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Research Article

Effect of Dietary Supplementation with *Lactobacillus* Strains in Non-Dairy Matrices on Colonic Metabolic Activities: A Clinical Trial

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Abstract

Background: Gut microbiota has beneficial effects on intestinal mucosa. It enhances immunity and produces metabolites that influence general health. This study aimed to evaluate the effect of dietary intervention with multi-strain potential probiotic *Lactobacilli* on functional metabolic colonic activities in healthy adult males. Furthermore, the study introduces dates and biscuits as non-dairy matrices for administration of probiotic *Lactobacilli*. **Methods**: The crossover study included eleven adult male volunteers, who were assigned to three different interventions consecutively, with each intervention lasting for 21 days. The participants were given biscuits with probiotics, followed by biscuits with placebo, and lastly dates with probiotics. Each phase was followed by a month of washout period. The consumed biscuits and dates contained probiotics with a total daily dose of 1.68×10^8 and 3.3×10^8 colony forming units, respectively. Before and after each test period, the volunteers were clinically examined and stool samples were collected. The samples were examined for *Lactobacillus* count, short chain fatty acids, secretory immunoglobulin A, ammonia contents, and stool pH using standard techniques. **Results**: After daily consumption of probiotics, a significant increase in fecal secretory immunoglobulin A and short chain fatty acids were observed, with associated significant decrease of fecal ammonia contents. As a whole, colonic metabolic functions were significantly improved. **Conclusion:** Biscuits and dates proved to serve as efficient matrices for delivery of live probiotics. The used probiotic *Lactobacillus* strains have positive impact upon metabolic functions of the colon in healthy adults.

Keywords: Ammonia; Gut microbiota; Non-dairy matrix; Probiotic *Lactobacillus* (LAB); Secretory Immunoglobulin A (sIgA); Shot Chain Fatty Acids (SCFAs)

Introduction

Human gastrointestinal tract is inhabited by indigenous microbiota forming unique microbial communities. The composition of these communities varies from one individual to another based on diverse factors including gender, age, geographic location, diet, host genetic makeup, and general health status of the individual [1]. Despite the diversity, gut microbiota was proven to have multiple beneficial functions to the host. The functions may be distant to the gut as its neurological effects. Being a component of the gut-brain axis, gut microbiota helps in regulating brain behaviour through bi-directional neuronal signaling [2]. Other functions of gut microbiota are detected within the gut. It has digestive functions such as fermenting otherwise non-digestible food items [3] and synthesizing vitamins and amino acids

[4]. Immunological functions include prevention of pathogen colonization [5] as well as the regulation and maturation of the immune system [6]. In return, secretory immunoglobulin A (sIgA) plays an essential role in maintaining gut microbiota homeostasis [7]. In other words, the host's immune system helps shaping the gut microbiota and the microbiota helps reinforcing the host's immune system. Therefore, the level of mucosal sIgA and degree of its specificity constitute a good marker of immunological events in the digestive tract [8].

Furthermore, gut microbiota plays a pivotal role in regulating multiple host metabolic pathways by producing a wide range of beneficial metabolites. Among these metabolites are Short Chain Fatty Acids (SCFAs), a family of carboxylic acids, the most abundant (95%) of which are acetate, propionate, and butyrate [9]. SCFAs result from anaerobic microbial fermentation in the large intestine [9,10]. They are associated with concomitant reduction of luminal pH, which inhibits pathogenic microorganisms and enhances the absorption of some nutrients [11]. In addition to their metabolic functions, there is growing evidence that SCFAs contribute to the reduction of colonic inflammation, preventing colon carcinogenesis, and promoting mucosal healing [12].

Ammonia represents another metabolite produced by the human gut. The gut-derived ammonia is produced via enzymatic de-amination of amino acids [13]. Afterwards, the produced ammonia together with carbon dioxide participates in producing urea through the hepatic-urea cycle [14]. Lastly, urea is filtered and cleared from blood through renal excretion [13].

Based on all the known benefits of gut microbiota, research was directed to introduce living non-pathogenic microorganisms as dietary supplements. Such microbial strains have been called probiotics. Results showed that ingestion of probiotics have well documented health advantages [15]. Further research aimed to embed probiotics within food substances to be consumed as an integral food ingredient. This brought up another challenge of developing novel probiotic carriers. The carrier should help the probiotic to colonize the colonic environment [16,17].

Therefore, the current study aims to evaluate the effect of dietary intervention with multi-strain indigenous potential probiotic *Lactobacillus* (LAB) on metabolic colonic activities in healthy adult males. It also introduces novel carriers in the form of biscuits and dates as non-dairy matrices and tests their efficacy.

Subjects and Methods

Study design

A crossover study was designed, where 3 different interventions were consecutively administered to the same participant. In the first treatment, the volunteers were assigned to receive one piece of biscuit (2 layers) containing the functional probiotic daily for three weeks (21 days). After a wash out period of one month, the second treatment was mediated where the same participants were assigned to receive placebo biscuits (free of probiotics) for another 21 days. After a second rest period of one month, the third treatment was mediated where the same participants were assigned to receive dates with probiotics. Each participant was asked to eat three pieces of functional dates daily for 21 days. At the end of the study, each subject served as his own control. For each intervention, the following outcomes were assessed: enumeration of fecal viable total LAB, assay of fecal secretory IgA (sIgA), quantitative determination of the fecal SCFAs, determination of fecal pH, and quantification of fecal ammonia concentration.

Subjects

The study included 11 adult healthy males and inclusion criteria included age ranging between 20-40 years, accepted the taste of the supplement and agreed to collect fecal samples at predetermined dates. Exclusion criteria included smoker individuals, individuals having gastrointestinal and/or chronic health problems, obese or undernourished subjects and individuals receiving any medications or antibiotics during the last month prior to study.

For all volunteers, detailed medical history was taken followed by thorough clinical examination including blood pressure measurement. Weight and height were measured using sensitive balance and the Body Mass Index (BMI) was calculated as kg/m^2, where kg is a person's weight in kilograms and m^2 is the height in meters squared. The same volunteers participated in three clinical trials, In Trial (1) and Trial (3), the volunteers ingested daily for 21 successive days probiotic formulation in biscuits and dates food matrix, respectively. In trial (2) the same volunteers ingested placebo biscuits. Flow diagram 1 illustrates the phases of the 3 trials.

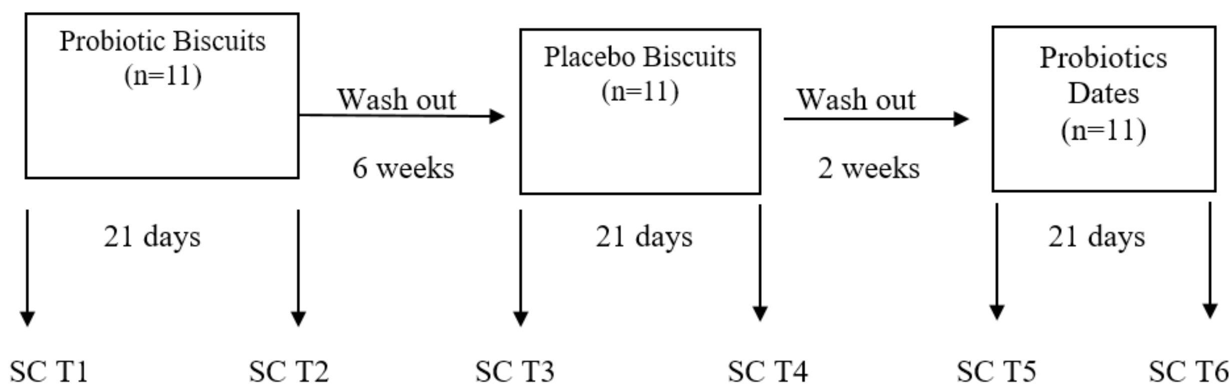

Diagram 1: Flow diagram showing the successive phases of supplementation. SC: Stool Collection.

Preparation of supplements

The potential probiotic LAB strains used in the clinical trials included *Lactobacillus plantarum* NRRL B-14768; AJ965482 [M02], *Lactobacillus rhamnosus* JCM 1136; D16552 [SM] and *Lactobacillus paracasei* JCM 8130; D79212 [K04] Each strain was subjected to Edible Film coating (EF) to prolong its viability during handling and as protectant against the harsh conditions during stomach transit. The film coating agents consisted of a blend of colloidal whey protein (2%), plasticizer alginate and glycerol and the standard technique was followed [18].

Multimix probiotic LAB formulation was prepared by gentle mixing of equal cell counts of the respective EF LAB strain, which was used as filling agent on a piece of biscuit, followed by covering with a layer of sesame butter to act as barrier against exposure to atmospheric air and the biscuit was covered immediately without delay with another piece of biscuit using honey as binding agent. The functional probiotic dates were prepared by injecting the multi-strain probiotic EF LAB directly inside dry dates freed from the seeds.

A layer of sesame butter was layered above the probiotic LAB immediately without delay followed by covering with a drop of honey. The functional biscuits and functional dates were packaged in bags under vacuum and kept at 40°C for the weekly supply to the participants. Placebo biscuits were not distinguished from probiotic biscuits except that they were free of probiotics. Table 1 presents the ingredients of the functional biscuits, functional dates and placebo biscuits.

Parameter	Mean (± S.D)
Number of participants	11
Age (year)	34.68 (± 8.94)
Body weight (kg)	87.18 (± 14.24)
Body height (cm)	178.73 (± 6.07)
BMI (kg/m^2)	27.27 (± 3.52)
Systolic blood pressure (mm/Hg)	121.88 (± 9.23)
Diastolic blood pressure (mm/Hg)	76.88 (± 5.94)

Table 1: Characteristics of the study subjects.

Microbiological testing

One whole biscuit or one date was homogenized with 90 ml of 0.85% (w/v) sterile saline. For viable cell counting, serially diluted cultures (100 µl aliquots) were cultured in MRS agar [1.5% (wt/vol)] [19] under aerobic condition without shaking and inoculated at 37°C for 48 h. The viability of the cell was expressed as colony-forming unit per functional biscuit or functional date. Table 1 presents the total LAB intake per serving portion.

Probiotic biscuits provided per portion 1.68 x 10^8 CFUs of equal quantities of *L. paracasei* and *L. rhamnosus*. On the other hand, probiotic dates (3 dates) provided 3.3 x 10^8 CFUs of equal amounts of *L. paracasei*, *L. rhamnosus* and *L. plantarum*. The mean weight for each portion eaten was 29.7±0.3 g in the probiotic biscuits group, 22.8±0.1 g for the placebo group, and 20.6±0.7 g in the dates group.

Stool sample collection

Six stool samples were collected from each volunteer at different predetermined collection periods which are illustrated in the flow diagram. For each treatment, the first sample was collected within 1-3 days before starting the intervention (pre-intervention) and the second sample at the last day of intervention (post-intervention). Collection of stool samples was repeated for each treatment with a total of 6 samples collected for each subject.

Fecal samples were collected in sterile containers. Immediately, aliquots were taken, placed in an icebox, and transported to the laboratory within one hour for bacterial count assessment. The remaining stool sample was placed in plastic vials and saved frozen at -40°C for further investigations.

Enumeration of fecal viable total LAB

Aliquots of stool samples (5 grams) were serially diluted and aliquots (100 µg) were plated on 1.5% MRS agar [19]. The plates were incubated at 37°C for 48-72 hours under anaerobic conditions using Gas Pak (H_2 and CO_2) anaerobic systems (Hartminke). The results were expressed as log10 CFUs per gram-wet weight of fecal material.

Assay of fecal secretory IgA (sIgA) by ELISA (fecal sIgA)

The vChrom ELISA kit (Bio Vender research Diagnostics, Cat # RIC6100, Germany) was used and the assay was carried out according to the instructions of the manufacturer. Secretory IgA concentration was expressed as µg per g stool.

Separation of fecal short chain fatty acids (SCFAs)

Fecal SCFAs were separated on HPLC instrument (Agilent, USA) equipped with 25 cm x 3 mm, i.d. column packed with LiChrosorb Si 60 of 5-mm particle size (Merck, Darmstadt, Germany).

Quantitative determination of fecal SCFAs

Fatty acid (FA) standards (acetic, propionic, and butyric acids) were obtained from Sigma-Aldrich, Inc., (St Louis, MO, USA). Fatty acid concentrations were determined by the external standard curve procedure. The analyses were performed with a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector (Little Falls, DE). The column was a 30-m capillary (0.32 mm i.d.) Omega wax (Supelco, Bellefonte, PA).

Chromatographic conditions were isothermal. Optimized injection, oven, and detection temperatures were carried out according to the instructions of the manufacturer. The carrier gas was hydrogen with a linear velocity of 45 cm/s and a 1:60 split ratio. The FAME were identified by their retention time and were quantified by the amount of internal standard recovered and by comparison with authentic lipid standards (Sigma, Deisenhofen, Germany). The FA concentrations were expressed as specific FAs (% weight/ total identified FA in the feces) and presented as µ mol/g.

Determination of fecal ammonia concentration

Colorimetric method was done using a microplate adapted method of the procedure outlined by Hsi-Chiang and Visek (1991) [20] using a microplate reader.

Fecal pH determination

pH was measured using a digital pH meter (Elico L 20/L610—India).

Statistical analyses

Quantitative data were expressed as arithmetic means with standard deviation. Fecal bacterial counts were transformed to the logarithmic scale. The collected data were compared within groups between baseline (T0) and the subsequent 21-day treatment. Statistical analyses were carried out for all the variables using the two-way ANOVA followed by the Tukey multiple comparisons test. $P \leq 0.05$ indicates significant statistical differences. Graphs were generated using GraphPad Prism (version 9, San Diego, CA, USA).

Results

Baseline data

The study included eleven adult male volunteers, aged 34.68±8.94 years. Baseline demographic and medical characteristics of the study subjects are presented in table 1.

Outcome data

Enumeration of fecal viable total LAB

The dietary intake of utilized probiotic LAB strains in biscuits or dates as matrices for 21 consecutive days resulted in significant increase of LAB count recovery in stool samples for both matrices ($p < 0.001$) as illustrated (Table 2, Figure 1).

Treatment	Probiotics Biscuit			Placebo Biscuit			Probiotics Dates		
	Pre	Post	*P*-value**	Pre	Post	*P*-value**	Pre	Post	*P*-value**
Fecal *LAB* count (log 10 CFU/g)	7.00 ±0.68	8.64 ±0.49	<0.001*	6.92 ±0.76	7.04 ±0.88	NS†	7.38 ±0.66	9.13 ±0.96	<0.001*
sIgA (µg/g feces)	812.41 ±97.14	1145.96 ±331.34	<0.010*	797.70 ±49.79	814.81 ±43.99	NS†	698.15 ±53.38	1369.44 ±127.25	<0.001*
Acetate SCFA (µ mol/ g)	24.82 ±4.66	41.77 ±9.44	<0.001*	19.38 ±5.19	25.14 ±2.74	NS†	17.79 ±4.77	49.37 ±4.71	<0.001*
Propionate SCFA (µ mol/ g)	11..89 ±3.86	19.52 ±6.24	<0.001*	10.40 ±3.35	11.42 ±2.36	NS†	9.75 ±3.35	22.85 ±2.91	<0.001*
Butyrate SCFA (µ mol/ g)	17.43 ±3.86	29.31 ±8.88	<0.001*	15.74 ±4.51	17.30 ±2.18	NS†	13.69 ±3.93	45.92 ±26.12	<0.001*
Total SCFA (µ mol/ g)	57.19 ±11.36	99.83 ±25.77	<0.001*	48.45 ±12.55	55.98 ±6.15	NS†	44.48 ±11.09	125.38 ±8.89	<0.001*
Ammonia (µg/g feces)	74.10 ±21.92	50.21 ±19.76	0.017*	76.52 ±15.65	67.21 ±18.94	0.023*	77.77 ±15.48	47.94 ±14.57	<0.001*
Fecal pH	6.32 ±0.42	6.19 ±0.53	NS†	6.26 ±1.26	6.17 ±0.74	NS†	6.17 ±0.74	5.64 ±0.29	0.013*

*P≤0.05; **Two-way ANOVA test; †NS: Non-Significant difference

Table 2: Mean and standard deviation of pre- and post-treatment values of study outcomes in the 3 intervention groups.

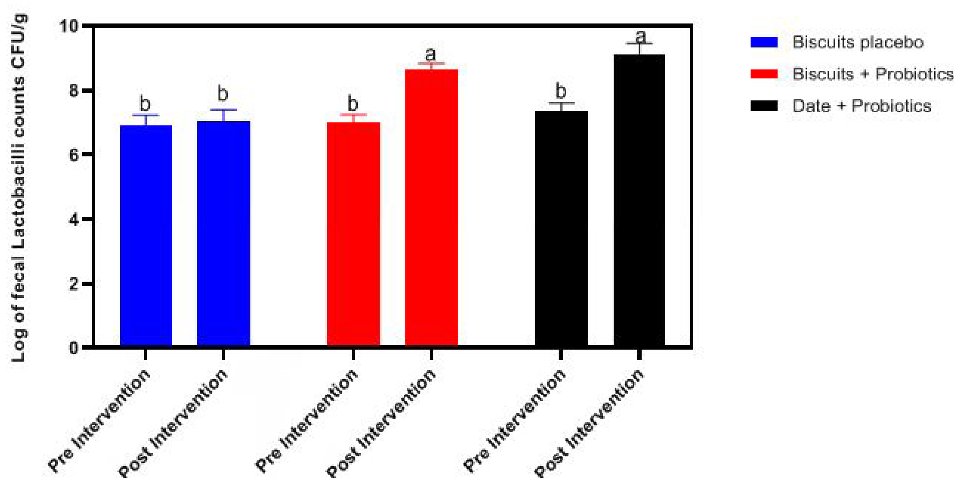

Figure 1: mean fecal LAB count (log 10 CFU/g) in pre and post interventions of probiotics with biscuits and dates versus placebo.

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Assay of fecal secretory IgA (Fecal sIgA)

Fecal sIgA production was increased with ingestion of probiotics in biscuits ($p<0.01$) and probiotics in dates ($p<0.001$) as illustrated in Table 2 and Figure 2.

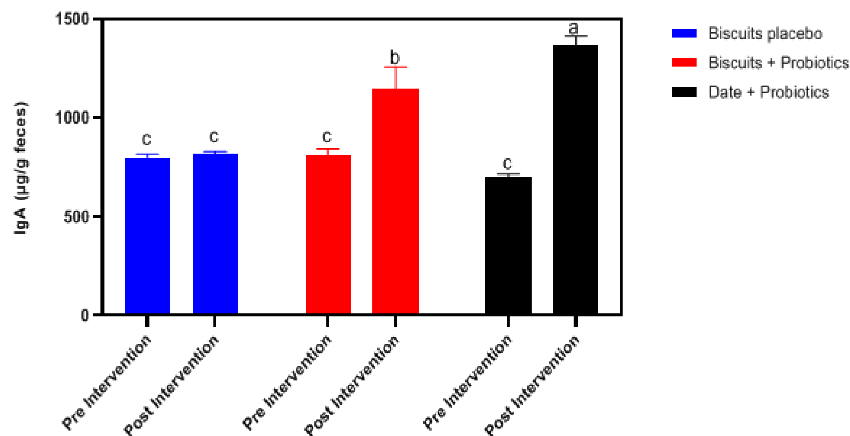

Figure 2: mean fecal sIgA in pre and post interventions of probiotics with biscuits and dates versus placebo.

Fecal Short Chain Fatty Acids (SCFA) production

All participants responded positively after the 3 week dietary intervention with functional biscuits or functional dates as evidenced by statistically significant increases in fecal acetate (Figure 3a), propionate (Figure 3b) and butyrate (Figure 3c) and total short chain fatty acids (Figure 3d) compared to the respective changes among the volunteers during the intake of the placebo biscuits, which were significantly increased with ingestion of probiotics in both biscuits and dates ($p<0.001$) (Table 2).

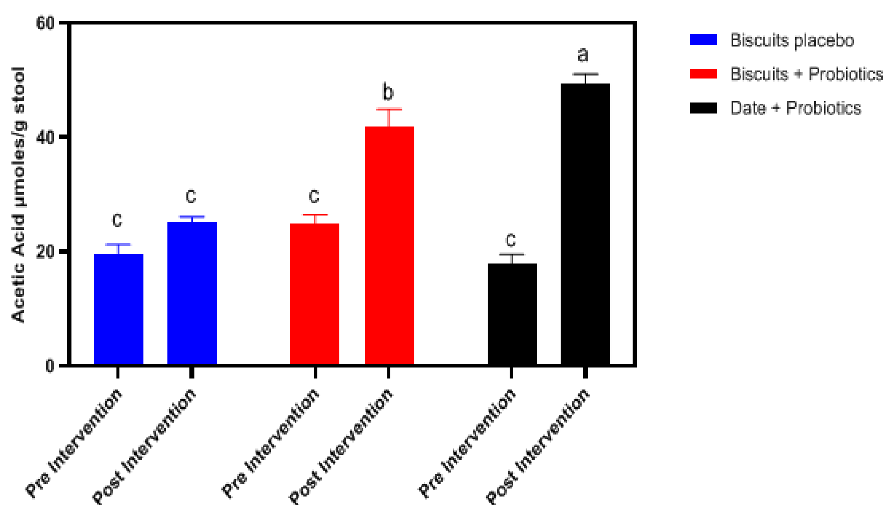

Figure 3a: Mean fecal acetic acid in pre and post interventions of probiotics with biscuits and dates versus placebo.

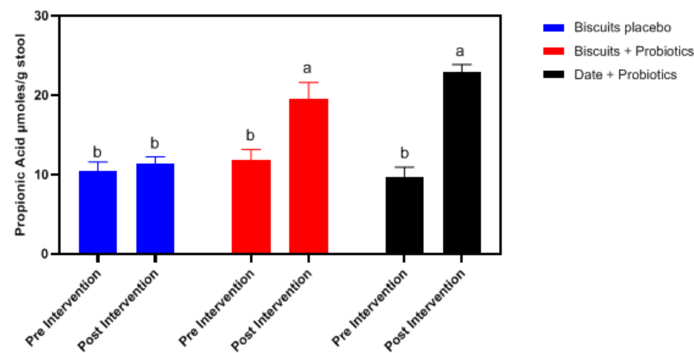

Figure 3b: Mean fecal propionic acid in pre and post interventions of probiotics with biscuits and dates versus placebo.

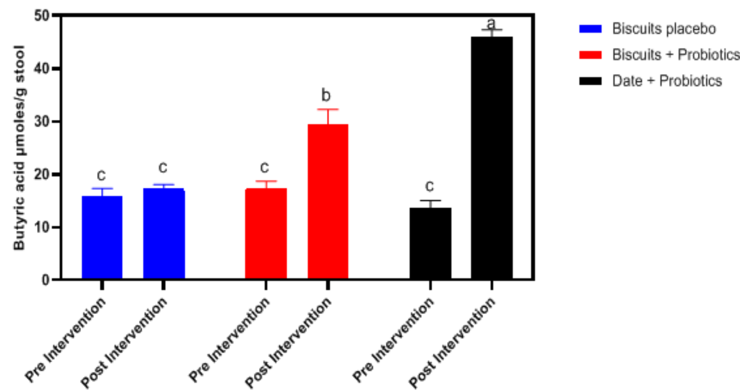

Figure 3c: Mean fecal butyric acid in pre and post interventions of probiotics with biscuits and dates versus placebo.

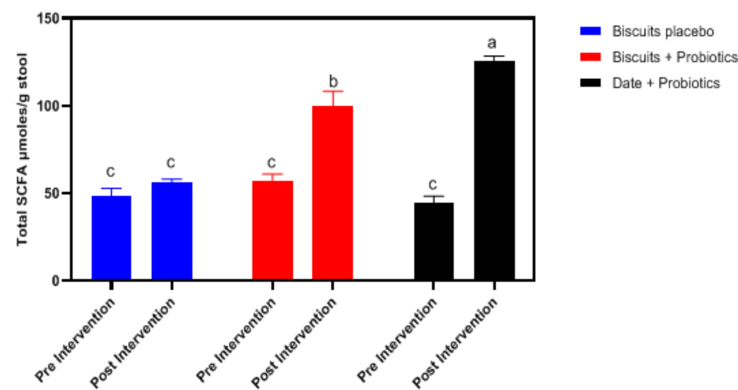

Figure 3d: Mean fecal total SCFA in pre and post interventions of probiotics with biscuits and dates versus placebo.

Quantifying fecal ammonia concentration

Fecal ammonia showed a significant decrease with ingestion of probiotics in biscuits (p=0.017) and probiotics in dates (p<0.001) (Table 2). In all the outcomes, placebo ingestion showed non-significant changes for LAB count, SCFAs, sIgA or pH values.

Determination of fecal pH

Stool pH was decreased with probiotics in both matrices but didn't reach significant levels.

Discussion

Probiotics are used to replenish beneficial bacteria in the body. However, probiotic food preparations still represent a challenge since different probiotic species possess sensitivity resistance towards the acidity of the substrate and GIT condition [21]. Therefore, the current study aimed to test the effect of dietary intervention with multi-strain indigenous potential probiotic *lactobacilli* (LAB) on metabolic colonic activities. In addition, because the tolerance of probiotic bacteria to gastric acidity and small intestine conditions are influenced by the carrier [22]. The current study presents dates and biscuits as novel non-dairy matrices for LAB strains.

To fulfill the aims of the study, 3 strains of *Lactobacillus* were used. Arguably, there are two main approaches for selecting a strain to be probiotic. The first is to use a species normally abundant at a site, and simply replenish or boost the total count of that species in subjects whose microbiota has shifted to being dysbiotic. The second approach is to select a strain(s) that has specific properties to counter an ailment. The *Lactobacillus* species found in the GIT have received tremendous attention due to their health-promoting properties [23]. The probiotic LAB strains used in the present study included "allochthonous" strains (*L. casei, L. plantarum* and *L. rhamnosus*) originally isolated from local fermented foods, which were present at different ecosystems, not from human origin.

The international scientific association for probiotics and prebiotics specified a list of dosages ranging from 1×10^8 to 1.8×10^{12} CFU twice daily depending on the strain and the disease based on at least one well-designed clinical trial showing a beneficial effect for a health-promoting or therapeutic outcome [24]. In the current study, probiotics were provided in doses of 1.68×10^8 and 3.3×10^8 CFU daily for probiotics with biscuits and probiotics with dates respectively, which are within advisable effective dose range.

Especially in studies using LAB, traditional yogurt and fermented milk are still the most common food matrix for probiotic consumption [22]. The addition of probiotics into dairy foods might improve their tolerance to the low gastro-intestinal pH level. Unfortunately, lactose intolerance among adults represents a significant downside to dairy-based foods consumption [21]. Therefore, the development of novel probiotic carriers is necessary for obtaining the full beneficial effect of probiotics [16,17]. In the present study, non-dairy matrices were introduced in the form of biscuits and dates.

Furthermore, probiotics in whole foods may not survive the manufacturing and storage processes. Therefore, parallel technologies have been utilized to maintain the viability of probiotics; among which is edible film coating or micro-encapsulation. In this technique, the probiotics are entrapped in microcapsule consisting of edible biopolymer [18] that has stable shelf life within heterogeneous food matrices [25]. The present study used Whey Milk Protein (WMP) as the colloidal biopolymer for LAB microencapsulation showing good stability for bacteria viability.

The primary outcome for assessing the effectiveness of probiotics is colonizing the colonic epithelia and increase in the recovery of the probiotic organism in the stool sample [16,17]. These factors are generally assessed by detecting the amount of viable probiotic strains in the stools [26]. In the current study, fecal LAB count increased significantly with biscuits and dates matrices (p<0.001) after 3 weeks consumption This indicated good gut colonization compared to placebo, which showed no changes in LAB counts.

Beneficial role of probiotics could be evaluated by measuring their metabolic activities through analysis of fecal sIgA, organic acid and ammonia concentrations [27].

Fecal sIgA concentration, which serves a marker of gut immune function, showed significant increase following the 3 weeks dietary intervention with probiotic functional biscuits (p<0.01) or functional dates (p<0.001). The results provided evidence of the efficacy of the ingested probiotics. Comparable results of a significant rise in fecal sIgA levels were previously reported after 3-week probiotic supplementation in preterm infants [28].

In the present study, the concentrations of fecal SCFAs (acetate, propionate and butyrate) revealed statistically significant increases in of among participants consuming probiotic supplements. The results denote that the intake of the used probiotics mix was associated with promotion in the production of these low molecular weight SCFA molecules. This indicates adequate colonization of our used probiotics and production of beneficial metabolites for the host.

Moreover, one of the fermentation intermediates of LAB is Lactic acid. It is a cross-feeding substrate for the colonic bacteria *Eubacterium hallii*; leading to production of either butyrate or

propionate [29]. The production of the SCFAs leads to lowering the pH of the colonic milieu to about 5.5 [30], which inhibits pathogenic microorganisms and increases absorption of some nutrients [11].

In our study, fecal pH was decreased in both treatments. The decrease was significant for probiotics with dates ($p < 0.05$).

Although the sample size was relatively limited (N=11), yet the results could be discriminated significantly according to the treatment. The main objective of the present study was to evaluate the efficiency of novel multi-strain probiotic LAB in non-dairy matrix on different colonic metabolic aspects of relevance to health. The results revealed significant discrimination in all the tested parameters according to the treatment, in part thanks to the subject group being quite homogenous regarding age, ethnic group, social and educational status. A trial on larger sample size including patients with disorder in the gastrointestinal tract is in progress.

Conclusion

The present study demonstrated the efficacy of the administration of the indigenous *Lactobacilli* strains as valuable probiotics with good bacterial recovery. They also have beneficial metabolic activities. The used matrices showed good shelf life and helped probiotics to withstand gastric acidity and intestinal conditions.

Declarations

Funding

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Competing Interests

All authors declare that they had not any financial or non-financial interests that are directly or indirectly related to the work submitted for publication.

Authors' Contributions

Laila Hussein designed the study and supervised all steps of the lab work, statistical analysis and writing the paper. Gamal Yamamah performed clinical work and wrote the manuscript. Amr Elbahnasawy, Mosab Gad, and Magda Soliman have prepared supplements, collected samples, and performed the lab work. Amr Elbahnasawy also performed the statistical analyses. Asmaa Ramadan performed microbiological work. All authors viewed and approved the final version of the manuscript.

Ethics Approval

The research protocol was conducted according to the guidelines laid down in the Declaration of Helsinki, and the trial was approved by the Medical Research Ethical Committee, National Research Center and registered as Clinical Trial number 16/422.

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Consent to Participate

Written informed consent was obtained from each participant.

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