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Changes in gut microbiota following supplementation with chitosan in adolescents with overweight or obesity: a randomized, double-blind clinical trial

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Abstract

Background Overweight and obesity have been associated with an altered intestinal microbiome. Recent investigations have demonstrated that fiber supplementation, including chitosan, can exert beneficial and protective effects on the composition of gut microbiota in humans diagnosed with overweight/obesity. However, there is still a great deal of heated debate regarding the impact of chitosan supplementation in overweight and obese adolescents. Therefore, the aim of this study is to clarify the effects of chitosan administration on the composition of the gut microbiome in overweight and obese adolescents.

Methods and analysis Sixty-four overweight and obese adolescents were subjected to supplementation with 3 g of chitosan for 12 weeks. Anthropometric indices and physical activity were measured at the beginning and at the end of the intervention. After DNA extraction and purification, the quantity of bacteria in the patients' stool samples was determined by real-time polymerase chain reaction (PCR). The RCT was registered on the Iranian Registry of Clinical Trials (www.irct.ir) website (IRCT20091114002709 N57; registration date: 2021 - 06 - 20).

Results Individuals who received chitosan supplementation experienced a significant decrease in the BMI z-score ($P < 0.001$). Administration of chitosan led to notable significant decrease in *the Firmicutes* ($P < 0.001$) populations and the ratio of *Firmicutes* to *Bacteroidetes* ($P < 0.001$) as well as a notable increase *in the Bacteroidetes* ($P = 0.008$) and *Akkermansia* ($P < 0.001$) populations, respectively compare to control group. Mean changes in *Lactobacillus* populations were marginally significant ($P = 0.05$). Chitosan administration did not alter the composition in *Bifidobacterium* populations ($P = 0.97$).

Conclusion The present study demonstrates beneficial effects of chitosan administration on some bacterial species associated with overweight and obesity in adolescents. Further research is needed to confirm our findings and clarify the impact of this intervention on the *Lactobacillus* population in the gut.

Keywords Chitosan, Obesity, Gut microbiome, Adolescence

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Introduction

Adolescence is one of the most rapid stages of human development that is accompanied by various physiological, social, and neural changes [1]. Individuals are considered adolescents if their age ranges from 10 to 19 years according to the World Health Organization [2]. Obesity among adolescents has become a major global health threat [3]. Childhood and adolescent obesity is frequently complicated early in life by the development of other cardiometabolic disorders, e.g., type 2 diabetes mellitus (T2DM), hypertension, dyslipidemia, metabolic syndrome, nonalcoholic fatty liver disease (NAFLD), as well as cancer [4–6]. Obesity and weight gain are the result of genetic and environmental factors, e.g., increased calorie intakes and inappropriate diets, smoking, geographical influences, lack of sleep, stress, and sedentary lifestyles [7].

Furthermore, recent investigations have demonstrated that changes in the composition of gastrointestinal (GI) bacteria (gut microbiome) can play an important role in the development of obesity among children and adolescents [8, 9]. Due to fermentation and production of short-chain fatty acids (SCFAs), e.g., acetate, propionate, and butyrate, the intestinal microbiota affects the host's energy metabolism and other aspects of weight gain, resulting in changes in glucose and lipid metabolism, as well as in the absorption of nutrients [8]. It has been reported that genetically obese mice have higher and lower amounts of SCFAs in their colon and feces, respectively, as compared to lean mice [10]. The two bacterial groups of *Firmicutes* and *Bacteroidetes* constitute the main bacterial species of the human GI tract. Recent assessments have highlighted that *Firmicutes* are abundant in individuals diagnosed with obesity [11]. Intake of fibers and other indigestible carbohydrate compounds are among the factors that can change the composition of the human gut microbiota [8].

Chitosan is a natural fiber that has attracted the interest of the scientific community due to its health benefits [12]. It is a cationic polysaccharide produced from the epidermis of crustaceans like shrimp and lobster or from the walls of fungi through distillation (hydrolysis of N-acetyl-D-glucosamine units)[13]. Investigations conducted have delineated that chitosan administration can results in changes of the gut microbial mass which play a relevant role in energy homeostasis and regulation of body weight[14]. Consequently, chitosan, as a potentially indigestible oligosaccharide for the host, can be metabolized by the gut microbiota and influence gut microbial composition, enhancing the production of SCFAs and bile acids [9, 15, 16]. Evidence highlights the inhibition of *Lactobacillus* and *Bifidobacterium* production after administration of chitosan. Moreover, it has been depicted that supplementation with chitosan increases the abundance of beneficial *Akkermansia* species in the rat intestine[9]. *Akkermansia* is involved in the proliferation of intestinal cells and interferes with metabolites resulting from high-fat diets, neutralizing their effects on the human health. Thus, because it reduces fat mass, adipose tissue inflammation, and insulin resistance, chitosan has recently been regarded as a potential prebiotic [8, 9]. In addition, chitosan supplementation increases the abundance of *Coriobacteriaceae*, which play an important role in the conversion of bile salts and steroids, as well as in the metabolism of dietary polyphenols [9]. However, some assessment involving laboratory animals have concluded that intake of chitosan does not alter the composition of the gut microbiota [17].

Although the pool of data is relatively limited, most investigations indicate that administration of chitosan is beneficial to human health and exerts protective effects on the composition of the gut microbiota. However, the findings of other assessment are contradictory hence there is still a great deal of heated debate regarding the impact of this natural product on the intestinal microbiota. Moreover, no study has been conducted so far to investigate the effect of this supplement on adolescents diagnosed with overweight/obesity. Therefore, taking into account the widespread prevalence of overweight and obesity in adolescents and the limited options for appetite and weight control in this age group, we conducted a randomized clinical trial (RCT) whose objective was to clarify the impact of chitosan supplementation on the composition of the gut microbiome in overweight and obese adolescents.

Material and methods

Participants

Adolescents with overweight or obesity who were referred to the Obesity Clinic of the Mofid Children's Hospital in Tehran, Iran, were chosen for participation in a double-blind RCT in 2021–2022, based on a predefined set of inclusion and exclusion criteria. The ethics committee of the Iran University of Medical Sciences approved the study. Moreover, the RCT was registered on the Iranian Registry of Clinical Trials (www.irct.ir) website (IRCT20091114002709 N57; registration date: 2021 -06- 20). The flow-chart of the study design and the schedule of the project are shown in Fig. 1. The study protocol was consistent with the ethical guidelines of the Declaration of Helsinki (World Medical Association Declaration of Helsinki, 1975).

Inclusion and exclusion criteria

We agreed upon the following inclusion criteria: (1) Willingness to participate and sign the informed consent

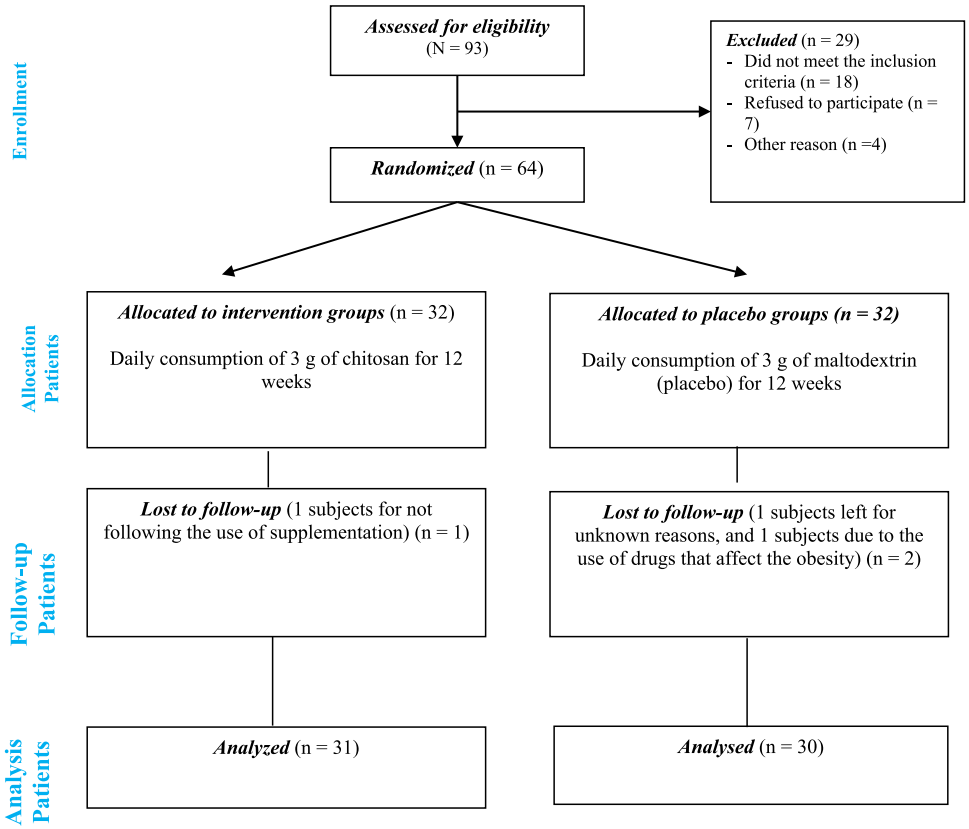

Fig. 1 Consort flow diagram for the trial

form after fully understanding the study's goals and methodology; (2) Adolescents of both genders, aged between 10 and 18 years, who were diagnosed with overweight or obesity; (3) body mass index (BMI) z-score of more than 1 but less than 3. We decided upon the following exclusion criteria: (1) Use of probiotics, prebiotics, or symbiotics supplements or of any foods fortified with these supplements within the last three months; (2) Administration of any antibiotics during the three months preceding the trial; (3) History of cardiovascular, hepatic, gastrointestinal (celiac disease, irritable bowel syndrome, and inflammatory bowel disease), renal, or metabolic illnesses (e.g., phenylketonuria, maple syrup urine disease); (4) History of surgery involving the GI tract; (5) Use of drugs or supplements that impact appetite, weight, or metabolism at least three months before the RCT, including drugs/herbal supplements that affect the metabolism of carbohydrates, proteins, or fats or drugs/herbal supplementsthat either enhance or decrease the appetite or food intake); (6) Adherence to weight loss diets or any type of intense physical exercise programs during the last 6 months; (7) Adolescent girls who were pregnant or lactating; (8) Smokers (more than 200 cigarettes smoked over the course of a lifetime or more than one cigarette smoked in the previous week); (9) Subjects with any allergy to chitosan, crabs and/or shrimp.

Participants with any acute sickness, any injury that may have had an impact on their health, those who used antibiotics throughout the RCT, those who failed to take the supplement for personal or other reasons, and those who migrated were disqualified from the RCT. Patients whose admission rate was lower than 80% were disqualified from the research as well. The admission rate of patients following the intervention period was computed using the formula below.

Acceptance rate = number of packages received at the beginning of the study/number of packages consumed at the end of the study * 100.

Sample size calculation

Given the absence of a study that investigated the effect of chitosan on weight loss in children and adolescents with overweight or obesity, we used the method of Reinehr et al. in order to calculate the sample size by considering the BMI z-score as the primary outcome, as these authors examined the effects of a lifestyle intervention (diet and exercise) on overweight and obese children [18]. In this way, considering the

difference of 0.15 units in the mean BMI z-score at the end of an intervention of 12 weeks and assuming that SD1 = 0.061 and SD2 = 0.078, and the type I of error probability level of 5% ($\alpha = 0.05$) and the type II error probability level of 20% ($\beta = 0.20$, power = 80%), the number of subjects was calculated based on the sample size formula at 24 participants in each group. Assuming that 30% of the enrolled patients are lost during the RCT, 64 participants (32 participants in each group) were finally included in the study.

Study design and intervention

In this 12-week, double-blind, randomized clinical study, 64 overweight or obese adolescents who met the inclusion criteria were randomly assigned to one of the two groups that received a chitosan supplement or placebo (maltodextrin). As most assessments have used 3 g/day of chitosan supplementation as the recommended dosage, we investigated the same dose [19–21]. Since the Food and Drug Administration (FDA) has not received any reports of this compound being hazardous to mammals [22], the participants enrolled in our RCT were given 1.5 g (twice daily for a total of 3 g) of chitosan powder (intervention group) or maltodextrin (placebo group) daily 30–60 min before lunch and supper for a period of 12 weeks. In order to accommodate for the likelihood that some people would not consume raw chitosan powder, standard fruit flavorings were added to these supplements. Parents were instructed to mix the suggested amount of powder per participant with 250 cc of water. The supplements were provided by Karen Pharmaceuticals and Vital-Food Supplements Company. The parents were given the powders at the start of the trial and at the end of the fourth and eighth weeks. They were also requested to provide empty cans and packages at the end of the fourth, eighth, and twelfth weeks in order to assess the acceptance rate of the supplements.

At the start of the trial, suggestions for moderate weight loss were given to all participants (0.5 to 1 kg per month). At the beginning of the study, all study participants received recommendations to adjust total energy intake per day based on energy intake calculated based on age, height, and BMI z-score. Energy intake was computed based on several factors, i.e., age, gender, height, and BMI z-score based on the methods described by Krauss, and a decrease of 200 kcal per person was taken into consideration[23]. The diet's caloric composition was calculated at 30% fats (7% saturation), 50% carbohydrates, 20% proteins, and 300 mg of cholesterol per day. Both groups received the same dietary advice, and it was requested that neither group use supplements or sources of probiotics, symbiotics, or prebiotics during this trial.

Randomization and allocation

Given that gender and BMI z-scores can have significant effects on the study's outcomes, stratified randomization and the permuted block randomization method with quadruple and binary blocks were used to ensure that these variables were distributed evenly among the groups. Using the www.sealedenvelope.com website, the quadruple block or double block were generated based on the sample size of 64 people. Due to the study's double-blind nature, sets of packets containing chitosan powder were made by someone other than the researcher prior to the study's commencement, with the placebo having a similar appearance to the chitosan powder.

In fact, the researchers were unaware of the groups into which the patients were randomly assigned throughout the assessment phase (anthropometric measures and laboratory testing), as well as the allocation of participants into each group (intervention and control group) until the conclusion of the intervention. The medication boxes were given unique codes, and the program was also used to generate appropriate codes in order to apply concealment throughout the randomization process. In this method of allocation concealment, neither the participants nor the researchers were aware of which group received the supplement or the placebo. The coding for the intervention was pre-assigned by the company supplying the supplements and placebos, ensuring complete blinding. These codes were discreetly labeled on the packaging. Upon enrollment, each participant was assigned a package based on a pre-generated random sequence, which was then provided to the parents. Furthermore, the randomization process was designed to be entirely unpredictable throughout the study, maintaining the integrity of blinding.

Evaluation of personal information

Face-to-face interviews were used to collect the following personal data at the start of the trial: name, age, sex, use of nutritional supplements, and history of other disorders (either of the enrolled subjects or of his parents). Using the Marshall and Tanner tables, a trained individual determined a person's maturity status [24].

Anthropometric and physical activity measurements

A number of anthropometric factors were assessed both before and after the trial. Adolescents were dressed simply and without shoes when their height and weight were recorded. The Seca digital scale (manufactured in Germany) was used to measure each subject's weight twice, with an accuracy of 0.01 kg. At the start and conclusion of the research, participants'heights were measured standing up with a tape measure, without shoes, with an

accuracy of 0.5 cm; measurements were taken twice each time, and the average was recorded. Weight in kilograms divided by height in meters squared was used to calculate BMI.

The BMI z-score, sometimes referred to as the standard deviation for the BMI score, is an assessment of relative weight and height based on a reference standard that takes into account age and gender. These scores are regarded as more relevant criteria for comparing the mean values of the group and are more appropriate for identifying longitudinal changes in body weight and obesity [25]. As a result, individuals'changes in body weight were evaluated using the BMI z-score. The International Physical Activity Questionnaire (IPAQ) in Persian was used to measure the amount of physical activity at the start and end of the RCT. The amount of physical activity was calculated as small continuous data taking into account the coefficients related to the activity, and recorded as Met-min/week. The Met coefficient (3.3. for walking, 4 for moderate activity, 8 for heavy activity) is multiplied by the duration of the activity in minutes and the number of days when the activity is performed during the week, and then the sum of Met coefficient defines the amount of physical activity in a week [26].

Gut microbiome assessment

In order to evaluate the composition of the gut microbiome, 10 g of stool samples were collected from the participants. The stool sample was taken with the help of a special dark-colored stool collection container and was immediately placed in a freezer at −70 °C. Bacterial DNA was extracted using the KPG-DNKtb commercial fecal DNA kit. The bacterial DNA sample was extracted from the stool sample based on the recommendations provided in the extraction kit. At first, 100–200 mg of stool were transferred into a sterile tube and 500 µl of homogenized buffer were then added to the sample. 500 µl of lysis buffer A were added to 250 µl of homogenized solution and vortexed for 5 s, then 50 µl of lysis buffer B were added to the previous solution and vortexed for 5 s. After that, the resulting solution was kept at room temperature for 5 min and vortexed every minute. 100 µL of lysis buffer C were then added to the previous solution and vortexed for 5 s. 200 µl of DNA precipitation buffer were added to the previous solution and vortexed for 5 s. Lysed DNA was transferred to high absorption columns and centrifuged at 8000 rpm for 1 min. To wash the DNA, 500 µl of washing buffer were added to it and centrifuged at 8000 rpm for 1 min. Afterwards, the columns were transferred into a 1.5 ml microtube and 60 µl of DNase-free water were added to it at 60 degrees Celsius and placed in an incubator for two minutes. The tubes were centrifuged at 12,000 rpm for 1 min. The extracted DNA samples were stored at − 20 °C. In order to ensure the correct extraction of the samples on the first day of extraction, samples were placed on agarose gel and DNA movement and band formation were checked using electrophoresis. We checked the ratio of the absorbance at 260 and 280 nm using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, U.S.A.) to evaluate the purity of extracted DNA. We considered $A_{260/280}$−1.8 for pure DNA.

A summary of primers and Tm for the five RT-PCR assays is provided in Table 1. All primers were based on S16 and S23 regions. RT-PCR was performed in duplicate in 96-well strips. The reaction mixture that was introduced into each well included 8 µl of Cybergreen containing polymerase (PCR Master Mix, TaKaRa, Japan), Rox Dye, 1 µl of forward and reverse primers, 1 µl of DNA/sample, and distilled water until a volume of 20 µl was reached. RT-PCR was performed using the The StepOne™ RT-PCR System (Applied Biosystems). RT-PCR

Table 1 Specifications of the primers used for each bacterial strain

Number	Primer	Sequence 5′→ 3′	Length	Tm
1	Bifidobacterium [26–28]	FP: GCGTGCTTAACACATGCAAGTC	22	59
2	Bifidobacterium [26–28]	RP: CACCCGTTTCCAGGAGCTATT	22	59
3	Firmicuits [29]	FP:GGAGCATGTGGTTTAATTCGAAGCA	25	56.6
4	Firmicuits [29]	RP: AGCTGACGACAACCATGCAC	21	57.3
5	Bacteroidetes [29]	FP: GGAACATGTGGTTTAATTCGATGAT	25	59.8
6	Bacteroidetes [29]	RP: AGCTGACGACAACCATGCAG	21	60
7	Lactobacillus [27]	FP: TACATCCCAACTCCAGAACG	20	56.4
8	Lactobacillus [27]	RP: AAGCAACAGTACCACGACCA	21	58.4
9	Akkermansia [29]	FP:CAGCACGTGAAGGTGGGGAC	20	59.1
10	Akkermansia [29]	RP:CCTTGCGGTTGGCTTCAGAT	21	59.4

FP: Forward *primer* 'RP: Reverse primer' Tm: melting temperature

cycles were: 10 min at 95 degrees, 45 cycles of 30 s at 95 degrees, 30 s at 56 degrees for *Firmicutes* and *Lactobacillus*, and 59 degrees for *Bacteroidetes*, *Bifidobacterium*, and *Akkermansia*, and finally 30 s at 72 degrees. The quantification of the amount of each bacteria was calculated by comparing the threshold cycle to the standard chart and based on the dilution series of any bacteria with the same device. We employed the formula previously published by Monte et al. [27]. Table 1 includes a list of the applied primers.

Dietary assessment

Interviews with the teenagers or their parents were conducted to evaluate their nutritional intake at the start and conclusion of the trial using a 24-h dietary recall questionnaire of three days (2 regular days and one off day). Nutritionist 4 software was used to identify relevant information, such as calorie consumption, macronutrients, and some micronutrients.

Statistical analysis

Quantitative variables were reported as mean (standard deviation) and categorical variables were reported as numbers (percentage). To compare the mean of quantitative outcomes between the two groups, Mann–Whitney test were used to compare the results between baseline and end of the intervention. Mann–Whitney, as well as Wilcoxon test, were used to analyze within-group data. Chi-square test or Fisher's exact test were used to compare qualitative factors between the two groups. SPSS software version 16 was used to obtain statistical analyses, and significant levels for all tests were considered as P-value < 0.05. Intention-to-treat (ITT) analysis was performed as well.

Results

Characteristics of the participants

The final analysis included 61 subjects diagnosed with overweight or obesity who were eligible for inclusion (31 in the intervention group and 30 in the placebo/maltodextrin group) (Fig. 1).

In the intervention group, the participants'mean age was of 13.51 years old compared to 13.12 years in the control group. There was no statistically significant difference in the BMI z-score ($P = 0.064$) and physical activity ($P = 0.778$) levels among the two groups at baseline. Supplementation with chitosan caused a significant decrease in BMI z-scores versus placebo ($P < 0.001$) (Table 2).

Energy and nutrients intake

The dietary intake of the participants is indicated in Table 3. We used the results of the 24-h dietary recall questionnaire to compare dietary intakes at the beginning versus the end of the RCT. Although the intake of energy ($P = 0.021$), proteins ($P = 0.031$) and total fats ($P = 0.018$) showed a significant decrease after the intervention in the chitosan supplementation group, dietary intakes did not change significantly between the chitosan group and the placebo group. Regarding the intake of other macronutrients and micronutrients, no statistically significant difference was observed between the two groups before and after the intervention.

Table 2 Baseline characteristics of participants

Variables	Groups, mean (SD)		P-value[a]
	Chitosan (n = 31)	Control (n = 30)	
Age, y	13.51 (2.15)	13.12 (2.02)	0.891
Male (n, %)	16 (51.6)	16 (53.3)	0.893
Female (n, %)	14 (48.4)	15 (46.7)	
Height (cm)	148.74 (9.15)	152.21(8.48)	0.130
Weight (kg)	56.12 (7.20)	57.67 (9.34)	0.702
BMI[b] (kg/m^2)	25.31 (1.79)	24.71 (1.86)	0.098
BMI (Z-score)	1.52 (0.26)	1.67(0.32)	0.064
Waist-circumference (cm)	88.48 (15.15)	93.40 (16.39)	0.196
Physical Activity (met.h/wk)	472.14 (225.33)	528.28 (327.04)	0.778
History of diseases (n, %)	14 (46.7)	14 (45.2)	0.906
Multivitamin use (n, %)	6 (19.4)	7 (23.3)	0.704

[a] Data obtained from Mann–Whitney Test for continuous variables and Chi-square for categorical variables

[b] BMI: Body mass index, DBP: Diastolic Blood Pressure, SBP: Systolic Blood Pressure, WC: waist circumference

Anthropometric and microbiome population characteristics

The effect of chitosan supplementation on the gut microbiome population in the intervention and control groups is shown in Table 2. Intake of chitosan caused a significant decrease in the population of *Firmicutes* ($P < 0.001$) and a significant increase in the population of *Bacteroidetes* ($P < 0.001$) and *Akkermansia* ($P < 0.001$) as well as a marginal significant increase in the *Lactobacillus* ($P = 0.051$). In addition, the ratio of *Firmicutes* to *Bacteroidetes* showed a significant decrease after chitosan administration ($P < 0.001$). Although the amount of *Bifidobacterium* species increased after the intervention, the variation in the quantity of this microbial agent was not significant ($P = 0.317$).

After adjusting for confounders (weight, BMI z-score, energy, baseline values for each bacteria), we detected a significant decrease in the quantity of *Firmicutes* ($P < 0.001$) populations and a notable increase in the amount of *Bacteroidetes* ($P = 0.008$) and *Akkermansia*

Table 3 Energy, macronutrient, and micronutrients intake at baseline and at the end of study

	Chitosan			Placebo			*P*-value[b]
	Baseline	After	*P*-value[a]	Baseline	After	*P*-value[a]	
Energy (kcal/d)	2081.43 (204.41)	1996.10 (233.05)	**0.039**	1961.79 (244.82)	1987.78 (311.85)	0.171	0.906
Carbohydrate (g/d)	276.95 (67.57)	271.35 (71.01)	0.754	262.18 (78.22)	262.58 (80.90)	0.177	0.433
Protein (g/d)	72.51 (19.99)	69.65 (21.96)	**0.031**	71.50 (14.40)	69.78 (15.31)	0.069	0.812
Fat (g/d)	59.43 (13.94)	55.65 (16.43)	**0.018**	55.17 (19.88)	57.94 (17.75)	0.254	0.812
SFA (g/d)	20.20 (6.11)	16.67 (5.49)	**< 0.001**	18.69 (4.97)	18.41 (5.66)	0.854	0.624
MUFA (g/d)	19.55 6.77)	17.49 (6.79)	0.053	16.87 (6.96)	17.33 (6.50)	0.430	0.988
PUFA (g/d)	20.76 (8.88)	17.58 (8.06)	**0.015**	19.64 (8.60)	20.40 (9.03)	0.629	0.319
Cholesterol (mg/d)	182.14 (81.94)	175.85 (66.63)	0.991	183.18 (64.99)	175.46 (67.97)	0.517	0.956
Fiber (g/d)	17.92 (6.45)	17.29 (6.88)	0.094	17.74 (8.13)	17.67 (8.50)	0.419	0.891
Vitamin B12 (mcg/d)	1.47 (0.90)	1.41 (0.70)	0.621	1.37 (0.74)	1.37 (0.78)	0.894	0.415
Folate (mcg/d)	249.88 (104.34)	237.25 (97.72)	0.122	280.98 (132.91)	270.07 (133.81)	**0.035**	0.466
Magnesium (mg/d)	207.40 (57.45)	196.33 (56.41)	0.469	200.38 (75.90)	200.37 (64.16)	0.393	0.971
Vitamin A (RE)	945.9 (557)	1022.9 (162.8)	0.312	894.5 (490.9)	963.2 (318)	0.447	0.593
Vitamin E (mg/d)	10.4 (4.9)	8.3 (6.1)	**0.036**	11.4 (5)	9.8 (7.4)	**0.042**	0.810
Vitamin C(mg/d)	90.8 (43)	90.1 (54)	0.701	86.1 (35.3)	97.6 (33.3)	0.670	0.502
Vitamin D (mcg/d)	8.7 (6)	9.1 (3.6)	0.481	8.8 (5.3)	9.6 (5.7)	0.268	0.471
Selenium (mg/d)	60.9 (39.1)	71.8 (34.1)	0.069	68.2 (30.5)	70.3 (41.7)	0.109	0.482
Zinc (mg/d)	12.8 (3.3)	9.7 (2.7)	0.001	9.5 (4.2)	9.3 (4.8)	0.802	0.059

Data obtained from Mann–Whitney test

Data are expressed as Mean (SD)

[a] P-values for comparison of within-group differences

[b] P-values for comparison of mean values between two groups

PUFA: Polyunsaturated fatty acid, SFA: Saturated fatty acid, MUFA: Monounsaturated fatty acid

Bold value means statistical significant of $p < 0.05$

($P < 0.001$) populations, respectively, in adolescents with overweight/obesity who were prescribed chitosan versus placebo. Moreover, there was a pronounced decline in the ratio of *Firmicutes* to *Bacteroidetes* following chitosan administration ($P < 0.001$). Although there was a tendency for *Lactobacillus* populations to increase after chitosan supplementation, the mean change in the amount of this microbial agent in the chitosan versus placebo group was estimated as borderline significant ($P = 0.05$). Moreover, chitosan prescription did not influence for the quantity of *Bifidobacterium* populations versus placebo ($P = 0.97$) (Table 4).

Table 4 Effect of chitosan supplementation on gut microbiome populations in the intervention and control groups

	Chitosan				Placebo				*P* – value[b]
	Baseline	After	Change	*P* – value[a]	Baseline	After	Change	*P* – value[a]	
Firmicutes (× 10 8 copies/g)	1.76 (0.22)	1.20 (0.46)	– 0.55 (0.43)	< 0.001	1.71 (0.19)	1.77 (0.27)	0.06 (0.25)	0.323	< 0.001
Bacteroidetes (× 10 8 copies/g)	1.20 (0.13)	1.97 (0.77)	0.77 (0.72)	< 0.001	1.16 (0.09)	1.25 (0.41)	0.09 (0.38)	0.967	0.008
Firmicutes/Bacteroidetes	1.48 (0.22)	0.67 (0.33)	– 0.86 (0.10)	< 0.001	1.47 (0.18)	1.54 (0.49)	0.006 (0.05)	0.530	< 0.001
Akkermansia (× 10 7 copies/g)	1.86 (0.09)	2.85 (0.66)	0.99 (0.64)	< 0.001	1.97 (0.13)	2.00 (0.55)	0.03 (0.51)	0.334	< 0.001
Bifidobacterium (× 10 7 copies/g)	1.95 (0.13)	1.97 (0.77)	0.04 (0.24)	0.317	1.96 (0.14)	2.02 (0.45)	0.06 (0.45)	0.399	0.972
Lactobacillus (× 10 7 copies/g)	1.57 (0.16)	1.84 (0.61)	0.26 (0.59)	0.021	1.60 (0.19)	1.65 (0.25)	0.04 (0.20)	0.369	0.051

Data are expressed as Mean (SD)

[a] P-values for comparison of within-group differences

[b] P-value changes for between-group differences using analyses of covariance, considering baseline values (BMI z-score and energy) as covariate

Discussion

Chitosan supplementation is associated with a significant decrease in BMI z-scores. Moreover, our investigation demonstrated that the amounts of *Firmicutes* significantly decreased, whereas *Bacteroidetes* and *Akkermansia* species increased significantly after administration of chitosan for 12 weeks versus placebo. Furthermore, the ratio of *Firmicutes* to *Bacteroidetes* displayed a significant decrease in our RCT. Although the amounts of *Lactobacillus* and *Bifidobacterium* species increased after supplementation with chitosan, these changes were not statistically significant.

Available dietary fiber supplements, i.e., prebiotics, have been prescribed in various studies to increase the amounts of accessible carbohydrates for the bacteria living in the gut, improve microbiome diversity and the production of fermentable metabolites [28]. It has been highlighted that prebiotics, e.g., inulin and *Ganoderma*, effectively prevent the development of obesity by reversing intestinal dysbiosis[29].

Several investigations have demonstrated that prescription of chitosan oligosaccharide partially reversed dysbiosis caused by high-fat diets and obesity in mice[30]. Compared to *Firmicutes*, *Bacteroidetes* encode more carbohydrate-degrading enzymes. For example, starch-degrading systems are widely available in *Bacteroidetes*. The function of chitosan is presumed to be similar to the actions of other oligosaccharide prebiotics, e.g., xylo-oligosaccharides. As chitosan can provide a better growth environment for *Bacteroidetes* versus *Firmicutes*, this type of dietary fiber can ensure suitable conditions for intestinal microbial agents to fully use carbohydrates [31]. The ratio of *Firmicutes/Bacteroidetes* in obese subjects reports significant decreasing changes[32]. Thus, since this parameter is modified in obese subjects, this may partially explain why chitosan administration was able to reduce *Firmicutes* abundance and modulate the *Firmicutes* to *Bacteroidetes* ratio [33]. In an assessment conducted by Hay et al. [30], mice who were fed high-fat and low-fat diets, respectively, experienced an increase in the abundance of *Bacteroidetes* (from 39.81% to 48.02%) and *Firmicutes* (from 09.09 10.0% to 38.21%) and an elevation of the ratio of *Firmicutes* to *Bacteroidetes*. After the mice were given chitosan, the animals who were fed low-fat diets experienced a decline in the aforementioned bacterial ratio, however, mice who were fed high-fat diets did not display alterations of the aforementioned ratio following consumption of chitosan. This finding may be explained by the fact that the mice who received high-fat diets experienced intestinal dysbiosis which was also enhanced by the pro-inflammatory stimuli of *Bacteroidetes*. Thus, chitosan supplementation could not effectively modulate this bacterial population. In another assessment [34], *Bacteroidetes* and *Firmicutes* expressed significant negative and positive correlations with peptides and adipokines related to the regulation of insulin action and insulin resistance as well as energy homeostasis, respectively.

In our research, *Akkermansia* species also increased significantly after 12-week chitosan administration versus placebo. The population of this bacterial species increases in the presence of prebiotic substances, e.g., fibers or indigestible carbohydrates [35]. Moreover, the decrease in the population of this microbial agent in the intestine is directly related to the presence of chronic diseases such as obesity, T2DM and inflammatory bowel disease [36]. Some studies have suggested that *Akkermansia* is one of the bacteria that can produce SCFAs[37]. Disturbance in the amounts of bacteria able to generate SCFAs may enhance the activity of pro- inflammatory signaling cascades in T2DM, obesity and other inflammatory diseases [38]. SCFAs can activate free fatty acid receptors which stimulate the secretion of appetite hormones such as leptin and peptide YY [39, 40]. Consistent with this hypothesis, several scientists have proven that in humans, at levels below a certain amount of *Akkermansia*, subjects are less likely to respond positively to a calorie-restricted diet[41].

Bifidobacterium is one of the first intestinal bacteria that develops in children and the most abundant intestinal bacteria in adults, exerting anti-tumor and pro-apoptosis effects [42]. This bacterial agent, along with *Lactobacillus*, is among the most prescribed probiotics. Co-administration of these two types of bacteria was able to significantly reduce body weight, BMI, waist circumference (WC), low-density lipoprotein cholesterol (LDL-cholesterol) and improve the quality of life of obese adults after 6 months of supplementation [43]. However, some *Lactobacillus* species are more likely to be overexpressed in children suffering from non-alcoholic fatty liver and obesity versus controls, whereas *Bifidobacterium* species were less abundant. Therefore, we may infer that the aforementioned microorganisms can exert protective effects against these diseases[44]. Regarding the effect of chitosan on *Bifidobacterium* and *Lactobacillus*, there are a few studies in this regard, and it is interesting to note that most of the assessments proposed chitosan as a potential preservative in the encapsulation process of these probiotics [45, 46]. For example, Tong et al. [47] investigated the combined effect of *Ganoderma lucidum* polysaccharide and chitosan in hamsters who were fed high-fat diets, demonstrating an increase in the abundance of *Bifidobacterium* after the intervention. However, these results cannot be attributed solely to chitosan because its net effect was not seen in the aforementioned publication.

In another research, it was seen that the consumption of 1 g of chitosan per kilogram of mouse weight increased the growth of *Lactobacillus* and *Bifidobacterium* [11]. In another animal investigation, administration of 60 mg/kg body weight of chitosan significantly increased *Bifidobacterium* populations compared to the control group, but no significant changes were observed in *Lactobacillus* populations after chitosan administration in the context of a high sucrose diet. Moreover, no significant changes in the numbers of these bacteria were observed in the control group. Therefore, it seems that chitosan administration can only moderate the harmful effects of high sucrose diets on the microbiome population [48]. Other studies that investigated the effect of chitosan in preclinical models concluded that chitosan significantly inhibits *Lactobacillus* species in direct relationship with *Bifidobacterium* species [9, 49]. The inconsistency regarding the impact on *Lactobacillus* and *Bifidobacterium species* may be caused by the differences between the molecular weights of chitosan used. Furthermore, an assessment depicted that different strains of *Lactobacillus* may exert different behaviors in dealing with chitosan. Thus, chitosan administration led to notable increases in the population of *Lactobacillus brevis* as compared to *Lactobacillus casei* [50]. It is well-accepted that *Lactobacillus* and *Bifidobacterium* are among the bacteria that influence gut health. However, more studies are needed to clarify the effect of chitosan on these bacteria and more specifically on their different species, as it is still unclear which microbial agent is predominantly influenced by chitosan.

Strengths and limitations

The main strength of this study is its RCT design and novelty, given that it was the first research involving humans to examine the effects of chitosan supplementation on gut microbiota in adolescents with overweight or obesity. However, there are some limitations to our investigation as well. The small number of study and also bacteria examined in the study and the fact that we did not consider different strains of the same bacterial species are among the main limitations of our RCT. In addition, the choice of screening gut microbiome using RT-PCR creates bias in the target selection. Another drawback of our research is that RT-PCR has low sensitivity for microbial agents. Moreover, we did not examine genetic factors that could have influenced the numbers and types of bacterial species in the gut. Thus, further research is needed to clarify the potential benefits of chitosan administration in obesity management and its impact on intestinal microbiota in adolescents with overweight/obesity [51, 52].

Conclusion

Our RCT suggests that the amounts of *Firmicutes* significantly decrease and the quantities of *Bacteroidetes* and *Akkermansia* notably increase following administration of 3 g of chitosan for 12 weeks versus placebo in adolescents with overweight or obesity. Moreover, the intervention reduced the ratio of *Firmicutes* to *Bacteroidetes*. However, although the amounts of *Lactobacillus* and *Bifidobacterium* species increased after chitosan was prescribed, the variation in their quantity was not statistically significant. However, the effects of chitosan supplementation must be considered as an adjuvant rather than a magic bullet for gut microbiota modulation and obesity management in adolescents with overweight or obesity.

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Author contributions
S.F., M.S. and Mh.S contributed to the conception, design, and statistical analysis. S.F., and H.S contributed to data collection and manuscript draft. M.S, A.S. and F.Sh supervised the study. S.F, K.V, P.R, M.-A.G. contributed to the manuscript draft and critical revision. All authors approved the final version of the manuscript.

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Availability of data and materials
Data available on request due to privacy/ethical restrictions.

Declarations

Ethics approval and consent to participate
This study was approved by the research council and ethics committee of Iran University of Medical Sciences, Tehran, Iran (NO: IR.IUMS.REC.1400.104). The study has been registered in IRCT (IRCT20091114002709 N57; registration date: 2021 - 06 - 20).

Consent for publication
An informed consent was obtained from all the individuals included in the study. All participants agreed to publish.

Competing interests
The authors declare no competing interests.

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