Microbiological and clinical effects of probiotic-related Zeger therapy on gingival health: a randomized controlled clinical trial

Xin Chen[1†], Yi Zhao[1†], Kun Xue[1], Mengyao Leng[1] and Wei Yin[1*]

Abstract

Background This single-blind randomized controlled trial was aimed to evaluate the microbiological and clinical effects of Zeger therapy on gingival health.

Methods Twenty-four adults with gingivitis were recruited and monitored micro-biologically and clinically at baseline (Day 0), 4 weeks (Day 29) after therapy. All volunteers received one-stage full-mouth supragingival scaling as basic oral health care for baseline, and then randomly divided into experimental (koumiss, $n = 12$) or control (none, $n = 12$) group. The koumiss was used once a day for 4 weeks.

Results The microbial diversity of the experimental group increased significantly after drinking koumiss ($p < 0.05$), mainly owing to increasing of Gram-positive bacteria ($p = 0.038$) and oral health-related microbes (*Rothia*, *Corynebacterium*, *Actinomyces*, *Saccharibacteria_TM7*, etc.), decreasing of Gram-negative bacteria ($p = 0.009$) and periodontal disease-related microbes (*Porphyromonas*, *Fusobacterium*, *Veillonella*, etc.), while the microbial diversity of the control group had no significant change ($p > 0.05$). However, there was no significant difference between the two groups in the clinical parameters ($p > 0.05$).

Conclusions Zeger therapy promotes the diversity of supragingival microbiome in adults with gingivitis and increases the abundance of some beneficial flora while decreasing some harmful without clinical parameters marked changing, which holds promise for improving of gingivitis and may be a valuable oral health care approach in the future.

Trial registration The clinical trial was approved by the Medical Ethics Committee of West China Hospital of Stomatology, Sichuan University, batch No. WCHSIRB-D-2021-428. Before patient registration began, the prospective clinical trial was registered in www.clinicaltrials.gov public repository in China under the registration number ChiCTR2200060555 on 04/06/2022.

Keywords Zeger therapy, Probiotics, Oral commensal microbiota, Gingival health, Gingivitis, Periodontal disease

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Introduction

Zeger therapy, entailing the consumption of koumiss, also known as kymyz or qymyz, a probiotic-rich beverage fermented from mare's milk, holds significance in traditional Asian steppe diets and features prominently in ethnomedicine. Initially localized to specific regions, the widespread adoption of koumiss ensued later, driven by its manifold benefits, including low cholesterol levels and a high content of polyunsaturated fatty acids. Consequently, koumiss has garnered increased attention, particularly in Europe, notably in countries like France and Germany [1]. Mare's milk, the primary ingredient in koumiss, bears a striking resemblance to human milk in terms of protein composition, sugar content, and salt levels [2]. The substantial sugar content in mare's milk undergoes fermentation by microorganisms, yielding lactic acid, alcohol, and various functional molecules, such as p-pyruvate, 20-HETE, 4-aminobutanoate, uracil, acetoacetate, and γ-linolenic acid, imparting a distinctive flavor, texture, and acidity to the beverage [1, 3]. Leveraging the unique attributes of koumiss, Zeger therapy manifests several advantageous effects, predominantly in three key domains. Firstly, it plays a pivotal role in regulating microbial community homeostasis by inhibiting potentially harmful genera like *Pseudomonas* and *Acinetobacter* [4], countering pathogenic *Escherchia coli.* and *Staphylococcus aureus* [5]. Secondly, its anti-inflammatory and immunomodulatory effects are evident in studies demonstrating enhanced intestinal immunity, reversal of thymus and spleen atrophy in immunosuppressed model rats, and an increase in leukocyte and lymphocyte counts, along with the CD4+/CD8+ratio in peripheral blood lymphocytes [6, 7]. Thirdly, its antioxidant properties are corroborated by studies indicating cholesterol reduction, atherosclerosis prevention [5, 6, 8], and protection against liver damage by enhancing antioxidant activity [9, 10]. However, research into the clinical and microbiological effects of probiotic-related Zeger therapy in oral health remains limited.

The current state of gingival health is significantly compromised by periodontal inflammatory diseases, constituting a burgeoning public health challenge that affects up to 90% of the global population and imposes substantial burdens on both health and the economy [11–13]. Gingivitis typically represents the initial stage of periodontal diseases, primarily driven by an imbalance in commensal microbiota within dental biofilms. Characterized by symptoms such as gingival hyperemia, swelling, pain, and gingival bleeding, gingivitis can progress to periodontitis, ultimately culminating in tooth loss among adults, thereby detrimentally impacting quality of life [14, 15]. Currently, various scientifically validated or exploratory approaches are employed to maintain gingival health and prevent gingivitis [16].

Notably, probiotic therapy has garnered considerable attention for its significant potential and promising prospects, albeit remaining insufficiently researched. Probiotics, as defined by the Food and Agriculture Organization of the United Nations/World Health Organization, refer to "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" [16]. Multiple systematic reviews and meta-analyses have underscored the substantial clinical benefits of probiotic supplementation for patients with gingivitis or other periodontal diseases, including improvements in plaque index (PI), gingival index (GI), probing pocket depth (PD), clinical attachment level (CAL), gingival bleeding index (GBI), and reductions in the levels of major subgingival periodontal pathogens such as *Porphyromonas*, *Fusobacterium*, and *Tannerella* [17–20]. However, research on the effects of probiotic-related Zeger therapy on gingival health remains sparse.

Hence, the hypothesis of this study posited that probiotic-related Zeger therapy would enhance microbiological and clinical outcomes in the treatment of gingival health. The objective of this investigation was to assess the microbiological and clinical effects of probiotic-related Zeger therapy in improving gingival health among individuals with gingivitis, employing a randomized controlled trial design.

Materials and methods

Ethics statement

The clinical trial was approved by the Medical Ethics Committee of West China Hospital of Stomatology, Sichuan University, batch No. WCHSIRB-D-2021-428. Before patient registration, the prospective clinical trial was registered in www.clinicaltrials.gov public repository in China under the registration number ChiCTR2200060555 on 04/06/2022. It was designed as a prospective, randomized, blank-controlled, single-blind clinical trial with volunteers recruited at the West China campus of Sichuan University. All the participants signed written informed consent before enrollment.

Materials preparation

The product used, Koumiss (Lot No.DJ1525240150, provided by Su Niteyouqi Baigali Creamery, Xilin Gol League, Inner Mongolia Autonomous Region, China) is a commercially available probiotic beverage made from fermented fresh mare's milk, rich in probiotics and a variety of bioactive components. The ingredients are 100% sour mare's milk. And it needs to be stored at 0–5℃ to ensure the activity, which is closely related to the effect it produces.

Sample size calculation

The lack of data assessing microbiologic outcomes in similar previous studies prevented accurate estimates of changes in microbiologic effects, so clinical outcome variables were used as proxies. Sample size was calculated for the secondaryoutcome variable changing in gingival index (GI) score, based on Vivekananda et al. (2010) [21]. Setting 1-β=90% and α=0.05, the formula $n=2*[(Z\alpha+Z\beta)\sigma/\delta]^2$ was used to calculate, with Zα=1.96 and Zβ=1.28. Regarding a standard deviation (σ) of 0.26 and a mean difference (δ) between the experimental and control group of 0.39, it turned out that 10 patients were needed in each group. Furthermore, 2 more people in each group wererequired for the experiment considering a 20% sample withdrawal rate. Therefore, 24 volunteers wererecruited for the experiment in total.

Inclusion criteria and exclusion criteria

Volunteers were selected based on the following inclusion criteria: [1] 18–35 years old [2], no radiographic signs of alveolar bone loss associated with periodontitis at baseline [3], gingival index (GI)>0 at baseline [4], in good general health, and [5] more than 20 natural teeth (excluding third molars).

Exclusion criteria are as follows: [1] severe systemic and hematological diseases [2], pregnancy or breast-feeding [3], physical or mental disorders that may affect oral health behavior [4], history of dairy allergy [5], use of non-steroidal or steroidal anti-inflammatory drugs, analgesics or antibiotics in the 4 weeks prior to the study [6], ongoing orthodontic treatment (including wearing orthodontics) and other unfinished dental treatment [7] acute oral lesions.

Experimental design

7 days before the beginning of the experiment, all subjects were enrolled for a unified dental examination according to the inclusion and exclusion criteria. Those who met the criteria were given a thorough upper gingival scaling by a professional dentist to remove plaque, stains and calculus.

Day 0 recalled the subjects, rechecked the inclusion and exclusion criteria, such as taking orthopantomogram for all first to exclude patients with invasive periodontitis. And baseline measurements were taken and clinical samples were collected for those who met the criteria. Baseline measurements included gingival index (GI), plaque index (PI) and gingival bleeding index (GBI) of 16,12,24,44,32 and 36 index teeth, along with probing depth (PD) of the whole mouth. GI was measured according to the method proposed by Loe and Silness, PI was referred to modified Silness-Loe, GBI was based on the method proposed by Ainamo and Bay, and PD was defined as the distance from the gingival margin to the bottom of the gingival furrow [22]. Before GI, GBI and PD while after PI were measured, all posterior teeth supragingival plaque were collected and stored at -80℃ for subsequent high-throughput sequencing analysis. All the above measurements were carried out by three professional dentists as examiners using instruments of uniform specifications. Kappa consistency test was performed for the examiners in advance (Kappa=0.65), and the subsequent examiners were consistent.

The subjects were randomly divided into experimental group and control group according to the random number table, with 12 subjects in each group. The experimental and control groups were denoted as E1 and C1 at baseline, and E2 and C2 at final visit, respectively. The examiners did not know the grouping of subjects, and did not participate in index recording and koumiss distribution.

Day 1-Day 28, a 2500mL package of pasteurized sealed koumiss was opened every day, divided into 12 150mL food-grade measuring cups with LIDS, and distributed to subjects in the experimental group at a uniform time and place, and they were instructed to drink koumiss as slowly as possible on the spot. Due to the unique taste of koumiss, the control group at the same time took blank treatment. During the intervention period, subjects were asked not to perform any oral procedures, maintain their oral hygiene habits, and refrain from taking antibiotics or additional probiotics.

Each weekend, the experimental group will conduct additional questionnaire survey on the adverse reactions of drinking koumiss.

Day 29 All subjects were recalled for clinical examination consistent with baseline examination and supragingival plaque collection. The flow chart was shown in Fig. 1.

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Sample collection

Sterile cotton swabs were used to wipe the buccal, lingual and occludal surfaces of the upper and lower mandibular posterior teeth on both sides until all the supringival plaque observed by naked eyes were collected[8]. The
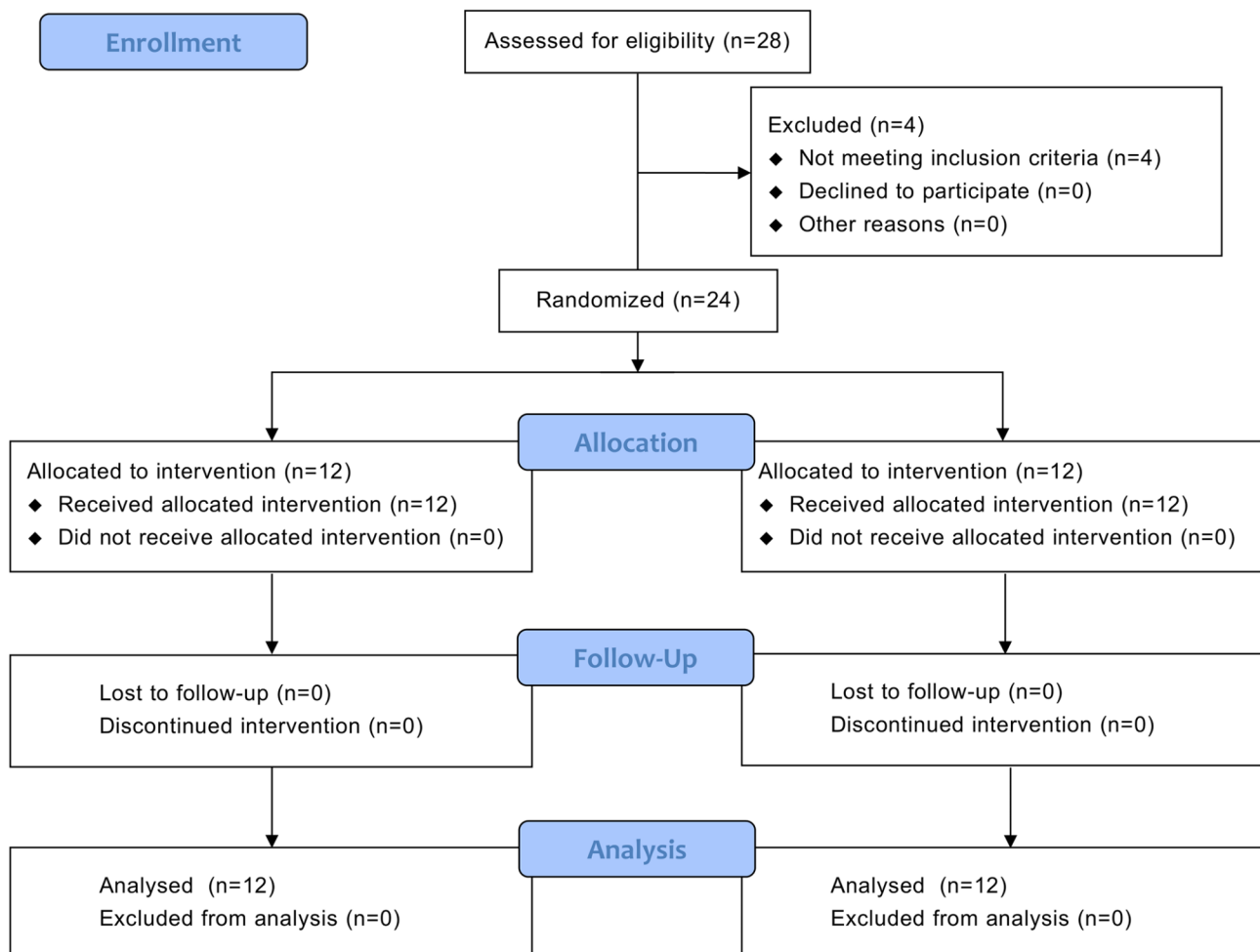
Fig. 1 Experimental design

cotton swabs were cut off and stored in 1.5 ml centrifuge tube with 0.5mlPBS buffer, respectively, and stored in the laboratory at -80℃ refrigerator within 2 h.

Genomic DNA extraction

The sample was thawed from −80℃ in advance, the cotton swab tip was removed, centrifuged at 15000r/min at 4℃ for 5 min, and the supernatant was discarded. DNA was extracted from both cotton swab and precipitate by CTAB method. Total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions, and stored at -20 ℃ prior to further analysis. The quantity and quality of extracted DNAs were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively [23].

Amplification of 16 S rRNA gene and sequencing

Using standard primers ACTCCTACGGGAGGCAG CA/ GGACTACHVGGGTWTCTAAT, hypervariable regions (V3-V4) of 16 S rRNA gene were amplified for each sample. In short, the sample was uniformly diluted to 20ng/ul. Amplification system (25μL) includes 5× Reaction buffer 5μL, 5×GC buffer 5μL, dNTP (2.5 mm) 2μL, Forwardprimer (10 μm) 1μL, Reverseprimer (10 μm) 1μL, DNA Template 2μL, ddH2O 8.75μL and Q5 DNA Polymerase 0.25μL. Amplification parameters were set as initial denaturation 98℃ 2 min, denaturation 98℃ 15s, rolling at 55℃ 30s, Extension 72℃ 30s, Final Extension 72℃ 5 min, 10 ℃ while held 25 to 30 cycles. The construction of the high-throughput sequencing library and sequencing tasks based on Illumina NovaseqPE250 platform were completed by Paiseno Biotechnology Co., LTD. (Shanghai, China).

Bioinformatic analysis of sequencing data

The expanded Human Oral Microbiome Database (eHOMD) was used for bioinformatic analysis. Initial screening was performed on the original off-board data of high-throughput sequencing based on sequence quality while retest the problem samples. Perform sequence denoising or OTU clustering according to QIIME2 DADA2 analysis process. According to the distribution of ASV/OTU in different samples, evaluate the α diversity level of each sample. At the ASV/OTU level, the distance matrix of each sample was calculated, and the differences in β diversity and significance among different groups were measured. At the level of taxonomic composition, we further measure the diversity of species abundance composition among different groups and try to find marker species.

Statistical analysis of clinical indicators

Data composed of oral clinical indicators, including baseline data and final data, were tested for normality and homogeneity of variance. Independent sample T test was used to statistically analyze the baseline level difference between the experimental group and the control group

Table 1 Baseline characteristics (mean ± SD)

	E1	C1	*P*-value[a]
Male/Female	6/6	4/8	0.680
Age(years)	20.67 ± 0.78	20.67 ± 0.78	0.737
GI(score)	0.74 ± 0.24	0.74 ± 0.29	0.615
PI(score)	0.70 ± 0.41	0.74 ± 0.31	0.463
GBI(%)	24.31 ± 9.54	18.40 ± 12.25	0.128
PD(mm)	2.26 ± 0.47	2.27 + 0.40	0.601
Inter-group difference analysis	-	-	0.904

[a]:Gender differences were based on Fisher's exact test, and other indicators were based on independent sample T test. Inter-group difference analysis of microbiological compositions was under high-throughput sequencing, based on the Weighted_Unifrac distance algorithm and the Adonis test. Set α = 0.05

and the level difference between the experimental group and the control group. Paired sample T test was used to statistically analyze the level difference of the experimental group and the control group before and after the trial, respectively. Software SPSS 25.0 was used.

Collection, sequencing and statistics of the koumiss sample

Koumiss samples were collected from the unsealed sour mare's milk within 28 days of intervention. An additional 5 ml of sour mare's milk was collected into a sterile centrifugal tube every day and stored at 4℃. After the intervention was completed, they were fully mixed, and 10 ml was extracted from them, which were sent together with the supringival plaque samples for nucleic acid extraction and 16 S rRNA high-throughput sequencing. The specific steps were the same as above. And the dominant species were counted at phylum and genus levels.

Result

Baseline characteristics

Twenty-four eligible subjects (10 males and 14 females, aged 19 to 22 years) were enrolled from 01/09/2023 to 30/09/2023. On Day 0 (baseline), gender, age distribution, oral clinical parameters and microbial parameters were similar between the two groups (Table 1), all without significant difference ($P > 0.05$). None of the subjects were lost to follow-up.

Result of 16 S high-throughput sequencing

Koumiss sample

There were 92,466 sequences obtained by analyzing the sample of koumiss. The DADA2 method was used for analysis, and 56,880 sequences were obtained through sequence quality control, denoising, linking and chimera removal. The length of sequence distribution ranged from 404 bp to 432 bp, with an average of 427 bp. The ASV value was 409 in total. One kingdom, 7 phyla, 16 classes, 29 orders, 44 families, 64 genus and 58 species were identified from the sample of koumiss. Abundant probiotics beneficial to gingival health were found in koumiss. *Lactobacillus* accounted for the highest proportion of microorganisms in the koumiss sample, reaching 76.67%. Previous double-blind randomized placebo-controlled pilot studies have shown that oral administration of *Lactobacillus reuteri*-containing lozenges is clinically and microbiologically effective in the treatment of chronic periodontal diseases, including gingivitis [24, 25]. The protective effects of Lactobacillus against gingivitis are mainly related to direct inhibition of pathogens, competitive inhibition of pathogen attachment, antibacterial biofilm, and direct or indirect attenuation of immune inflammatory responses [26]. The second genus is *Roseomonas*, with 9.59%, and studies have shown that

it has a therapeutic effect in regulating the skin microbiota and exhibits a possible anti-inflammatory effect [27, 28]. This property may be helpful in the treatment of gingivitis, but there are no studies on the oral microbiota and further research is needed. In addition, there are many other probiotics that may be beneficial for gingival health. For example, *Lactococcus*, which accounts for 2.12%, has shown in previous studies that its whey bioconversion products can reduce inflammation-related cytokines, increase antioxidant stress markers [29]. It can also produce nisin, which prevents oral biofilm formation and disrupts pre-formed 24- and 48-hour biofilms, reducing the level of pathogens in biofilms and promoting plaque diversity levels closer to "health" level [30]. Bifidobacterium, which accounts for 0.09%, has similar effects to *Lactobacillus*, an auxiliary effect on the treatment of gingivitis, significantly reducing the levels of pathogens and pro-inflammatory cytokines [31, 32].

Supragingival plaque samples

Sequence processing analysis A total of 48 samples were analyzed, resulting in 4,345,611 sequences, with an average of 90,534 sequences per sample. The DADA2 method was used for analysis, and 2,491,993 sequences were obtained after sequence quality control, denoising, linking and chimera removal, with an average of 51,917 sequences per sample. The length of sequence distribution ranged from 48 bp to 437 bp, with an average of 423 bp. A total of 7522 ASV values were calculated, and the average ASV value of each sample was 157.

Diversity analysis Previous studies have shown that patients with periodontal diseases (including gingivitis) have a higher diversity and biomass of microbial communities, along with an increased proportion of Gram-negative and anaerobic bacteria in the gingival microbial population [34–37]. In our findings, by α-diversity analysis (Fig. 2A) and β-diversity analysis (Fig. 2B), we obtained the results that after the intervention, the diversity of the microbial community in the experimental group increased with statistically significant differences, and there was no significant change in the control group. This is contrary to the previous research findings, but the possible reason is analyzed as follows. After the intervention, compared with the control group, the Gram-positive bacteria in the experimental group increased by relatively +749.44% ($p=0.038$), and the Gram-negative bacteria decreased by relatively −883.88% ($p=0.009$). And after the intervention, compared with the control group, the aerobic and facultative anaerobic bacteria in the experimental group increased by relatively +164.40% ($p=0.356$), and the obli-
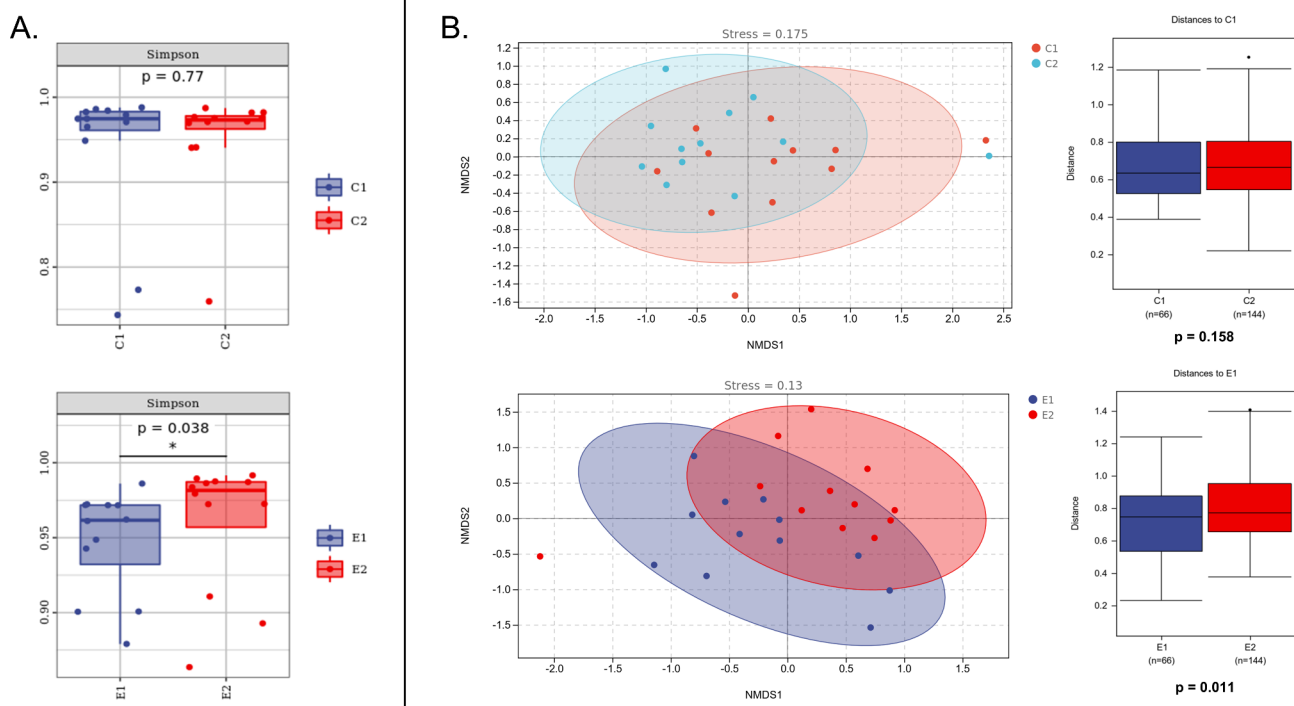

Fig. 2 Diversity analysis. **A**. α-diversity index: Chao1 and Observed Species index are selected to represent richness, Shannon and Simpson index to represent diversity, and Faith's PD index to represent evolution-based diversity. Pielou's Evenness index was used to characterize evenness, and Good's Coverage index was used to characterize coverage. **B**. Non-metric multidimensional scaling (NMDS) analysis(left), group difference analysis(right): the distance algorithm chosen was weighted_unifrac
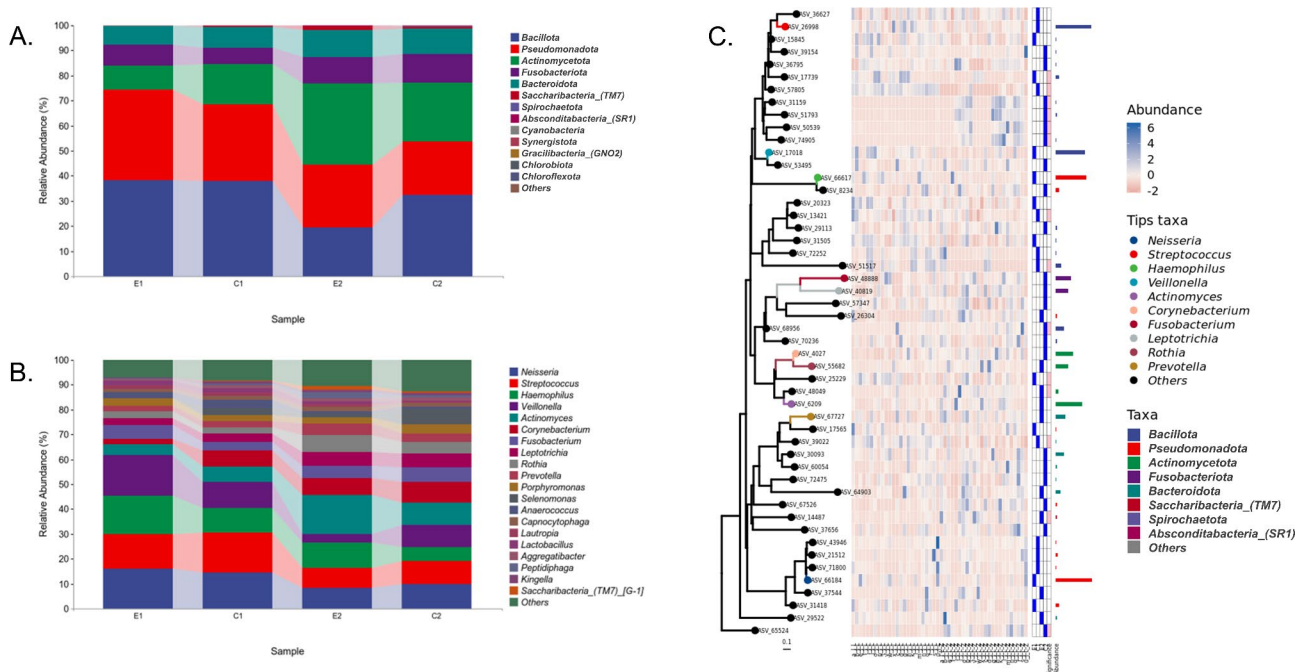
Fig. 3 Bacterial community analysis. **A**. Relative abundance structure (phylum, top 20). **B**. Relative abundance structure (genus, top 20). **C**. Phylogenetic tree plot (top 20)
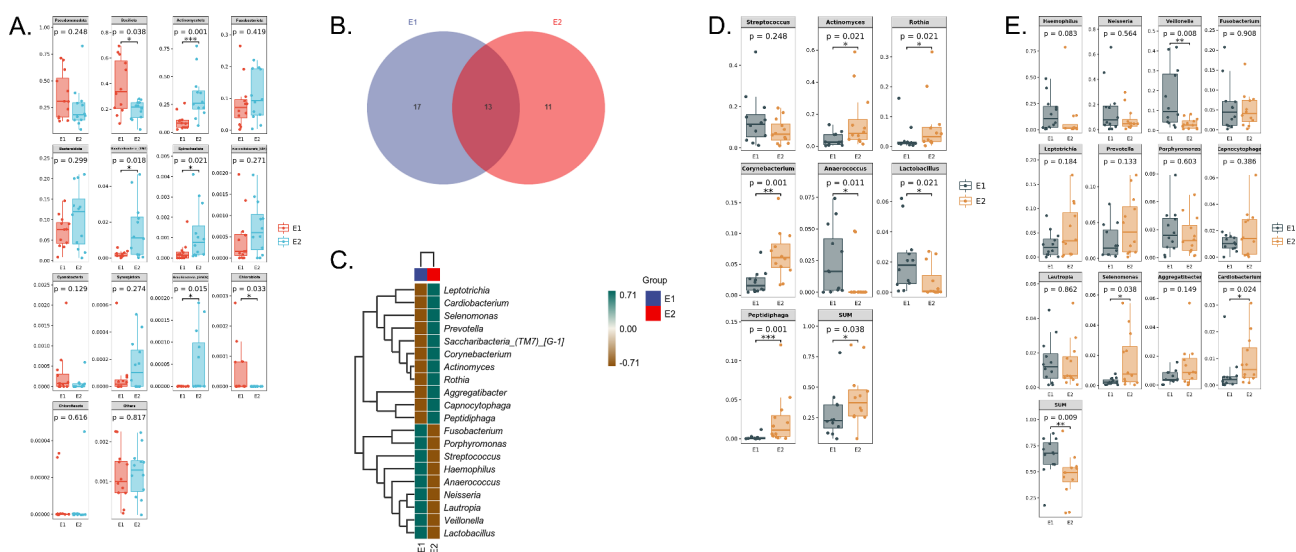

Fig. 4 Species difference analysis in the experimental group before and after intervention. **A**. Difference test boxplot (phylum, top 20). **B**. Venn diagram (the frequency of occurrence = 1). **C**. Heat map (genus, top 20). **D**. Difference test boxplot of Gram-positive bacteria (genus, top 20). **E**. Difference test boxplot of Gram-negative bacteria (genus, top 20)

gate anaerobic bacteria decreased by relatively −292.73% ($p = 0.106$). These indicates that in this study, the increased diversity of the experimental group was mainly derived from Gram-positive bacteria, aerobic and facultative anaerobic bacteria, while the proportion of Gram-negative bacteria and anaerobic bacteria decreased. These are healthy changes for gingival microecological stability, consistent with previous studies [34–37].

Bacterial community analysis Figure 3(A, B) showed the dominant bacteria (top 20) of supragingival plaque before and after intervention at the phylum and genus level by using the relative abundance structure. At the phylum level, the top 5 microorganisms with the highest abundance ratio are *Bacillota, Pseudomonadota, Actinomycetota, Fusobacteriota,* and *Bacteroidota*. Regarding *Bacillota*, the previous literature is controversial. S. Huang at al. and A. L. Griffen at al. observed a higher proportion

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of Firmicutes around the gingival tissue in healthy individuals [36, 38], and L. Abusleme at al. observed *Bacillota* in patients with periodontal diseases (including gingivitis) was significantly increased [34]. In this experiment, we measured a relative decrease (-252.45%) of *Bacillota* in the experimental group ($p<0.05$) compared to the control group. On the basis of previous studies about the difference between gingival microbes in healthy people and patients with gingivitis or periodontitis, we found that after the intervention, the experimental group had a relative decrease in gingival disease-related bacteria *Fusobacteriota* (-64.45%, $p>0.05$), and a relative increase in gingival health-related bacteria *Pseudomonadota* (+2.85%, $p>0.05$) and *Actinomycetota* (+406.14%, $p<0.05$). In addition, in the experimental group, some gingival disease-related microorganisms *Bacteroidota*, *Spirochaetota*, *Synergistota*, and *Chloroflexota* increased after the intervention compared with the control group [34–36]. But considering that the increase of these microorganisms is not large, and they are not absolute pathogenic bacteria in the progression of gingival disease, it may be normal fluctuations of oral flora changes.

At the genus level, the top 10 microorganisms with the highest abundance ratio were *Neisseria*, *Streptococcus*, *Haemophilus*, *Veillonella*, *Actinomyces*, *Corynebacterium*, *Fusobacterium*, *Leptotrichia*, *Rothia*, *Prevotella*. Observed a higher amount in healthy people than in patients with gingival disease, the bacteria relatively increased in the experimental group are Rothia (+49.95%, $p<0.05$), Corynebacterium (+687.71%, $p<0.01$) and Actinomyces (+565.17%, $p<0.05$) [35]. These changes suggest that Zeger therapy can promote the development of gingival flora closer to a healthy environment. Associated with gingival disease, the bacteria relatively decreased in the experimental group are *Porphyromonas* (-149.26%, $p>0.05$), *Fusobacterium* (-116.45%, $p>0.05$) and *Veillonella* (-390.04%, $p<0.05$). Among them, *Porphyromonas* and *Fusobacterium* are considered as the main pathogenic bacteria of periodontal diseases (including gingivitis) [35]. Although their statistical significances are not obvious, their obvious clinical significances call for a larger sample size and further study. In addition, it is interesting to note that although outside the top10, *Saccharibacteria (TM7)* was significantly relatively higher (+442.18%) in the experimental group ($p<0.05$) compared with the control group. Previously, its role in oral health and disease was thought to be unknown [39], and it has tended to be considered as a potential pathogen of periodontal diseases. But a recent study has shown that, contrary to the suspected pathogenic role, *TM7* protects the host from bacterial-induced inflammatory damage [40]. Therefore, the changes in gingival microbiome caused by Zeger therapy are beneficial for gingival health.

Figure 3C used a phylogenetic tree plot to show the position of each ASV/OTU in the evolutionary tree and the evolutionary distance between each other, and reflected their composition, abundance, taxonomy and other information through heat maps and bar charts.

Bacterial difference analysis in the experimental group before and after intervention Based on the above analysis, it was concluded that the experimental group had significant changes in the supragingival plaque habitat before and after intervention, while the control group did not. Therefore, we further conducted species difference analysis on the experimental group and tried to find marker species.

Statistical differences in relative species composition at phylum(A) and genus (D, E) levels were tested before and after treatment in the experimental group using the difference test boxplot in Fig. 4.

Figure 4B shows that E1 and E2 have 13 common ASV/OTU by using Venn diagram, E1 has 17 exclusive species, and E2 has 11 accordingly.

In order to further compare the differences in species composition between the experimental groups before and after intervention, and to display the species abundance distribution trend of each sample, the heat map was used for species composition analysis in Fig. 4C. Heat maps are made using abundance data from the top 20 genera with average abundance. It was found that the abundance of *Leptotrichia*, *Cardiobacterium*, *Selenomonas*, *Prevotella*, *Saccharibacteria_(TM7)_[G-1]*, *Corynebacterium*, *Actinomyces*, *Rothia*, *Aggregatibacter*, *Capnocytophaga*, *Peptidiphaga* and other genera increased after intervention, while *Fusobacterium*, *Porphyromonas*, *Streptococcus*, *Haemophilus*, *Anaerococcus*, *Neisseria*, *Lautropia*, *Veillonella*, *Lactobacillus* and other genera decreased after intervention.

Results of clinical indicators

δE and δC are based on the paired sample T test, except that the changes in PD in the control group before and after intervention were clinically significant (95% CI, -0.63 to -0.11; $p<0.01$), there were no significant differences in other indexes. Based on independent sample T test, δE-δC showed no significant difference in the four clinical indicators between the two groups. However, we found that GI, PI and GBI showed a trend of increasing after intervention in both groups, while PD showed a trend of decreasing (Table 2).

Discussion

Koumiss, renowned for its extensive historical usage, harbors a plethora of advantageous constituents including abundant polyunsaturated fatty acids, small-molecule amino acids, lactose, lactic acid, and a profusion of

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Table 2 Comparison of clinical indicators between final visit and baseline

	GI(score)	PI(score)	GBI(%)	PD(mm)
δE[b]				
Mean±SD	0.19±0.41	0.16±0.37	4.51±17.61	-0.16±0.45
95% CI	(-0.07,0.45)	(-0.08,0.39)	(-6.68,15.70)	(-0.45,0.13)
p-value	0.134	0.175	0.394	0.249
δC				
Mean±SD	0.06±0.42	0.07±0.34	5.90±20.38	-0.37±0.40
95% CI	(-0.21,0.32)	(-0.14,0.29)	(-7.05,18.85)	(-0.63,-0.11)
p-value	0.632	0.476	0.338	0.009*
δE-δC				
Mean±SD	0.13±0.17	0.08±0.15	1.39±7.78	0.21±0.18
95%CI	(-0.22,0.48)	(-0.22,0.39)	(-17.52,14.73)	(-0.16,0.57)
p-value	0.941	0.394	0.420	0.895

[b]:δE: E2-E1, δC: C2-C1, δE-δC: (E2-E1)-(C2-C1)

probiotics. These beneficial components engage in interactions with the oral microenvironment, thereby inducing alterations in the composition of the oral microbiota. Such modifications hold promise for potentially enhancing the oral health of patients with gingivitis. These interactions manifest through diverse mechanisms, with some mediated by the molecular constituents of koumiss, others involving molecules possessing antibacterial properties, and yet others serving as substrates for the metabolism of beneficial microorganisms. Moreover, the probiotics present in koumiss may facilitate certain functions, thereby modulating the microenvironment of the oral microbiota. Recent research has revealed that *TM7*, utilizing the arginine deiminase system (ADS), metabolizes arginine to produce adenosine triphosphate (ATP), thereby conferring protection against acid stress to both *TM7* and its host bacteria. Acid stress arises commonly in the human oral cavity due to bacterial carbohydrate metabolism. Within the oral cavity, *TM7* engages in a symbiotic association with *Actinomyces*, wherein *Actinomyces* deficient in ADS may benefit from enhanced survival conditions through symbiotic interaction with *TM7* [41]. Notably, koumiss is known to be abundant in small-molecule amino acids, and our investigations have demonstrated a significant elevation in the abundance of both *TM7* and *Actinomyces* following koumiss consumption. Consequently, *TM7* utilizes small-molecule arginine present in koumiss to foster a symbiotic relationship with *Actinomyces*, thus ameliorating the oral gingival microbial milieu and reinstating microbial homeostasis. This intriguing phenomenon warrants further investigation to validate its potential implications.

In koumiss, probiotics have the capacity to effectively colonize and proliferate within the bacterial milieu, leading to the depletion of nutrients crucial for the growth of pathogenic bacteria. Moreover, these probiotics produce antimicrobial substances, such as acetic acid and lactic acid, which exhibit inhibitory effects specifically against Gram-negative bacteria. On the host front, probiotics in koumiss can modulate host responses by down-regulating pathways associated with tissue damage, such as reversing epithelial damage induced by inflammation through the up-regulation of structural proteins. Additionally, they may up-regulate pathways that suppress pathogen growth or virulence [16]. For instance, koumiss has been shown to stimulate the secretion of the anti-inflammatory cytokine IL-10, thereby exerting anti-inflammatory and immunomodulatory effects [6, 7]. Furthermore, *Lactobacillus helveticus NS8*, a constituent of koumiss, significantly inhibits the activation of NF-κB, upregulates IL-10, downregulates IL-17-producing T cells, and promotes the proliferation of beneficial commensal microorganisms while inhibiting harmful microorganisms, thereby preserving microbial balance and health [42]. However, while these mechanisms have been extensively investigated in the gut, their specific effects within the oral cavity remain understudied. Therefore, further research is warranted to elucidate the intricate mechanisms underlying the therapeutic efficacy of koumiss for promoting gingival health.

High diversity in the oral microbiome is often indicative of a dysbiotic community. Interestingly, our findings reveal that while koumiss treatment has the potential to improve gingival health, it also increases oral microbiome diversity. According to Abusleme et al.'s study on the gingival microbiome in healthy individuals, gingivitis patients, and periodontitis patients, oral microbial diversity is higher in gingivitis patients compared to healthy individuals, but lower in periodontitis patients than in gingivitis patients [35]. This suggests that the diversity of the oral microbiome does not strictly correlate with the progression and severity of periodontal disease. This raises the question: although increased oral microbiome diversity can reflect ecological changes and is often viewed as an indicator of dysbiosis, could it be that, in certain cases (such as gingivitis patients), an appropriate range of increase is not necessarily detrimental and may prevent the progression of periodontal disease? This does not contradict the idea that increased diversity in the oral microbiome of healthy individuals indicates dysbiosis; it merely represents a different context. For healthy individuals, an increase in bacterial abundance is unfavorable. However, for gingivitis patients with pre-existing microbial imbalance, a moderate elevation in bacterial abundance may potentially prevent the progression to periodontitis. Our results indicate that most of the microbes contributing to the increased diversity are beneficial oral bacteria. Given that the gingival microbiome of periodontitis patients is widely dominated by pathogenic bacteria, the heightened diversity resulting

from koumiss consumption may mitigate the predominance of these harmful bacterial species.

Periodontal diseases, including gingivitis, arise from an imbalance in the commensal oral microbiota, triggering the host's immune response and resulting in inflammation and tissue damage [13, 33]. The diverse microenvironments within the oral cavity harbor unique microbial communities governed by intricate signaling pathways, as well as host and environmental factors [15]. Considering the ability of Zeger therapy to modulate gingival microbial diversity, it is conceivable that it could also confer benefits in addressing other oral microbial imbalance-related conditions, such as caries, halitosis, and oral mucosal diseases. However, this study is constrained by the limited sample size, which may compromise the certainty of our findings. Furthermore, no significant differences were observed in our clinical parameters before and after the intervention in either the experimental or control groups, except for periodontal probing depth (PD) in the control group ($p < 0.01$). This discrepancy may be attributed to the mild gingival inflammation exhibited by our target population of gingivitis patients, which may not manifest significant changes in clinical parameters over the short-term duration of 1–28 days. Although we utilized 16 S high-throughput sequencing of the microbiota to enhance sensitivity and accuracy, enabling the detection of rapid changes in oral microbiota associated with gingival health, further investigation is warranted to elucidate the long-term effects of koumiss consumption on gingival health over an extended period, such as several years.

Conclusion

Zeger therapy increasing microbial diversity, mainly attributable to increasing proportion of Gram-positive bacteria and decreasing of Gram-negative bacteria, thought to be beneficial for gingival health while improved no significant change in clinical parameters. It also promotes gingival health-related microbes increasing (*Rothia, Corynebacterium, Actinomyces, Saccharibacteria_TM7*, etc.) and gingival disease-related microbes decreasing (*Porphyromonas, Fusobacterium, Veillonella,* etc.).

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Author contributions
Conceptualization, methodology, project administration, investigation, validation, formal analysis, data curation, resources and writing—original draft preparation, XC and YZ; investigation, validation, resources and visualization, KX and ML; supervision, funding acquisition, project administration, writing—review and editing, WY; All authors have read and agreed to the published version of the manuscript.

Data availability
The datasets generated and/or analysed during the current study are available in the BioProject database (ID: PRJNA1145633), http://www.ncbi.nlm.nih.gov/bioproject/1145633.

Declarations

Ethics approval and consent to participate
Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the volunteers to publish this paper. The clinical trial was approved by the Medical Ethics Committee of West China Hospital of Stomatology, Sichuan University, batch No. WCHSIRB-D-2021-428. Before patient registration began, the prospective clinical trial was registered in www.clinicaltrials.gov public repository in China under the registration number ChiCTR2200060555 on 04/06/2022.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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