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Original Article

Possible correlation between *Lactobacillus paracasei* X12 intake and tumor characteristics in the rat model of colorectal cancer

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Abstract

Introduction: There are many different therapeutic approaches to control colorectal cancer (CRC) which is recognized as one of the deadliest diseases in the world today. One of the most recent of which is the probiotic interventions to change the gut microbiome. Probiotics can be related to the control of gastrointestinal cancers in a variety of ways. Therefore, the present study aimed to investigate the relationship between supplement use with macroscopic and physiological changes of tumor in 1,2-dimethylhydrazine (DMH) -induced rats.
Methods: The male Wistar rats were divided into three groups. *Lactobacillus paracasei* X12 was administrated (40 weeks) to the DMH-induced rats. DMH injection (30 mg/kg BW) was used for 12 weeks to induce CRC. The "AgNOR method" was applied for the evaluation of cell proliferation. Real-time polymerase chain reaction (PCR) was used to measure the gene expression of apoptotic markers.
Results: The findings of this study indicated that *L. paracasei* X12 intake prevented weight loss caused by CRC ($P=001$), and probiotic consumption could significantly prevent tumor growth. Additionally, a significant correlation was observed between apoptosis markers and weight of animals, and a strong negative correlation ($P=000$) between apoptosis parameters and tumor characteristics (incidence, volume, and multiplicity of adenomas). A close association between cell proliferation and tumor characteristics was illuminated as well ($P<001$).
Conclusion: This study revealed a strong correlation between tumor incidence and growth and probiotic intake in CRC. Moreover, it could be believed that cancer prevention is a far more essential and cost-effective way than its cure.

Introduction

In the history of deadly diseases, colorectal cancer (CRC) has been thought as one of the leading causes of death in the world, which has led to the emergence of a variety of problems and bearing heavy burdens on the global public health.[1,2] There are still many concerns regarding cancer treatment in that the definite cause of cancer has not yet been discovered, and consequently, there is no definitive cure for it as well.[1,3] Gastrointestinal cancers, especially cancers of the large intestine, are at the heart of such frequent and deadly cancers.[4] Therefore, preventive intervention research to reduce the incidence and progression of CRC appears to be much more significant than therapeutic implications.[5]

Recent developments in the fields of diets and nutritional interventions have opened new doors to a renewed interest in studying their association with cancers, especially gastrointestinal diseases.[6,7] Therefore, special attention to dietary interventions can be one of the most critical factors in reducing or controlling CRC cancers.[6]

On the other hand, the gut microbiome - as a novel and mysterious target - can provide many benefits for the host.[8] Recent literature has suggested that interventions that can alter the composition of the gut microbiome can have beneficial effects on the cancers.[9] On this note, there is a great deal of evidence linking microbiome and CRC.[9,10] Nutritional interventions like probiotics such as *Lactobacillus* species have beneficial effects on the host.[11-

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[13] Therefore, there is no consequence that recently, the use of probiotics for the prevention and even treatment of chronic diseases, including cancers, is at the heart of the concerns.[14,15]

The indefinite growth and proliferation of tumor cells in CRC results in a severe weight loss[16] as well as an increase in the apoptosis resistance, which is a crucial mechanism in reducing the growth and development of tumors.[17] Also, there is clarifying evidence that the use of probiotics may have beneficial effects on apoptotic pathways[18] and the prevention of severe weight loss.[19]

Numerous studies have reported the beneficial roles of probiotics in improving CRC *in vivo* and *in vitro*[18,20,21]; however, the generalizability of much-published research on this issue is problematic in that challenges remain in their mechanisms of action, and their apparent role in the control and recovery of cancer has not yet been identified.[22] What is more, there are some debates witnessed on the part of probiotics in CRC.[23]

One of the most significant current discussions indicated that the consumption of *Lactobacillus paracasei* could reduce the incidence of cancers *in vitro* and *in vivo*.[24-26] Besides, the controversy about the beneficial effects of *L. paracasei* has been reflected in the reports of various diseases, including its tumor-suppressive effects.[26,27] Based on the reports from other studies and our previous study, the present study aimed to investigate the correlation between the weight changes and the rate of apoptosis with tumor characteristics (size, incidence, multiplicity, and volume of the tumors) following the consumption of *L. paracasei* X12.

Methods

Animals

Six-week-old male Wistar rats (140-180 g, n=36) were obtained from the Pasteur Institute of Iran. All animal procedures were under the Principles of Laboratory Animal Care (NIH Publication 1986) and were approved by the Animal Experimentation Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran. The animals were then housed in standard cages (four rats per cage) in a room with controlled temperature (22–25°C) and humidity (40–60%) under a 12h:12h light-dark cycle. They were also allowed ad libitum access to food and water following which they were randomly assigned to three groups (n=12 per group) as following:

1) Healthy Control (HC), which received single weekly doses of 1 mM EDTA saline. 2) DMH treated rats' control (DC), which received a single dose of DMH (Sigma, St. Louis, MO, USA) subcutaneously (SC) and sterile normal saline intragastrically. 3) Probiotic treated rats (DP), in which the rats were injected with DMH. Finally, the rats were fed *L. paracasei X12* daily for forty weeks.

Experimentally Induced Tumors in Animals

Animals were given subcutaneous injections of DMH (dissolved in 1 mM EDTA-normal saline, pH 7.0) twice a week for 12 consecutive weeks at a single dose level of 30 mg/kg BW.[28]

Study design

The probiotic was administrated for 40 weeks. Body weight (BW) was measured every eight weeks. Eventually, the rats were anesthetized with sodium pentobarbital (65 mg/kg BW, IP) and were sacrificed by cervical dislocation at the endpoint. After laparotomy, the colon and rectum tissues were removed and cut longitudinally. Following all, they were thoroughly washed with cold saline.

Preparation of supplement

Regarding the process of the probiotic development, *L. paracasei X12* was purchased from TBZMED Biotechnology Research Center (Tabriz, Iran) and was collected after incubation in MRS broth at 37°C and centrifuged at 3000 rmp for four minutes.[29] Then, it was washed with sterile phosphate-buffered saline (PBS). Ultimately, the numbers of the viable bacteria were adjusted to 2×10^9 colony-forming units (CFU)/rat/day and administrated orally every day for each rat in the DP group.

Surgery and macroscopic measurements of tumors

The animals were sacrificed under an overdose (65 mg/kg BW) of sodium pentobarbital anesthesia and cervical dislocation at the endpoint.[30] After laparotomy, the colon and rectum tissues were removed, their colons were harvested and washed with cold saline, and the macroscopic changes were evaluated (Figure 1). Finally, the tumors count, volume, and multiplicity were counted and measured using Vernier caliper (0.1-mm graduation).

Real-time PCR analysis

As previously described, total RNAs of cells were extracted from the rats' colon and were applied for complementary DNA (cDNA) by PrimeScript RT Reagent Kit (Takara Bio Inc., Tokyo, Japan). cDNA template was the basis of the design, and specific primers were used in this regard as well. Finally, all amplification reactions were carried out on ABI-step I plus (Applied Biosystems, California, USA).

Cell proliferation

The "AgNOR method" has been applied for prognostic and diagnostic purposes of tumor pathology. AgNORs were identified by silver nitrate staining. AgNORs staining was performed based on Murray et al.[31]

Statistical analysis

Results were obtained from 8-12 rats in each group, and their related data were analyzed using SPSS (version 24). The repeated-measures ANOVA test was used to examine weight changes. The Pearson correlation coefficient test was also applied to study the correlations between

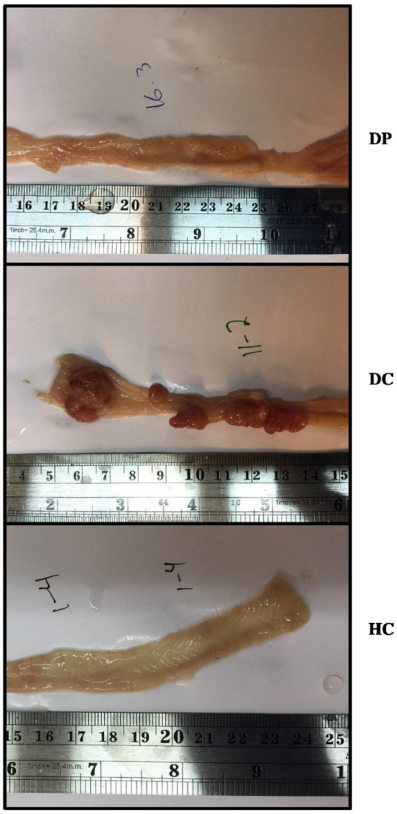

Figure 1. Effects of *L. paracasei X12* on the macroscopic appearance of tumor incidence, multiplicity, and volume in the colon of rats. HC: healthy control group; DC: DMH group; DP: treated group by the *L. paracasei* X12.

variables. Data were considered statistically significant at $P < 0.05$.

Results

Changes in body weight

The weight gain of the animals did not differ notably among the three groups at the beginning of the intervention (Figure 2). In comparison with the control group, the BW of the DC group dramatically decreased at the end of the last weeks (weeks 16, 32, and 40). The administration of *L. paracasei X12* could prevent severe weight loss in these weeks ($P<0.001$). Interestingly, the most significant weight loss was observed after the 32nd week in the DMH-injected group. Moreover, weight loss in the treated group (DP) was much lower than the DC group ($P<0.001$).

The correlation between weight gain and apoptosis

As shown in Table 1, there are indicative correlations between weight gain and apoptotic parameters, especially in the last weeks. For example, there is a positive correlation between weight loss and the increased level of caspase-8, protein kinase B (Akt-1), and Janus kinase 1 (Jak-1) ($P<0.001$). However, no significant correlation was found between BW and concentrations of caspase-3,

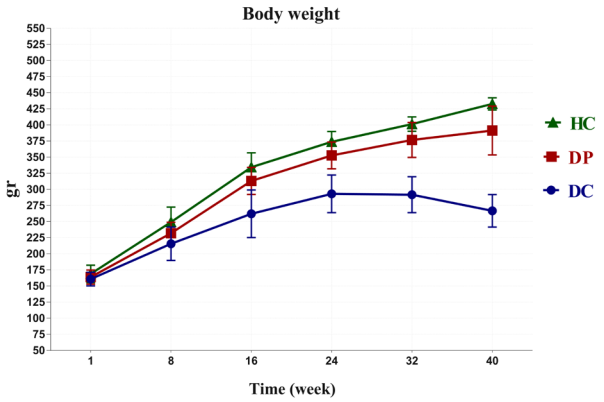

Figure 2. The effect of *L. paracasei* supplementation on weight gain. HC: healthy control group; DC: DMH group; DP: treated group by the *L. paracasei* X12. DMH: 1,2-dimethylhydrazine Repeated measure test was applied. $P<0.05$ was regarded as statistically significant.

caspase-9, and Bax. Besides, based on anti-apoptotic markers, the most active correlation was between Bcl-2 and weight gain in the last week.

The correlation between tumor characteristics and weight gain

Using Pearson's correlation coefficient test, a negative correlation between weight gain and tumor characteristics was observed (Figure 3). Weight loss was strongly correlated with an increase in tumor incidence ($P<0.001$). There was also a close association between the number of adenomas and BW ($P<0.001$). Eventually, a good correlation could be found in a positive and negative relationship between tumor volume and weight gain.

The correlation between tumor characteristics and apoptosis

As expected, there was an interesting correlation between tumor characteristics and apoptosis, the overall results of which are summarized in Table 2. For example, although there were no significant correlations related to a few markers, strong correlations were observed between caspase-8 and Akt-1 and Jak-1 with tumor incidence rates ($P<0.001$). Besides, there was a close relationship between the volume and the number of tumor cells with these parameters ($P<0.001$). Equally important, it could be indicated that there is a positive correlation between cell proliferation (AgNOR) with tumor characteristics (incidence, volume, and multiplicity of adenomas) (Figure 4).

Discussion

As mentioned above, there is an ample evidence that probiotic use may have beneficial effects on the prevention or even control of CRC. Therefore, it is the primary

Table 1. The correlation coefficient among apoptosis variables and animals' weight

		Weight (week 32)	Weight (week 40)
Caspase-8	r	-0.845**	-0.884**
	P	<0.001	<0.001
Caspase-3	r	-0.199	-0.216
	P	0.274	0.236
Caspase-9	r	0.296	0.313
	P	0.100	0.081
Akt-1	r	-0.854**	-0.894**
	P	<0.001	<0.001
Bax	r	-0.149	-0.177
	P	0.416	0.333
Bcl-2	r	0.428*	0.449**
	P	0.015	0.010
Jak-1	r	-0.820**	-0.858**
	P	<0.001	<0.001
Bax/Bcl2	r	0.408*	0.431*
	P	0.020	0.014

Akt-1: protein kinase B; Bcl-2: B-cell lymphoma 2; Bax: Bcl-2-associated X protein; Jak-1: Janus kinase 1.
Pearson correlation coefficients computed correlations between two variables.
Data were expressed as means ± SD. P<0.05 was considered statistically significant.

Table 2. The correlation coefficient among apoptosis variables and tumor characteristics

		Volume	Incidence	Multiplicity
Caspase-8	r	0.846**	0.980**	0.858**
	P	<0.001	<0.001	<0.001
Caspase-3	r	0.041	0.347	0.087
	P	0.823	0.052	0.636
Caspase-9	r	-0.513**	-0.248	-0.463**
	P	0.003	0.171	0.008
Akt-1	r	0.923**	0.950**	0.948**
	P	<0.001	<0.001	<0.001
Bax	r	-0.048	0.254	-0.021
	P	0.794	0.160	0.910
Bcl-2	r	-0.256	-.553**	-.278
	P	0.157	0.001	0.123
Jak-1	r	0.894**	0.955**	0.919**
	P	<0.001	<0.001	<0.001
Bax/Bcl-2	r	-0.230	-0.535**	-0.254
	P	0.205	0.002	0.161

Akt-1: protein kinase B; Bcl-2: B-cell lymphoma 2; Bax: Bcl-2-associated X protein; Jak-1: Janus kinase 1.
Pearson correlation coefficients computed correlations between two variables.
Data were expressed as means ± SD. P<0.05 was considered statistically significant.

purpose of this paper to draw attention to the possible relationships and correlations between macroscopic and physiological characteristics of the colorectal tumors and weight changes following the consumption of *L. paracasei X12* in DMH-induced rats.

In the current study, the use of *L. paracasei* X12 helped to discover that it could prevent severe weight loss in animals. As stated, the presence of tumor cells and their growth has been closely correlated with weight loss. Further, more severe weight loss was observed by increasing the number and size of tumor masses, and these results were reversed following the administration of the supplementation. In a study, Li and Li[20] have shown that the weight of the animals with CRC was dramatically decreased. According to the reports, the main reasons for severe weight loss caused by cancers is the sarcopenia (the loss of muscle mass).[32,33] Another important cause of weight loss is the increased consumption of glucose by tumor cells. On the same note, one study reported that the weight of mice with colorectal tumors was significantly reduced. In this regard, it was indicated in several reports that decrees of muscle mass were one of the main factors for mortality of CRC.[34]

Contrary to expectations, another implication was the possibility for the weight loss to originate from impaired host metabolism (glucose, lipids, and proteins).[35,36] As mentioned above, the use of probiotics could improve the weight loss caused by cancer.[37] This finding is consistent with those of many other studies suggesting the beneficial role of probiotics such as *L. paracasei* in enhancing metabolism, appetite, food intake, weight control, and energy.[29,30,38] which may be related to the high correlation between *L. paracasei* X12 consumption and weight management in this study. Therefore, it can be assumed that the administration of probiotics in DMH-induced rats could have an active role in improving metabolism and controlling weight gain.

Also, an increase in the number of adenomas or an increase in their volume along with excessive proliferation (increased energy requirement as well as uncontrolled consumption of glucose) was closely associated with weight loss, especially in the last weeks.[39,40] A high correlation between weight gain at weeks 32 and 40 with cell proliferation (mAgNORs and pAgNORs) was implied following the probiotic intake. In a critical study,[41] it has been illustrated that the administration of *L. salivarius* for 40 weeks (5×10^{10} CFU) could dramatically alleviate the volume, size, and multiplicity of the adenomas. It was illustrated in the same work that *L. salivarius* intake may manipulate the gut microbiome and immune regulation of the rats with CRC. Thus, one of the most important reasons for maintaining weight could be the inhibition of tumor growth and cell proliferation which could lead to regulating the metabolism of macronutrients.

On the other hand, the results accumulated by this paper well represented a close relationship between weight gain with apoptosis. As it is known, apoptosis is a critical process in the mechanisms related to tumor incidence and growth.[42] Moreover, the beneficial role of probiotics in reducing and controlling tumor growth by regulating main factors in the apoptotic pathways has

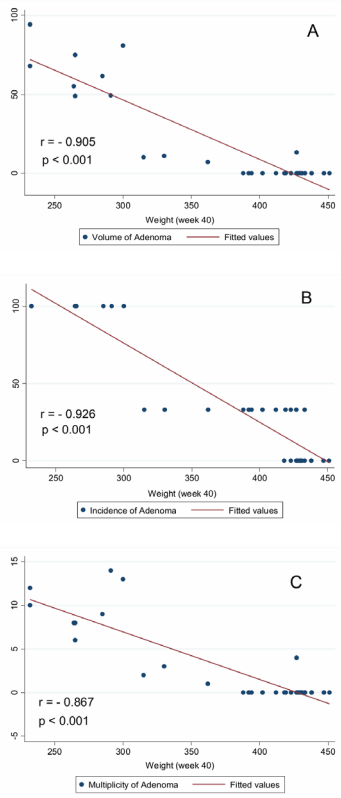

Figure 3. The correlation coefficient between the animal's weight and tumor characteristics (incidence, volume, and multiplicity of adenomas). (A) Correlation coefficients between volume and of adenomas with bodyweight (week 40). (B) Correlation coefficients between the incidence of adenomas with bodyweight (week 40). (C) Correlation coefficients between the multiplicity of adenomas with bodyweight (week 40). HC: healthy control group; DC: DMH group; DP: treated group by the *L. paracasei* X12.

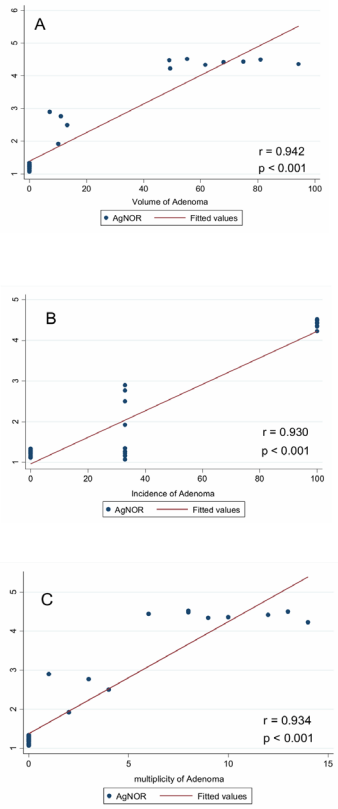

Figure 4. The Correlation coefficient between cell proliferation and tumor characteristics (incidence, volume, and multiplicity of adenomas). (A) Correlation coefficients between volume and of adenomas with cell proliferation (AgNOR). (B) Correlation coefficients between the incidence of adenomas with cell proliferation (AgNOR). (C) Correlation coefficients between the multiplicity of adenomas with cell proliferation (AgNOR). HC: healthy control group; DC: DMH group; DP: treated group by the *L. paracasei* X12.

been demonstrated.[18,43,44] These notions are consistent with those of other studies such as that of Baldwin et al[45] suggesting that *L. acidophilus* and *L. casei* (10^8 CFU/mL) consumption could augment apoptosis (40%) by activation of caspase-3 of a CRC cell line. According to the findings of this study, there was also a high correlation between the size and the number of tumors with anti- and apoptotic markers. Considering the evidence, improving immune response probably decreases cell proliferation, gut inflammation suppression, and regulation of metabolism, all of which could be the possible mechanisms involved in this study.[46,47] However, it cannot be confidently stated that there is a direct relationship between the incidence of a tumor and the rate of apoptosis, and there are supporting reports in this regard as well. Overall, it is difficult to indicate a clear pathway between apoptosis and tumor growth. More research to reveal related mechanisms is warranted.

Given the crucial and determining role of the gut microbiome in gastrointestinal cancers' association - which has received much attention in recent years[9,48] – a possible explanation could be that the effects of probiotics such as *L. paracasei* X12 may have been altered by the changes in the gut microbiome combination and population that have been well documented in some studies.[48,49]

The results of this study, which is the continuation of the previous research, represents a strong correlation between weight changes as well as markers of apoptosis with physical characteristics of the tumor. Besides, the consumption of *L. paracasei* X12 was able to prevent severe weight loss in rats with CRC. It has also been well demonstrated that the use of *L. paracasei* X12 is closely related to the reduction of tumor cell incidence and suppression of tumor growth. What is more, there was a close relationship between cell proliferation in tumors and weight changes. Although the present study clarified a promising and negative correlation between probiotic consumption with tumor growth, there are some limitations, most notably the lack

of consideration of microbial changes due to the use of *L. paracasei* X12. However, there were some limitations to this study, as well. Perhaps the most notable limitation was the need to investigate and analyze changes in the gut microbiome as an essential part of the host intestine - as it has been reported to have many functions and have not been addressed in this study.

It could be assumed that probiotics consumption appears to be a promising way to reduce the incidence of CRC and even to treat and control it. Further research is needed to elucidate the extent and type of association of the gut microbiome with the spreading and incidence of cancer, especially with signaling pathways. As dietary supplements, probiotics could have great potential in controlling and managing CRC.

Conclusion

The most prominent finding to emerge from this study is that there was a high correlation between apoptotic markers, weight gain, and macroscopic characteristics of tumors. Considering the increasing prevalence of CRC in the world community, the heavy burden of cancers on the world public health, and the lack of definitive treatment for this issue, the path is paved for the need for the prevention and the reduction of the incidence of cancer. Therefore, the use of non-pharmacological treatments such as probiotics that have shown promising results could attribute to a small part of this larger goal. Moreover, there is a clear need for more well design researches to clarify more evidence on possible roles of probiotic and its mechanisms for suppression of the colorectal tumors. Clearly, much more research is needed, especially concerning human beings.

Conflict of Interest

There is nothing to declare.

Ethical Approval

The Research and Ethics Committee of Islamic Azad University, Tabriz Branch, approved this study's ethicality with no.: 5D997543

Author's contributions

SAM was the writer of the study protocol, study design, keeping rats, and performed the experiments and drafting the manuscript. AYK held the responsibility to analyze and interpret the data. BA was involved in developing and editing the document. All authors have given their final approval of the present version to be published.

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