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Research Article

The Effects of Qualitative and Quantitative Protein Malnutrition on Cecal Microbiota in Wistar Rats with or without Neutrophil Suppression*

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Abstract: Gastrointestinal microbiota has been claimed to be affected by the diet consumed. In this study the effects of severe qualitative and quantitative protein malnutrition on cecal microbiota of male Wistar rats with or without neutrophil depletion were investigated. A total of 43 animals were divided randomly into 7 groups. Group I (control) was given a complete chow diet, groups II, III and IV received almost protein free (N-free) diet and groups V, VI and VII received a 20% gelatin containing diet for 35 days ad libitum. In addition, groups I, II and V were given physiologic saline, groups III and VI were given normal rabbit serum while groups IV and VII were given anti-rat neutrophil antibody containing rabbit serum (antineutrophil serum) by intraperitoneal route weekly. At the end of experiment, the animals were euthanized; the ceca were removed aseptically and total aerobe and anaerobe microorganisms, lactobacilli and Enterobacteriaceae were isolated from cecal contents. The evidences indicated that the growth of cecal aerobe microorganisms and lactobacilli was influenced by the quantity and quality of the dietary protein, while total anaerobes and Enterobacteriaceae remained unaffected. The count of cecal lactobacilli was decreased by protein malnutrition. The differences between the control group and both malnourished groups given SP were confirmed statistically ($P < 0.0001$). In addition, the comparisons among different malnourished groups showed that the mean count of lactobacilli in group III was significantly higher than that of groups II, IV, V, VI and VII ($P < 0.05$, $P < 0.01$, $P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively). Also, for total aerobe microorganisms the differences between group IV and groups VI and VII and between group II and group VI were confirmed ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). We conclude that dietary protein could influence the cecal microbiota, and this effect could be modified by actual immune status of animals.

Key Words: Diet, qualitative and quantitative protein malnutrition, neutrophil suppression, cecal microbiota, rat

Nötrofil Baskılanmış ve Baskılanmamış Wistar Sıçanlarında Kalitatif ve Kantitatif Protein Yetersizliklerinin Sekal Mikroorganizmalara Etkileri

Özet: Gastrointestinal mikrobiyotanın diyetten etkilendiği bilinmektedir. Bu çalışmada nötrofil baskılanmış veya baskılanmamış erkek Wistar sıçanlarında kalitatif ve kantitatif protein malnutrisyonunun sekum mikrobiotasına etkileri araştırıldı. Toplam 43 hayvan rasgele 7 gruba ayrıldı. Grup I (kontrol) normal yem, grup II, III ve IV protein içermeyen diyet, grup V, VI ve VII % 20 jelatin içeren diyetle 35 gün süreyle ad libitum beslendi. Ayrıca, grup I, II ve V'e fizyolojik tuzlu su, grup III ve VI'ya normal tavşan serumu ve grup IV ve VII'ye anti-rat nötrofil antikoru içeren tavşan serumu haftada bir kez i.p. yolla verildi. Deney sonunda hayvanlar ötenazi edildi ve aseptik şartlarda sekumlar çıkartıldı. Sekum içeriklerinde total aerob ve anaerob bakteri, lactobacilli ve Enterobacteriaceae sayıları belirlendi. Çalışma sonuçları diyeter proteinlerin miktarının ve kalitesinin sekal aerob ve lactobacilli konsantrasyonlarını etkilediğini, buna karşın total anaerob ve Enterobactericeae konsantrasyonlarını etkilemediğini gösterdi. Protein malnutrisyonu sekal lactobacil sayısında azalmaya neden oldu. Kontrol grubu ile fizyolojik tuzlu su uygulanan malnutrisyon grupları arasındaki fark önemliydi ($P < 0,0001$). Ayrıca malnutrisyonlu gruplar arasında yapılan karşılaştırmada grup III'ün ortalama lactobacilli sayısının grup II, IV, V, VI ve VII'dekinden daha yüksek olduğu görüldü (sırasıyla $P < 0,05$, $P < 0,01$, $P < 0,05$, $P < 0,01$ ve $P < 0,05$). Çalışmanın sonuçları, diyetteki proteinin sekal mikrobiyotayı etkilediğini ve bu etkinin hayvanın bağışıklık sisteminin fonksiyonel durumu tarafından değiştirilebildiğini göstermektedir.

Anahtar Sözcükler: Diyet, kalitatif ve kantitatif protein malnutrisyonu, nötrofil baskılanması, sekal mikrobiota, sıçan

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Introduction

Protein-calorie malnutrition is not only an important health problem of underdeveloped countries, but also can be seen frequently in developed countries as a result of certain critical diseases, and is associated closely with immune dysfunction and concomitant infections in man and animals (1). In protein-calorie malnutrition the incidence of infections is very high. One of the main gateways for infectious agents is the gastro-intestinal system which builds a stable barrier to translocation of microorganisms from lumen into tissues under physiological conditions (2,3).

The main components of the gut barrier to pathogenic agents include gastric acid, pepsin, biliary salts, intestinal motility, intestinal epithelium, mucus, mucosa-associated immune system, normal intestinal microbiota and their products (3). Under healthy circumstances gastric acid, bile secretion and intestinal motility prevent the colonization of the bacteria in the upper intestine, where the concentration of bacteria is generally less than 10^5 colony forming units (CFU) per milliliter of contents (3,4). It is known that nutritional deficiencies of protein, calorie or other dietary constituents can profoundly influence morphology and physiology of the digestive system (2). The chemical nature of the diet has long been considered to exert a controlling influence on the bacteria (3,5-7) and their metabolites in the intestine (8). Moderate to severe protein-calorie malnutrition are known to be associated with decreased gastric, biliary, pancreatic and intestinal secretions. Intestinal motility is also markedly impaired (2,4). Because of these changes in structure and functions of gastrointestinal system the distribution and type of normal microbiota in intestinal lumen could be affected inevitably. In protein-calorie malnutrition colonic type microbiota can spread and proliferate in the upper small intestine that may be accompanied by diarrhea and a variety of metabolic disturbances, including steatorrhea, vitamin deficiencies and nutrient malabsorption (2,3). Cecal microbiota is disrupted in mice fed a protein free diet, and these mice are found to be more susceptible to bacterial translocation than mice given an adequate diet (9,10). The feces of the people living on the high carbohydrate diet had fewer bacteriodes and more enterecocci than those of people on a Western diet including more fat and animal protein (5). It is also known that there is more risk for breast and colon cancer in developed countries where dietary consumption of fat is high and the fiber is low, conditions that probably are also related to the intestinal bacterial microorganisms (4).

Several studies have been concerned with the effects of food restriction and protein-calorie malnutrition on the intestinal microbiota in man and animals (9-12). However, the role of definite dietary constituents like dietary proteins, their quality and quantity as well as the status of the actual immune system in the maintenance of the normal intestinal microbiota still have an important question mark. The aim of this experimental study was to determine the effects of severe qualitative and quantitative protein malnutrition on the cecal microbiota in male Wistar rats.

Materials and Methods

This study has been approved by the Animal Ethics Committee of the Adnan Menderes University. A total of 43 young male Wistar rats, ca. two months old and weighing in mean 176 g ± 19 g, were used. They were bred for 3 years as a closed colony at our institute. The animals were divided into 7 groups randomly, each consisting of 6 or 7 animals. During the study, including adaptation period, animals were held under definite conditions with 12/12 h light/dark cycles, 28 ºC ± 1 ºC external temperatures and 50-70% relative humidity in individual cages. Food and water were given ad libitum. Following a one-week adaptation period, animals in group I were given a complete chow diet for mice and rats consisting of 23% raw protein (Best Yem, Gebze, Turkey) and served as control, while experimental groups were given either an almost protein free diet (N-free diet, groups II to IV) or a diet consisting of 20% gelatin as protein source for 35 days (groups V to VII) (Table 1). Experimental diets were purchased from Altromin (Lage, Germany). The control diet had also been used for the rearing of animals. The control group and one of the malnourished groups received physiologic saline (SP) in each dietary regime, while other experimental groups received either normal rabbit serum (NRS) or anti-rat neutrophil antibody containing serum (ANS) (Table 2). All injections were made weekly by intraperitoneal route. After 35 days, animals were euthanized under ether anesthesia, then ceca were removed aseptically and cecal contents were weighed, then transferred into sterile tubes immediately, diluted (10^{-1} to 10^{-8}) in 0.1% sterile

Table 1. The compositions of semi-synthetic experimental diets (% DM) [Altromin®].

Ingredients	N-free diet	Gelatin containing diet
Gelatin	0	20
Starch	73	53
Saccharose	10	10
Mineral premix[1]	6	6
Soy oil, Refine	5	5
Cellulose, Pulver[2]	4	4
Vitamin Premix[3]	2	2
Summ	100	100

[1] Mineral premix (60 g/kg diet): $CaCO_3$ 14 g; $CaHPO_4$ 14 g; K_2HPO_4 (sicc.) 10 g; NaCl 8 G; Na_2HPO_4 (sicc.) 7 g; $MgSO_4$. 7 H_2O 5 g; Fe(II)-gluconate. 2 H_2O 1480 mg; $MnSO_4$. 4 H_2O 450 mg; $ZnCO_3$ 40 mg; $CuSO_4$. 5 H_2O 19 mg; NaF 10 mg; KI 0.5 mg; Na_2MoO_4 . 2 H_2O 3.5 mg.

[2] Cellulose powder Nr. 123, Firma Schleicher and Schüll, D-3354 Dassel.

[3] Vitamin premix (20 g/kg diet): Vitamin A 15,000 IU; Vitamin D_3 500 IU; Vitamin E 150 mg; Vitamin K_3 10 mg; Vitamin B_1 20 mg; Vitamin B_2 20 mg; Vitamin B_6 15 mg; Vitamin B_{12} 0.03 mg; Nicotinic acid 50 mg; Pantothenic acid 50 mg; Folic acid 10 mg; Biotin 0.2 mg; Choline 1000 mg; p-aminobenzoic acid 100 mg; Inosit 100 mg; Vitamin C 20 mg; Rice starch *ad* 20 g.

Table 2. The group design.

Groups	Interventions
Group [Control]:	Animals were given chow diet and physiological saline (PS)
Group II [N-free+PS]:	Animals were given N-free diet and physiological saline (PS)
Group III [N-free+NRS]:	Animals were given N-free diet and normal rabbit serum (NRS)
Group IV [N-free+ANS]:	Animals were given N-free diet and antineutrophil serum (ANS)
Group V [Gelatin+PS]:	Animals were given 20% gelatin containing diet and physiological saline (PS)
Group VI [Gelatin+NRS]:	Animals were given 20% gelatin containing diet and normal rabbit serum (NRS)
Group VII [Gelatin+ANS]:	Animals were given 20% gelatin containing diet and antineutrophil serum (ANS)

peptone water and mixed gently with vortex. From each of the dilutions 0.05 mL sample was plated onto selective media. Plate Count Agar (Merck, VK 525963) was used to determine total aerobes with dilutions of 10^{-2} to 10^{-6}. For total anaerobes Columbia Blood Agar (Acumedia, 7125) was used with dilutions of 10^{-3} to 10^{-8}. *Lactobacilli* were counted on Rogosa Agar (Merck, V 273813) by using dilutions of 10^{-2} to 10^{-7}. For Enterobacteriaceae Levine Agar (Acumedia, 7103) was used with dilutions of 10^{-1} to 10^{-5}. Plate Count Agar and Levine Agar were incubated aerobically at 37 °C for 24 hours. Rogosa Agar and Columbia Blood Agar were incubated in anaerobic jar (Merck, 116387, 2.5 l volume) with gas pack (Oxoid, BR 038) at 37 °C for 72 hours. After incubation, colonies were counted according to colony morphology and recorded as CFU per g of contents (13,14).

Statistical Analyses

The data were analyzed by one and two way analyses of variance (M-ANOVA) (15). If differences between groups occurred, the Tukey test was used to find out from which group the difference(s) originated. The results are given as the mean of $\log_{10}$ transformed data of groups with their standard deviations and minima and maxima.

Results

The gathered data are summarized in Table 3.

If both malnourished groups given PS (groups II and V) were compared with the adequate-fed controls by ANOVA, it was seen that only the mean counts of the lactobacilli were affected by malnutrition significantly ($P < 0.0001$). The results of the *post hoc* tests revealed that the mean lactobacilli counts in cecal samples of rats decreased as a result of both the dietary qualitative and quantitative protein malnutrition ($P < 0.0001$) (Figure 1).

Two way analyses of variance was run to analyze the effects of the diet and neutrophil suppression on cecal microbiota of rats by using the data of both qualitative and quantitative malnourished groups given PS, NTS or ANS. The results revealed that the effects of the experimental interventions on the counts of aerobe

Table 3. Counts of bacteria from cecum samples of Wistar rats with or without severe qualitative and quantitative protein malnutrition and neutrophil suppression [Log_{10} CFU / g wet weights of cecal content samples].

Groups	N	Total Anaerobe	Enterobacteriaceae	Total Aerobe	Lactobacilli
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
		(Min – Max)	(Min – Max)	(Min – Max)	(Min – Max)
Group I	7	8.48 ± 0.67	4.99 ± 0.73	7.04 ± 0.91	7.64 ± 0.47
[Control]		(7.26 – 9.45)	(4.08 – 6.08)	(5.88 - 8.92)	(6.75 - 8.15)
Group II	6	7.47 ± 1.31	5.96 ± 1.43	6.31 ± 1.07	5.20 ± 0.88
[N-free+PS]		(5.68 – 8.56)	(4.72 – 7.90)	(5.62 – 8.48)	(4.30 – 6.60)
Group III	6	7.43 ± 1.67	5.84 ± 2.33	6.87 ± 0.95	6.68 ± 0.67
[N-free+NRS]		(5.72 – 9.38)	(2.30 – 7.76)	(5.51 – 7.82)	(6.20 – 7.15)
Group IV	6	7.68 ± 0.93	4.68 ± 0.37	5.72 ± 0.06	4.81 ± 0.35
[N-free+ANS]		(6.08 – 8.41)	(4.08 –5.08)	(5.66 – 5.82)	(4.30 – 5.15)
Group V	6	7.91 ± 1.14	5.32 ±1.18	7.04 ± 1.22	5.11 ± 0.47
[Gelatin+PS]		(6.45 – 8.87)	(3.78 – 6.78)	(5.92 – 8.62)	(4.78 –5.64)
Group VI	6	8.47 ± 0.51	5.46 ± 1.57	7.70 ± 1.41	4.85 ± 0.49
[Gelatin+NRS]		7.81 – 8.97	3.73 – 7.97	5.88 – 9.34	4.30 – 5.26
Group VII	5	7.79 ± 1.10	5.30 ± 0.91	6.44 ± 0.44	4.96 ± 0.26
[Gelatin+ANS]		6.41 – 8.72	3.78 – 6.00	5.82 – 6.78	4.78 –5.15

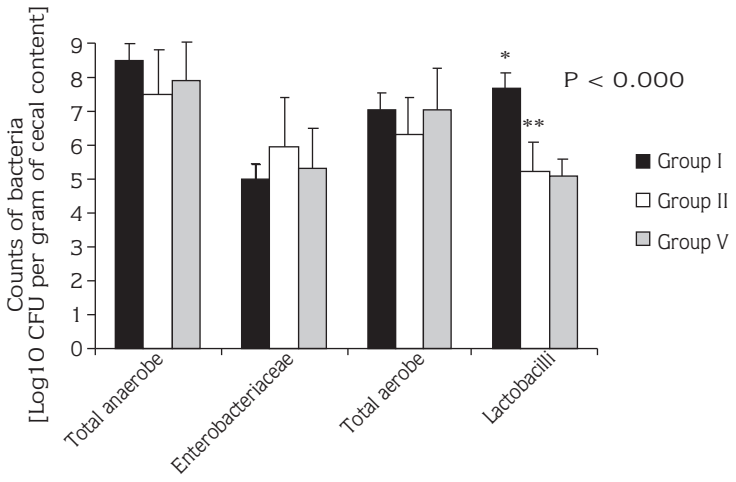

Figure 1. Colony counts of the groups of bacteria from cecum samples of male Wistar rats fed adequate or severe qualitative and quantitative protein deficient diets.

bacteria were not confirmed statistically (p= 0.0605, F= 2.45). However, there was a significant interaction between diet and neutrophil suppression; the growth of cecal aerobe microorganisms was influenced if the animals were exposed to both malnutrition and neutrophil suppression concomitantly ($P < 0.05$). The results of the post hoc tests revealed that for neutrophil suppression the differences in mean total aerobe counts between groups of rats given NTS and ANS were significant ($P < 0.05$). The differences originated mainly from the group fed with an almost N-free diet and given ANS, in general. Taking the effects of both immune suppression and diet into consideration it was seen that the differences between the group IV and groups VI and VII as well as between the group II and group VI were statistically confirmed ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). Experimental interventions influenced also the mean counts of cecal lactobacilli. Neither the effect of dietary regimens ($p = 0.0611$, $F = 4.15$) nor the effect of the immune status ($p = 0.0875$, $F = 2.91$) on the mean cecal lactobacilli counts could be confirmed statistically. However, there was a bi-directional significant interaction between diet and neutrophil suppression in this respect ($P < 0.05$). Post hoc tests revealed that for neutrophil suppression the differences in mean counts of lactobacilli between groups given ANS and NTS were significant ($P < 0.05$). When the effects of both dietary regimen and neutrophil suppression were taken into consideration, it was also seen that the differences originated mainly from the group III. So, the differences in mean cecal lactobacilli counts between group III and groups II, IV, V, VI and VII were confirmed statistically ($P < 0.05$, $P < 0.01$, $P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively). However, the counts of cecal anaerobe microorganisms and Enterobacteriaceae were not affected by the experimental interventions in this study. There was also no interaction between dietary regimen and innate immune status in these respects (Figure 2).

Discussion

In preliminary studies, the effects of the diet on the microbiota of the gastrointestinal tract and feces as well as bi-directional interactions between nutritional value of the diet and intestinal microbiota in man and animals are well documented (5,10,13,16). The possible roles of the diet and gastrointestinal microbiota in health status of the host organisms are also widely discussed (3,4,9). Because of the close relationships among diet, gastrointestinal microbiota and health status, any change in feeding pattern or dietary conditions could inevitably influence the composition of the intestinal microbiota. This was the

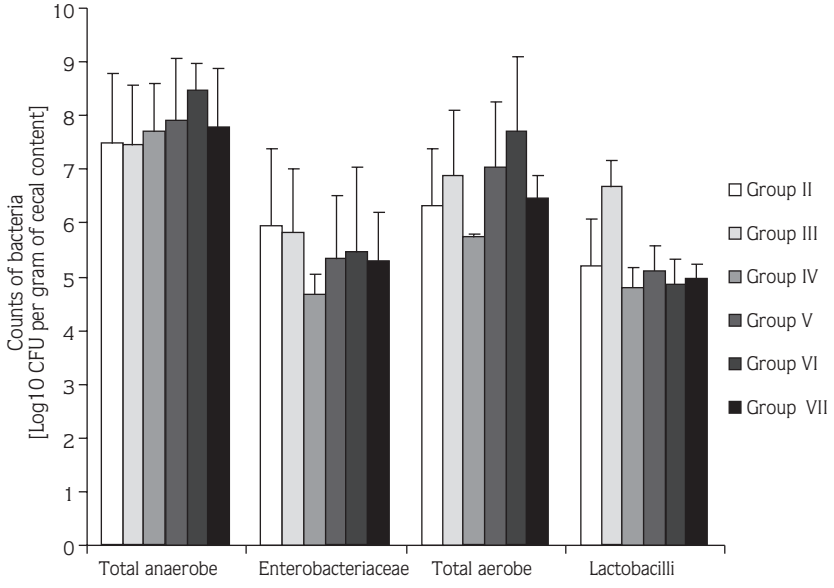

Figure 2. Colony counts of the groups of bacteria from cecum samples of male Wistar rats with or without severe qualitative and quantitative protein malnutrition and neutrophil suppression.

main standpoint for studies involved in the search of the consequences of fasting, dietary restriction or malnutrition of different etiology, including protein and/or calorie malnutrition (9-12,17). However, the role of the quantity and quality of dietary proteins as well as the role of the actual specific and nonspecific immune status of the individuals subjected to dietary challenges in maintenance of gastrointestinal microbiota remains unclear. Because qualitative or quantitative protein malnutrition suppresses the specific immune system (18,19) and causes severe lymphopenia (20-23) while qualitative malnutrition could result in neutrophilia as it was seen in animals given a diet containing gelatin as protein (22,23), the role of the nonspecific immune system would be more important in this respect.

In the present study, total aerobe microorganisms and lactobacilli in the cecal samples were found to be most affected by the quantity and quality of the dietary protein; however, total anaerobes and the Enterobacteriaceae remained unaffected.

The mean counts of lactobacilli decreased as a result of both qualitative and quantitative dietary protein inadequacy significantly when compared to the controls. There were also certain significant differences in relation to the aerobes and lactobacilli among qualitatively and quantitatively malnourished groups per se. If the effects of the neutrophil suppression are considered, the differences in mean counts of both aerobes and lactobacilli between malnourished groups given NTS and ANS were confirmed ($P < 0.05$). Compared to the gelatin-fed animals, lower aerobe and higher lactobacilli counts were observed in ceca of rats fed almost N-free diet, in general. Although qualitatively low priced, the gelatin in the diet seems to be important in maintaining the population of total aerobes, but not lactobacilli population.

Lactobacilli, a very important co-population of the gastrointestinal microbiota for healthy life of the host, play a definite role in maintaining of a normal microbiota within the gut by suppressing the colonization of pathogenic microorganisms and their adhesion to the mucosa (24). Its suppression, under others, could possibly be responsible for the increase in translocation of pathogenic microorganisms from lumen into tissues as well as the high morbidity and mortality rate seen in malnutrition.

These results are not comparable with those gathered from clinical cases with different etiologies of protein-calorie malnutrition, which are almost always complicated with other kinds of deficiencies or imbalances and a variety of parasitic, bacterial or viral infections or infestations, as well as the time of exposure. In an experimental study on CD-1 mice fed with an almost N-free diet for 21 days, time-dependent significant increases in cecal total aerobic bacteria and Gram-negative enteric bacilli counts were reported (9). The results of another study on adult female Crl:CD-1[ICR]BR mice also showed that both the feeding of an almost protein-free diet for 14 days and starvation for 3 days caused an increase in Gram-negative enteric bacilli population and a decrease in lactobacilli and strict anaerobes (10). However, the diet was used by those investigators consisted of 20% fat, whereas the diet in present study contained only 4% fat. Tannock and Savage (11) reported that deprivation of food, water and bedding for 48 hours decreased the cecal lactobacilli counts of CD-1 and C57BL mouse strains, whereas coliforms increased significantly. However, no important change could be observed by these authors in Ha/ICr mice. These strain-specific differences in reaction of intestinal microbiota to the dietary and environmental challenges could be dependent on the actual immune status of animals. Then, all these animals are known to be immune-compromised in a different manner. This evidence is in accordance with our findings so far that the influence of dietary proteins on the cecal bacteria of male Wistar rats was also dependent on neutrophil suppression.

Based on the evidences gathered in this study as well as in other studies it is concluded that intestinal microbiota could be influenced by dietary components including proteins. Furthermore, it seems that this influence could be modified at least by the host's actual immune status, which may also be the reason of strain-specific differences in reaction of gastrointestinal microbiota to malnutrition found in preliminary studies by others. This problem requires further detailed investigations for a sufficient explanation.

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