real-time PCR kit with the following conditions: predenaturation at 95℃ for 5 min, then denaturation T 95℃ for 20 sec followed by annealing\extension at 55-60°C for 40-45 sec.

Data analysis

The free online tool SPSS v.22 was used to analyze the current study data. Nominal data were described by number and percentage and compared

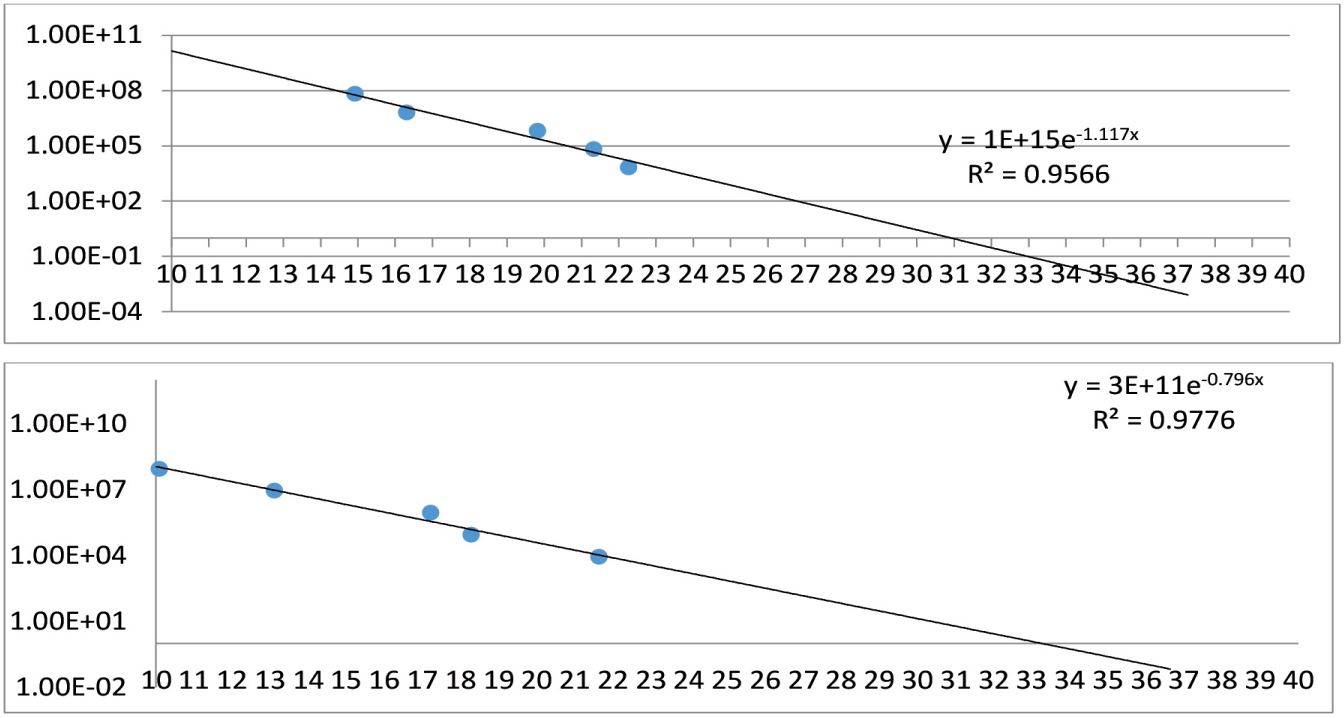
Table 1. Primer sequence and annealing temperature

Bacteria	Primer	Sequence 5—3	Annealing Temperature	Reference
Lactobacillus	Lac F	TGGAAACAGGTGCTAATACCG	58	This study
	Lac R	CCATTGTGGAAGATTCCC		
Bifidobacterium	Bif F	CCACCGTTACACCGGGAA	62	This study
	Bif R	GGGTGGTAATGCCGGATG		

using X^2. Mean ± SD was used for numeric data. While two numeric variables comparison was done by T-test, the ANOVA F test was used to compare three or more numeric variables. A significance level of of α=0.05 was applied to the test.

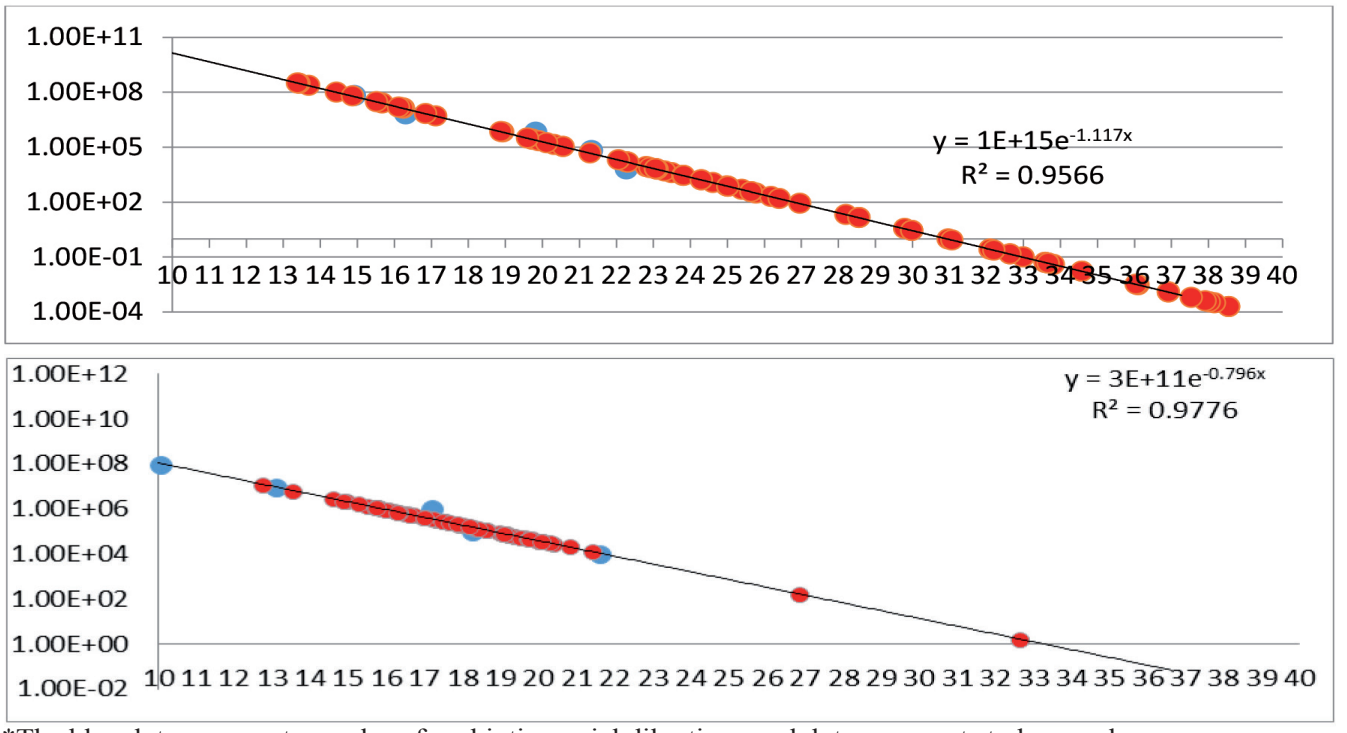
Results

A real-time PCR experiment was performed and a standard curve was graphed (Fig. 1a and b). The CT values in the results for *Lactobacillus* were shown sequentially from the highest concentration to the lowest concentration, as follows:10.09, 13.11, 17.22, 18.28, 21.64. Moreover, the CT values in the results for *Bifidobacterium* were shown sequentially from the lowest concentration to the highest concentration, as follows: 14.92, 16.31, 19.82, 21.33, 22.27.



*The blue dots represent samples of probiotics serial diluutions

Fig. 1. Absolute real-time PCR standard curves: (a) *Bifidobacterium*, (b) *Lactobacillus*



*The blue dots represent samples of probiotics serial diluutions, red dots represent study samples

Fig. 2. Estimation of bacterial numbers by absolute curve: (a) for *Bifidobacterium*, (b) for *Lactobacillus* standard samples blue, samples of the trial under study red dots

All the results and the numbers mentioned in the standard curve experiment were calculated on the basis that the sample taken for probiotics and DNA extraction was from 200 mg feces.

The number of bacteria was calculated automatically by a PCR device based on the standard curve that wasgenerated from the standard samples whose numbers were already known. The process was based on the threshold cycle (Ct) value that the device read, as shown in Fig. 2a and b.

The results obtained in this studyshowed a difference in the mean values of the number of Lactobacillus bacteria between the two groups of samples. The mean value ranged from 1145452.95 to 610680.26. Likewise, a difference was observed in the mean values of the number of Bifidobacterium bacteria between the two groups of samples. The mean value of the Bifidobacterium count ranged from 63405999.00 to 737988.22 as shown in Table 2.

The study results showed that the number of *Lactobacillus* bacteria was affected by cancer, also the count connotes the stages of chemotherapy taken by the patient in the treatment, as shown in Table 3.

A difference in the mean value of the *Lactobacillus* bacteria number was shown between the three groups of chemotherapy stages. The mean value ranged from 3943762.84 to 515170.73. Comparing the mean value of *Lactobacillus* in the treatment stage group (Table 3) with the control group (Table 2) significant differences were observed in the number of *Lactobacillus* bacteria in the recently diagnozed patients in comparison with the other two cancer treatment sample groups (Table 3).

An increase was observed in the mean value in the case of the recently diagnozed group compared to the control group. As for the mean value of the dose phase group and the doss completed group, its values were near the control group values**.**

The number of *Bifidobacterium* was affected by cancer. Also, the count relates to the stages of chemotherapy treatment received by the patient, as shown in Table 4.

Table 4. Concentration of *Bifidobacterium* by stage of cancer treatment (Recent diagnosis, Doses phase, Doses finished)

Cancers types	N	Concentrations of bacteria	
		Bifidobacterium spp.	
		Mean	SD
Recent diagnosis	4	3906720.94 [a] A	7488592.20
Doses phase	13	21619.06 [c] B	45535.76
Doses finished	5	65561.86 [b] B	74415.51
Total	22	737988.22	3217361.60

*Small letters compared vertically between groups.

Differences in the mean value of the *Bifidobacterium* number were observed between the three groups of chemotherapy stages. The mean value ranged from 3906720.94 to 21619.06. The numbers of *Bifidobacterium* between all three groups at

Table 2. Concentration of bacteria by sample group (natural, cancer, diabetes, and hypertensive patients).

Types	N	Concentrations of bacteria			
		Bifidobacterium spp.		*Lactobacillus spp.*	
		Mean	SD	Mean	SD
Normal	17	63405999.00 [a] A	110063588.90	610680.26 [a] B	718968.86
Cancers	22	737988.22 [b] B	3217361.60	1145452.95 [b] A	2387038.08

*Small letters compared vertically between groups, Capital letters compared horizontally between two types of bacteria for each disease.

Table 3. Concentration of *Lactobacillus* in the stages of cancer treatment (Recent diagnosis, Doses phase, Doses finished)

Cancer types	N	Concentrations of *Lactobacillus spp*.	
		Mean	SD
Recent diagnosis	4	3943762.84 [a]	5088436.21
Doses phase	13	515170.73 [b]	499836.55
Doses finished	5	545538.80 [b]	417258.02
Total	22	1145452.95	2387038.08

*Small letters compared vertically between groups

the different stages of cancer treatment (Table 4) showed statistical differences, comparing the mean value of *Bifidobacterium* in the treatment stage group (Table 4) with the control group in Table 2.

There was an increase observed in the mean value of the recently diagnozed group compared with the number of bacteria in the control. As for the mean values of the dose phase group and the doses finished group, their values showed a strong decrease in the number of bacteria in comparsion with the control.

The current study results showed that the mean values for *Bifidobacterium* and *Lactobacillus* were very close in the patients with recent diagnosis. The mean values for *Bifidobacterium* and *Lactobacillus* were 3906720.94 and 3943762.84, respectively. *Bifidobacterium* and *Lactobacillus* numbers showed no significant differences at the recent diagnosis stage. In the case of doses phase, the mean value of *Lactobacillus* was higher than that of *Bifidobacterium* bacteria. The mean value for *Bifidobacterium* and *Lactobacillus* bacteria were 21619.06 and 515170.73, respectively. The numbers of *Bifidobacterium* and *Lactobacillus* at the doses stage differed significantelly. While in the case of finished dose, the mean value of Lactobacillus was higher than that of *Bifidobacterium*. The mean values for *Bifidobacterium* bacteria and *Lactobacillus* were 65561.86 and 545538.80. At the finished dose stage the numbers of *Bifidobacterium* and *Lactobacillus* showed statistically significant differences.

Discussion

The precise number of target DNA molecules was determined by absolute quantitative estimation, while the DNA standards utilized a standard curve. The curve was graphed using sequentially diluted standards from known concentrations and a relationship was formed linearly, with the logarithm of the primary amount of total template DNA and the ct. The accuracy of the absolute quantification process depends on the amplification competence of the target and the calibration standard curve, which must be considered in the workflow analysis (Taur and Pamer, 2016). The standard curve method is one of the most accurate methods of determining the sample bacterial cell numbers. This is in agreement with the method used in this study in determining the number of bacteria.

The intestine and its related microorganisms prove to play a crucial role in ailments, particularly in a wide range of syndromes due to the interactions between the host's physiology and intestinal microbiota (Woting and Blaut, 2016). Gut microbiota share with its host and habit to the micro-environment in which it live, which is the digestive tract. Intestinal microbiota develops concurrently with the host adapting to the environment which it inhabits, i.e. the digestive tract (Montandon and Jornayvaz, 2017). Nevertheless, there are many open questions, for instance, if the changes in gut microbiota are the reason or the result of diseases. Since bacteria in the intestine have a great influence on human health and are believed to cause certain illnesses, hence, their use in treating numerous chronic diseases. Additional research is required to combat opposition to bacteria-related diseases of the intestine (Zhang *et al.,* 2015).

The notable destruction of microbial populations due to cancer treatment explains why the microbiome is central to understanding the development of infectious complications arising in the intestinal tract (Taur and Pamer, 2016). Using culture methods, *Eubacterium spp., Bacteroides vulgatus, Streptococcus hansenii, Ruminococcus spp., Faecalibacterium prausnitzii,* and *Bifidobacterium spp* have been observed in the microbiota of human feces . The species *Eubacterium aerofaciens* and *Lactobacillus S06* have been observed to be extensively higher in human beings with low CRC risk. Also, it was observed that *Bifidobacterium* might also additionally enhance chemotherapeutic efficacy (Zou *et al*., 2018).

The reduction of bacterial total number after cnacer chemotherapy was estimated to be higher than any change in the number of copies observed in the controls (Zwielehner *et al.,* 2011). Interestingly, many studies have shown that *Lactobacillus* or *Bifidobacterium* consumption may reduce the production of toxic metabolites by reducing the concentrations of fecal deoxycholic acid and dehydroxylation of primary bile acids (Kahouli *et al.,* 2013).

Conclusion

In this study, it was observed that the numbers of *Lactobacillus* were generally higher in cancer patients compared to control samples, while *Bifidobacterium* in cancer samples declined significantly compared to the control samples. Furthermore, the results indicated that *Lactobacillus* and *Bifidobacterium* decreased at the onset of chemotherapy and even after the completion of chemotherapy doses.

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