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Optimal storage time and temperature of human oral samples to minimize microbiome changes: A scoping review

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ABSTRACT

Improper storage times and temperatures negatively impact the quality of biospecimens with oral microbiomes. This study aimed to determine the optimal storage time and temperature for maintaining the integrity of human dental plaque and saliva samples' microbial profiles. A comprehensive search yielded 5433 studies, with 12 meeting inclusion criteria. The number of studies on the storage time and temperature for plaque or saliva samples was extremely limited, with large variability in study designs and analytical tools. The best approach for dental plaque and saliva samples was to immediately freeze fresh specimens at − 80 °C or lower until DNA extraction, with a recommended storage time not exceeding 1–2 years, regardless of temperature. Checkerboard DNA-DNA hybridization-based studies suggested dental plaque storage at − 20 °C for 6 months, but a shorter duration was advised. Based on 16 S rRNA gene sequencing studies, dental plaque samples could be stored at − 80 °C for 6 months in 75 % ethanol or Bead Solution. Dental plaque and saliva samples could be stored at room temperature for 1–2 weeks without significant microbiome changes if stored in appropriate media. Further well-designed randomized controlled studies with longer-storage duration are necessary to establish more definitive guidelines.

1. Introduction

The main habitats of microbial communities present in the oral cavity are dental plaque and saliva [1]. In healthy oral cavities, these stable and symbiotic microbial communities coexist and maintain homeostasis. However, an increase in oral pathogenic bacteria can cause oral infectious diseases, including dental caries and periodontal diseases, leading to a homeostatic imbalance [2–4]. Additionally, oral microorganisms including periodontal pathogenic bacteria exhibit close relationships with various systemic diseases, such as diabetes [5], atherosclerosis [6], rheumatoid arthritis [7], and non-alcoholic fatty liver disease [8] and with systemic inflammatory processes [9,10].

When studying the role and association of oral microbial communities with various human diseases, storage of oral samples is usually inevitable because immediate analysis directly after sample collection is not always possible. Such scenarios occur in many microbiome studies, including studies with large sample sizes, long-term longitudinal studies, studies on large populations or very rare diseases, and studies that procure samples from human biobanks [11]. Samples derived from the oral cavity, including plaque and saliva, are collected and stored in biobanks for varying durations, and those can be given to researchers for future studies upon request [12].

If samples of oral microbiomes are stored at suboptimal temperatures or for too long prior to DNA extraction, they become degraded by microorganisms, enzymes, and oxidative processes, resulting in poor-quality samples and subsequent confounding factors and experimental bias [13,14]. Roesch et al. [15] demonstrated the importance of storage time and temperature by comparing the bacterial diversity between fecal samples that were immediately frozen after collection and others that were kept at room temperature (RT) for a period of time. Katsoulis et al. [14] reported that the storage of plaque samples at 4 °C for up to 6 weeks prior to analysis using the checkerboard DNA–DNA hybridization

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technique did not adversely affect microbial DNA stability. It was based on the fact that there was no significant difference in total bacterial DNA, absolute DNA counts and relative amounts of target microbiomes between the storage group for 6 week at 4 °C and the non-storage group. However, they also reported the loss of bacterial DNA occurred when stored for more than 6 months even at − 20 °C, because there was significant difference in the storage group for more than 6 months compared to the non-storage group regarding these evaluation variables. On the other hand, do Nascimento et al. [16] reported that plaque samples could be successfully stored for up to 6 months at − 20 °C or − 80 °C, based on the finding that the storage protocol showed no significant differences in total and individual microbial counts compared to the non-storage group. Although freezing can prevent nucleic acid degradation by nucleases or enzymes, freezing itself or repeated freeze-thaw cycles may cause DNA fragmentation, indirectly resulting in DNA damage and loss [16,17]. There appears to be no consensus regarding the acceptable storage time and suitable storage temperature of samples containing oral microbiomes. Additionally, microbiological analysis methods have undergone many changes and rapid developments over the past few decades. For example, bacterial culture or checkerboard DNA–DNA hybridization was commonly used in the past. However, the development of next-generation sequencing has led to the advent of new technologies, enabling more accurate analysis of microbiomes with an incomparably broader spectrum than previous techniques. Therefore, there is a further need to review and establish appropriate storage temperatures and acceptable storage times according to the changing analytic methods.

Thus, this scoping review aims to assess the available literature regarding the optimal storage temperature and acceptable storage time necessary to minimize damage to and degradation of the oral microbiome in human dental plaque and saliva samples, thereby maximizing the quality and accuracy of the analysis results.

2. Materials and methods

2.1. Focused questions

For this scoping review, the following two questions were constructed according to the population, intervention, comparison, and outcomes [18] framework:

(i) What is the optimal storage temperature and the acceptable storage time that minimizes changes in oral microbial profiles in human dental plaque samples?

- Population: human dental plaque
- Intervention: storage duration, storage temperature, and storage conditions
- Comparison: immediately processed dental plaque samples (i.e., not stored) with dental plaque samples stored under various combinations of times and temperatures
- Outcomes: bacterial genomes, microbial DNA, 16 S RNA gene sequencing, microbiome composition

(i) What is the optimal storage temperature and the acceptable storage time that minimizes changes in oral microbial profiles in human saliva samples?

- Population: human saliva
- Intervention: storage duration, storage temperature, and storage conditions
- Comparison: immediately processed saliva samples (i.e., not stored) with saliva samples stored under various combinations of times and temperatures
- Outcomes: bacterial genomes, microbial DNA, microbial RNA, microbiome composition

2.2. Literature search strategy

The PubMed, Embase, and Cochrane Library databases were searched using different combinations of the following keywords: (i) “dental plaque,” “oral,” “dental,” “mouth” AND “biofilm,” “bacteria,” “microbial composition,” “bacterial DNA,” “microbial DNA,” “bacterial RNA,” “microbial RNA,” “microbial genome,” “microbiota,” “microbiome” AND “storage,” “preservation,” “temperature,” “storing period,” “storing duration,” “storing condition,” “storing time” and (ii) “saliva,” “mouth” AND “bacteria,” “microbial composition,” “bacterial DNA,” “microbial DNA,” “bacterial RNA,” “microbial RNA,” “microbial genome,” “microbiota,” “microbiome” AND “storing time,” “storing condition,” “storing duration,” “storing period,” “temperature,” “preservation,” and “storage condition.” Table 1 shows the search strategy with queries for human dental plaque-related and human saliva-related studies. The year of publication was not restricted, and only studies published in English were included. The database searches were completed on October 4, 2022. The titles and abstracts of the resultant studies were independently screened by two authors (HJK and SYK). Then, full-text versions of potentially relevant studies were thoroughly reviewed, and their reference lists were hand-searched. Any

Table 1
Searching strategy with queries for the human dental plaque- and saliva-related studies.

Search number	Query for dental plaque-related study	Query for saliva-related study
1	dental plaque[Title/Abstract]	saliva* [Title/Abstract]
2	dental plaque[MeSH Terms]	saliva[MeSH Terms]
3	oral[Title/Abstract]	mouth* [Title/Abstract]
4	dental[Title/Abstract]	mouth[MeSH Terms]
5	mouth[Title/Abstract]	#1 OR #2 OR #3 OR #4
6	mouth[MeSH Terms]	bacteria* [Title/Abstract]
7	#1 OR #2 OR #3 OR #4 OR #5 OR #6	microbial composition* [Title/Abstract]
8	biofilm* [Title/Abstract]	"DNA, Bacterial"[Mesh]
9	bacteria* [Title/Abstract]	microbial DNA[Title/Abstract]
10	microbial composition* [Title/Abstract]	"RNA, Bacterial"[Mesh]
11	"DNA, Bacterial"[Mesh]	microbial RNA[Title/Abstract]
12	microbial DNA[Title/Abstract]	"Genome, Microbial"[Mesh]
13	"RNA, Bacterial"[Mesh]	microbial genome* [Title/Abstract]
14	microbial RNA[Title/Abstract]	"Microbiota"[Mesh]
15	"Genome, Microbial"[Mesh]	Microbiota* [Title/Abstract]
16	microbial genome* [Title/Abstract]	Microbia* [Title/Abstract]
17	"Microbiota"[Mesh]	Microbiome* [Title/Abstract]
18	Microbiota* [Title/Abstract]	#6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17
19	Microbia* [Title/Abstract]	storing time* [Title/Abstract]
20	Microbiome* [Title/Abstract]	storing condition* [Title/Abstract]
21	#8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18 OR #19 OR #20	storing duration* [Title/Abstract]
22	#7 AND #21	storing period* [Title/Abstract]
23	storage* [Title/Abstract]	"Temperature"[Mesh]
24	preserv* [Title/Abstract]	temperature* [Title/Abstract]
25	temperature* [Title/Abstract]	preserv* [Title/Abstract]
26	"Temperature"[Mesh]	storage condition* [Title/Abstract]
27	storing period* [Title/Abstract]	#19 OR #20 OR #21 OR #22 OR #23 OR #24 OR #25 OR #26
28	storing duration* [Title/Abstract]	#5 AND #18 AND #27
29	storing condition* [Title/Abstract]	
30	storing time* [Title/Abstract]	
31	#23 OR #24 OR #25 OR #26 OR #27 OR #28 OR #29 OR #30	
32	#22 AND #31	

disagreements regarding study selection were resolved through discussion and consensus.

2.3. Eligibility criteria and selection

The eligibility criteria were as follows: (i) any published original articles, case reports, case series, or short communications, and (ii) studies published in English. Studies were excluded if they were (i) review articles or part of a book or book series and (ii) irrelevant or performed on nonhumans. Additionally, duplicate studies were removed, as well as those where the full-text version was unavailable.

2.4. Data extraction and synthesis

Data that were considered to influence the purpose or main findings of our scoping review and useful meta-information were extracted from all eligible studies and organized into tables. The main extracted domains were as follows: bibliography (study type, title and authors, publication year, name of journal/book, affiliation, and country of origin), demographic data (number of participants, age, gender distribution, and any restriction for some systemic conditions), sample-related data (sample type, instructions prior to sample collection, collection method, and transport media), storage-related data (study groups, storage time, storage temperature, and storage media), microbial analysis-related data (targeted microbial profiles, time of DNA/RNA extraction, DNA/RNA extraction method, and microbial analysis tools), outcome-related data (main findings regarding changes of microbial profile), and useful meta-information (oral hygiene and others).

2.5. Quality assessment

The quality of the included articles was assessed using the Timmer scale [19]. This 19-item checklist has proven to be a reliable and useful tool for systematic reviews of basic science articles, particularly in the context of clinical research [20]. Each item carries a potential score of up to 2 points: full compliance, 2; partial compliance, 1; and noncompliance, 0. Two of the 19 checklist items were automatically excluded because they were irrelevant to basic science (blinding of investigators and blinding of subjects). Additional points (maximum = 5) are allocated based on study design and randomization. Hence, the total possible score was $(17 \times 2) + 5 = 39$. The article quality scores were calculated by dividing attained scores by the maximum possible score and ranged between zero and one. The quality assessments were independently conducted by two authors (HJK and SYK). Any discrepancies were resolved through discussion and consensus.

3. Results

3.1. Study characteristics

Our search strategy identified a total of 5433 studies (plaque-related, 3982; saliva-related, 1451). Next, we excluded those studies irrelevant to the purpose of our study, duplicates across databases, studies not published in English, studies on nonhumans, review articles, studies that were part of a book or book series, and those where full-text versions were unavailable (plaque-related, 3974; saliva-related, 1445). Fourteen studies (plaque-related, 8; saliva-related, 6) remained after applying the eligibility criteria, of which two were both plaque- and saliva-related. Thus, 12 studies were finally included in our scoping review (Fig. 1).

The number of studies on the storage conditions for plaque or saliva samples to be used for human oral microbiome investigations was very limited compared with the number of studies on the storage of fecal samples for human gut microbiome investigations, which is a major area of interest in human microbiome studies [15,21–25] and showed large variability in study designs and analytical tools used to assess microbial profiles.

Table 2 presents the main characteristics of all 12 studies included in our scoping review, including the number and age of the participants, sample type, study groups, storage time, storage temperature, DNA extraction method, method of microbial analysis, and changes in microbial profiles. Supplementary Table 1 presents additional information, including the study type, country of origin, systemic and oral conditions, period of antibiotic restriction, instructions prior to collection, collection method, transport and storage media, targeted microbial profiles,

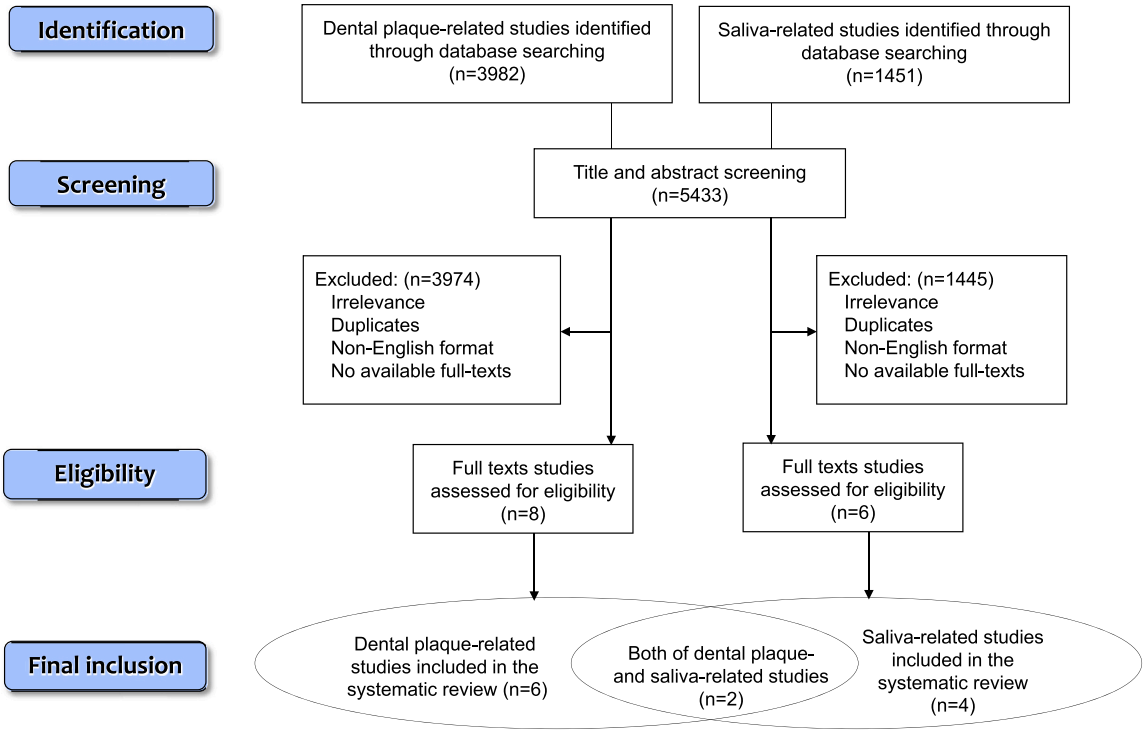

Fig. 1. Schematic diagram of retrieved and selected studies from the PubMed, Embase, and Cochrane Library databases for this scoping review.

Table 2
Study characteristics and main findings of the included studies in this scoping review.

Study	No. of partici-pants	Age	Sample type	Study groups	Storage time	Storage temperature	DNA extraction method	Method of microbial analysis	Outcomes (Change of microbial profile)
Zhou et al. (2019) [11]	20	> 18Y M:F (not specified)	Supragingival plaque (full mouth)	**Group 1**: Use of 75% ethanol as storage media and bead beating DNA Isolation kit **Group 2**: Use of 75% ethanol and chemical lysis QIAamp DNA Mini Kit **Group 3**: Use of Bead Solution as storage media and bead beating DNA Isolation kit **Group 4**: Use of Bead Solution and chemical lysis QIAamp DNA Mini Kit	• Day 0 • 1 M • 3 M • 6 M	-80 °C	• PowerSoil DNA Isolation Kit (bead beating) • QIAamp DNA Mini Kit (chemical lysis)	Amplification of 16 S rRNA gene V4 region using PCR, Sequencing on the Illumina MiSeq platform	The abundance, the shared and overall diversity of samples did not significantly vary over 6 months of storage at −80 °C.
Adler et al. (2018) [26]	8	5.9-6.3Y M:F= 3:1	Supragingival plaque	**Group 1**: Immediate storage at −20 °C **Group 2**: VMG II transport media at RT for 2 W and then at −20 °C **Group 3**: RNAprotect Bacteria at RT for 2 W and then at −20 °C	Less than 3 W	• −20 °C for no media group, • 2 W at RT and then at −20 °C for media using group	PowerBiofilmTM DNA Isolation Kit (MoBio)	Amplification of 16 S rRNA gene V3–V6 region using PCR, Pyrosequencing using 454 GS FLX Titanium chemistry	(1) The overall diversity of bacteria was similar regardless the storage condition. (2) The abundance of bacteria was influenced by storage environment. Group 2 (VMG II transport media at RT for 2 W and then at −20 °C) showed similar abundance compared with the immediately-frozen dental plaque samples (−20 °C), whereas Group 3 (RNAprotect Bacteria at RT for 2 W and then at −20 °C) showed significant difference.
do Nascimento et al. (2012) [33]	36	Mean 37Y M:F (not specified)	Subgingival plaque (1st molars)	**Group 1**: Processing after collection immediately **Group 2**: Processing after storage at –20 °C for 6 M **Group 3**: At –20 °C for 12 M **Group 4**: At –20 °C for for 24 M	• Day 0 • 6 M • 12 M • 24 M	-20 °C	Lysis buffer containg lysozyme, ethanol and etc.	Checkerboard DNA–DNA hybridization	(1) Storage time has an important impact on the results of hybridization signals. (2) Hybridization signal was lower in the 12 M- and 24 M-storage samples than in the immediately processed samples or 6 M-storage samples −20 °C.
Katsoulis et al. (2005) [28]	7	Not specifed	Subgingival plaque (5 sites showing PD ≥ 6 mm)	**Group 1**: Processing on the same day **Group 2**: Processing after 6 W-storage at 4 °C **Group 3**:	• Day 0 • 6 W • 6 M • 12 M	• 4 °C • −20 °C	TE buffer, lysozyme, sodium dodecyl sulfate (SDS), proteinase K and etc.	Checkerboard DNA–DNA hybridization	(1) Total bacterial DNA was identified less (2.4 times) in the 6 M- and 12 M-storage samples at −20 °C than in the immediately

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Table 2 (*continued*)

Study	No. of partici-pants	Age	Sample type	Study groups	Storage time	Storage temperature	DNA extraction method	Method of microbial analysis	Outcomes (Change of microbial profile)
				Processing after 6 M-storage at −20 °C **Group 4**: Processing after 12 M-storage at −20 °C					processed samples. (2) There was no differences in total DNA between immediately processed samples and 6 W-storage samples at 4 °C. 3) There was no differences in total DNA between the 6 M- and 12 M-storage samples at −20 °C.
Katsoulis et al. (2005) [14]	7	Not specifed	Subgingival plaque (5 sites showing PD ≥ 6 mm)	**Group 1**: Processing on the same day **Group 2**: Processing after 6 W-storage at 4 °C **Group 3**: Processing after 6 M-storage at −20 °C **Group 4**: Processing after 12 M-storage at −20 °C	• Day 0 • 6 W • 6 M • 12 M	• 4 °C • −20 °C	TE buffer, lysozyme, sodium dodecyl sulfate (SDS), proteinase K and etc.	Checkerboard DNA–DNA hybridization	(1) The proportional distributions and each pathogen remained constant in the immediately processed samples and 6 W-storage samples at 4 °C but not in the 6 M- and 12 M-storage samples at −20 °C. (2) All bacterial samples for DNA extraction should be processed following a standardized storage protocol (i.e. maximum 6 W-storage samples at 4 °C).
Wilson et al. (1984) [30]	10	12-35Y M:F (not specified)	Subgingival plaque	**Group 1**: Non-frozen immediate culture **Group 2**: Storage in liguid nitrogen for 30 min **Group 3**: Storage in liguid nitrogen for 8-10 W **Group 4**: Storage in liguid nitrogen for 1-2Y	• 30 min • 8-10 W • 1-2Y	• Not frozen (immediate culture) • Liquid Nitrogen	Not applicable	Culture	(1) Colony-forming units (CFUs) of bacteria recovered after the storage in liquid nitrogen were reasonably similar to those of a non-frozen sample. (2) Some sensitive organisms showed significantly different CFUs after recovering compared to those of a non-frozen sample.
Luo et al. (2016) [29]	10 (4 adults and 6 children)	19-29Y and 1-4Y M:F= 1:1	Plaque	**Group 1**: Use of LDTM as transport media and storage at −80 °C for 2 days and then at −20 °C until DNA extraction **Group 2**: Use of OMNIgene as transport media and storage at RT	• 2 Days • 5 Days • 7 Days	• LDTM: −80 °C for 2 days and then −20 °C until DNA extraction • OMNIgene: at RT	DNeasy blood and tissue kit with QIAcube	Amplification of 16 S rRNA gene V6 region using PCR, Sequencing on the Illumina sequencing platform	The recovered DNA amounts were similar between the plaque samples stored in OMNIgene at RT and samples stored in LDTM at −80 °C for 2 days and then at −20 °C.
			Stimulated saliva (Adults) & swab (children)	**Group 1**: Storage at RT **Group 2**: Storage at RT for 2 days and then at −20 °C **Group 3**: Storage	• 2 Days • 5 Days • 7 Days	• RT, • RT and then −20 °C • −20 °C • −80 °C and then −20 °C	DNeasy blood and tissue kit with QIAcube	Amplification of 16 S rRNA gene V6 region using PCR, Sequencing on the Illumina sequencing platform	(1) Bacterial diversity was not affected by storage temperature but affected by transport media. (2) The relative

(*continued on next page*)

Table 2 (*continued*)

Study	No. of partici-pants	Age	Sample type	Study groups	Storage time	Storage temperature	DNA extraction method	Method of microbial analysis	Outcomes (Change of microbial profile)
				at RT for 5 days and then at −20 °C **Group 4**: Storage at RT for 7 days and then at −20 °C **Group 5**: Storage at −20 °C **Group 6**: Storage at −80 °C for 2 days and then at −20 °C until DNA extraction					abundance in some phyla was reduced in saliva stored at RT for 5, 7 days and on dry ice compared with that stored at −20 °C. (3) Saliva stored in OMNIgene at RT, the community remained stable for at least 1 W.
do Nascimento et al. (2014) [16]	10	Mean 21.7Y M:F= 1:1	• Unstimulated saliva • Supragingival plaque(1st molars) added to the saliva	A total of 17 groups; **Group 1**: Immediately processed samples **Group 2-5**: Storage at RT, 4 °C, −20 °C, and −80 °C for 2 W **Group 6-9**: Storage at RT, 4 °C, −20 °C, and −80 °C for 4 W **Group 10-13**: Storage at RT, 4 °C, −20 °C, and −80 °C for 6 M **Group 14-17**: Storage at RT, 4 °C, −20 °C, and −80 °C for 12 M	• Day 0 • 2 W • 4 W • 6 M • 12 M	• RT • 4 °C • −20 °C • −80 °C	Not specifed	Checkerboard DNA–DNA hybridization	(1) Samples stored up to 6 M at cold temperatures (4 °C, −20 °C, and −80 °C) showed similar counts to those immediately processed. (2) 12 M-storage group showed a reduction in the total and individual microbial counts and microbial incidence irrespective of temperatures. (3) Samples should be processed immediately or within 6 M at cold temperatures.
Furuhashi et al. (2022) [31]	5	28-37Y M:F= 2:3	Unstimulated saliva	**Group 1** (control): immediate extraction of DNA **Group 2**: liquid nitrogen and then 2 W storage at −80 °C **Group 3**: 2 W storage at −80 °C **Group 4**: 2 W storage at −15 °C.	• Day 0 • 2 W	• Liquid nitrogen and then −80 °C • −80 °C • −15 °C	TE buffer, lysozyme, sodium dodecyl sulfate (SDS), proteinase K, and etc.	Amplification of 16 S rRNA gene V1-V2 region using PCR, Sequencing on the Illumina sequencing platform with MiSeq Reagent Kit v3	Storage temperatures (−80° C or −15° C) and flash-freezing using liquid nitrogen before storage did not affect the salivary microbial profiles.
Marotz et al. (2021) [32]	12	Not specifed	Unstimulated saliva	**Group 1**: Immediate storage of swab or aliquot at −80 °C **Group 2**: Immediate frozen of saliva aliquot **Group 3-5**: Saliva aliquot storage in 95% ethanol of 1:1, 2:1 or 4:1 ratio and left at RT for 1 W **Group 6**: Storage of swab in 95% ethanol and left at RT for 1 W	• Day 0 • 1 W	• RT • −80 °C	Qiagen PowerSoil MagAttract DNA kit	Amplification of 16 S rRNA gene V4 region using PCR, Sequencing on the Illumina sequencing platform with MiSeq Reagent Kit v2, qPCR	Microbial composition of saliva samples was well maintained over time and across temperatures when samples were stored in 95% ethanol.

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Table 2 (*continued*)

Study	No. of participants	Age	Sample type	Study groups	Storage time	Storage temperature	DNA extraction method	Method of microbial analysis	Outcomes (Change of microbial profile)
				Group 7: 95% ethanol eluent stored at RT for 1 W **Control**: Storage in 95% ethanol at −80 °C (gold standard)					
Vogtmann et al. (2019) [34]	103	≥ 18Y M:F (not specified)	Unstimulated saliva	**Group 1**: OMNIgene ORAL Collection Device and immediate storage at at −80 °C **Group 2**: Scope mouthwash and immediate storage at −80 °C **Group 3**: Scope mouthwash and storage at RT for 4 days and then at −80 °C	• Day 0 • 4 Days	• RT • −80 °C	MO-BIO PowerMag Soil DNA Isolation Kit	Amplification of 16 S rRNA gene V4 region using PCR, Sequencing on the Illumina HiSeq	(1) Scope mouthwash samples were stable at RT. (2) The relative abundance of the top 25 genera was generally similar between the samples frozen immediately at −80 °C and those left at RT for 4 days and stored at −80 °C. (3) There were significant differences for some taxa between
Karched et al. (2017) [27]	4	34-41Y M:F (not specified)	Stimulated saliva	A total of 3 groups according to the used samples **Group 1**: Whole saliva **Group 2**: Salivary pellet (after centrifugation) **Group 3**: Salivary supernatant (after centrifucation) For all groups, immediate analysis, 1 W, 2 M, and 6 M storage at −80 °C.	• Day 0 • 1 W • 2 M • 6 M	-80 °C	MasterPureTM DNA purification kit	UV spectrometry method using NanoDropTM 1000, DNA purity was assessed by A260/280 ratio, qPCR quantification of the 6 oral species,	(1) The quantities of most bacterial species remained stable over the 6 M. (2) DNA purified from saliva can be preserved at −80 °C without decrease in DNA concentration for at least 6 M.

Abbreviations: A260/280 nm, ratio of absorbance at 260 and 280 nm; CFU, colony-forming unit; LDTM, liquid dental transport medium; M, months; NA, not applicable; NS, not specified; PCR, polymerase chain reaction; PPD, periodontal pocket depth; qPCR, quantitative polymerase chain reaction; RT, room temperature; SDS, sodium dodecyl sulfate; TE, Tris-EDTA; W, weeks; Y, years

and measured outcomes. The number of participants across all 12 studies ranged from 4 to 103 (1–10, eight studies [14,16,26–31]; 11–20, two studies [11,32]; and >20, two studies [33,34]). Seven studies documented the systemic conditions of the participants [11,16,26,27,31,33,34]: the participants in five studies were systemically healthy [11,16,27,33,34], those in one study had minor gastrointestinal symptoms not requiring therapeutic intervention [31], and those in the remaining study suffered from diseases not directly related to oral conditions (eczema, ear infection, heart murmur, asthma, and bronchitis) [26]. Regarding the assessment of oral conditions, four studies reported the absence of active oral lesions in all participants [11,16,29,33], and there was one study on children where a minority of the participants had carious lesions [26]. In two plaque-related studies by the same authors, the study population comprised patients diagnosed with chronic periodontitis with at least five sites with a probing depth of 6 mm [14,28].

In plaque-related studies, subgingival plaque samples were collected in four studies [14,28,30,33] and supragingival plaque in three studies [11,16,26]. One study did not specify the sample collection site [29]. In saliva-related studies, unstimulated saliva samples were more frequently used [16,31,32,34] than stimulated saliva samples [27,29]. Dental plaque was collected using a sterile curette or scaler in five studies [14,16,28–30] and via swabbing in two studies [11,26]. In one study, paper points were used for collecting plaque [33], and a cytology brush was additionally used in another study [29]. Saliva samples were mostly collected via self-expectoration [16,27,31,32,34] and the remainder by swabbing [29,32], although one study used both methods concurrently [32]. The most frequently used transport media in plaque-related studies was Tris-EDTA (TE) buffer and NaOH solution [14,28,33], although one study used TE buffer only [11]. Other transport media included OMNIgene (DNA Genotek, Ontario, Canada), liquid dental transport medium (LDTM; Anaerobe Systems, CA, USA), VMG II made as suggestion by Moller et al. [35]), and RNAprotect Bacteria (Qiagen, Venlo, Netherlands) [26,29]. One study did not use any transport media for plaque samples and added them directly to the saliva

samples to increase the microbiome yield [16]. Transport media were not used in three saliva-related studies [27,31,32]. However, three studies used TE buffer and NaOH solution [16], OMNIgene or Scope mouthwash (Procter & Gamble, Ohio, USA) [34], and OMNIgene or LDTM [29], respectively, as transport media for saliva. Most plaque-related studies stored the samples in the transport media [14,26, 28–30,33]. One plaque-related study compared storage media, namely, 75% ethanol and PowerSoil Bead Solution [11]. In three saliva-related studies, the storage media were the same as the transport media [16, 29,34]. In contrast, two of the studies did not use any transport or storage medium [27,31], and in one study, the swab itself or the saliva eluent was stored in 95 % ethanol [32].

The storage time in the plaque-related studies varied from immediate processing without sample storage (Day 0), to 30 min, to 1–2 years, and most of the studies included groups of samples that were stored for 6 months [11,14,16,28,33] or 12 months [14,16,28,30,33]. The storage time in the saliva-related studies ranged from immediate processing without storage (Day 0) to 12 months, and most studies included short storage periods, such as 2, 4, 5, 7, and 14 days [29,31,32,34]. The most common storage temperatures were RT, 4 °C, − 20 °C, and − 80 °C, although the samples of one plaque-related study [30] and one saliva-related study [31] were stored in liquid nitrogen. Storage at − 20 °C was the most common in the plaque-related studies [14,16,26, 28,29,33], followed by storage at − 80 °C [16,29]. On the other hand, the most common storage temperature among the saliva-related studies was − 80 °C [16,27,29,31,32,34], followed by storage at − 20 °C [16, 29]. One study included a storage temperature of − 15 °C [31].

The results of the targeted microbial profiles, time of DNA extraction, DNA extraction method, and microbial analytical tools are also presented in Table 2. Except for one study that observed colony-forming units (CFUs) and microbial proportions [30], all others targeted microbial DNA, and there were no microbial RNA studies. Only one study extracted the microbial DNA prior to storage [27], and DNA extraction was performed after a predetermined storage time in all other studies [14,16,26,28–34]. The most frequently used technique for microbial analysis was polymerase chain reaction (PCR) amplification of the 16 S rRNA gene [11,26,29,31,32,34]. The V4 region was the most commonly amplified [11,32,34], and V1–V2 [31], V3–V6 [26], and V6 [29] amplification was performed in one study each. The checkerboard DNA–DNA hybridization technique was used in four studies [14,16,28, 33], of which two targeted the DNA of 40 microbial strains [14,28], one targeted the DNA of 13 strains [33], and the other targeted 38 microbial species and five *Candida* species [16]. In another study, quantitative PCR (qPCR) was performed for six oral microbial species, and DNA purity was evaluated by UV spectrometry (NanoDrop 1000, Thermo Fisher Scientific, MA, USA) [27], while the culture method was used in another study [30]. Among the six studies utilizing 16 S rRNA gene sequencing, five used an Illumina sequencing platform (Illumina, CA, USA) [11,29, 31,32,34], and the remaining study used 454 GS FLX Titanium pyrosequencing [26]. Various commercial kits were used for DNA extraction among the 11 studies targeting microbial DNA [26,27,29,32,34]. In some studies, DNA was manually extracted using TE buffer, lysozyme, ethanol, sodium acetate, etc. [14,28,31,33]. The DNA extraction method was not specified in one of the studies using the checkerboard DNA–DNA hybridization technique [16].

3.2. Quality assessment of included studies

The Timmer scale was used to assess the quality of the 12 included studies (Table 3). Since the majority met the criteria outlined in the checklist in full, with only a small number partially meeting the criteria, all studies were classified as exhibiting high quality (quality score: 0.75–0.89).

3.3. Effect of storage time changes in microbial profiles

3.3.1. Plaque samples

Three plaque-related studies reported changes in microbial profiles due to the effect of storage time [14,28,33]. These studies all used the checkerboard DNA–DNA hybridization technique and a storage temperature of − 20 °C [14,28,33]. do Nascimento et al. [33] reported that the hybridization signal was lower in the samples stored for 12 and 24 months, respectively, compared with the samples that were immediately processed or stored for 6 months. Two studies reported a reduction in the amount of total bacterial DNA following sample storage for 6 and 12 months, respectively, compared with the immediately processed samples [14,28]. However, the same studies reported no significant differences between samples stored for 6 weeks and samples that were immediately processed samples, even at a storage temperature of 4 °C, and suggested that the samples be stored at 4 °C for up to 6 weeks [14, 28].

On the other hand, three plaque-related studies reported that storage time did not significantly change the microbial profiles [11,29,30]. Zhou et al. [11] observed no significant differences during a 6-month storage period at − 80 °C. However, the longest storage time in that study was 6 months. Wilson et al. [30] reported that the bacterial CFUs of samples stored in liquid nitrogen for various periods (30 min, 8–10 weeks, and 1–2 years) were fairly similar to those of nonfrozen samples, although some sensitive bacteria showed a change of the CFUs. They suggested that storage in liquid nitrogen could be a useful alternative to immediate culture. Luo et al. [29] also reported that the storage time did not significantly affect the amount of recovered DNA; however, they used a different transport medium for each temperature condition, and the maximum storage time was 1 week.

3.3.2. Saliva samples

None of the saliva-related studies reported that storage time caused a significant effect [27,32]. Two reported that the microbial profiles remained stable and were unaffected by storage time [27,32]. One of these used 95 % ethanol as the sample storage medium and compared it with samples that were immediately frozen and samples that were stored at RT for 1 week, reporting that the microbial composition remained stable over time and across temperatures [32]. The other study compared immediately processed samples with the samples stored for 1 week, 2 months, or 6 months at − 80 °C and found that the amount of bacterial DNA remained stable for all periods (i.e., up to 6 months) [27].

3.3.3. Mixed samples of plaque and saliva

One study added the plaque sample to the saliva sample, reporting reduced microbial counts and microbial incidence following 12 months of storage regardless of the temperature (RT, 4 °C, −20 °C, and −80 °C) [16].

3.4. Effect of storage temperature on changes in microbial profiles

3.4.1. Plaque samples

Only two plaque-related studies investigated changes in microbial profiles due to storage temperature, and both reported no significant effect [26,29]. One of these studies found no significant difference when comparing the overall bacterial diversity between immediately frozen samples and samples stored at RT for 2 weeks and then stored at − 20 °C [26]. The other study used different transport media for each storage condition and did not observe any significant differences in the amount of recovered DNA between the samples stored at RT and the samples stored at − 80 °C for 2 days and then at − 20 °C [29]. However, in both of these studies, the maximum storage time until DNA extraction was short, either within 3 weeks [26] or for 1 week [29].

Table 3
Quality assessment of included studies.

Quality assessment	Adler et al. (2018) [26]	do Nascimento et al. (2012) [33]	do Nascimento et al. (2014) [16]	Furuhashi et al. (2022) [31]	Luo et al. (2016) [29]	Marotz et al. (2021) [32]	Katsoulis et al. (2005) [28]	Katsoulis et al. (2005) [14]	Karched et al. (2017) [27]	Vogtmann et al. (2019) [34]	Wilson et al. (1984) [30]	Zhou et al. (2019) [11]
1. Question / objective sufficiently described?	2	2	2	2	2	2	2	2	2	2	1	2
2. Design evident and appropriate to answer study question?	2	2	2	2	2	2	2	2	2	2	2	2
3. Subject characteristics sufficiently described?	2	1	2	2	2	0	1	1	1	2	1	2
4. Subjects appropriate to the study question?	2	2	2	2	2	2	2	2	2	2	2	2
5. Controls used and appropriate? (if no control, check no)	2	2	2	2	2	2	2	2	2	2	2	2
6. Method of subject selection described and appropriate?	1	2	2	2	2	2	2	2	2	2	0	2
7. If random allocation to treatment groups was possible, is it described? (if not possible, check n/a)	2	2	2	2	2	2	2	2	2	2	2	2
8. Outcome measure well defined and robust to measurement bias? Means of assessment reported?	2	2	2	2	2	2	2	2	2	2	2	2
9. Confounding accounted for?	2	1	2	2	2	1	1	2	2	2	2	2
10. Sample size adequate?	1	2	2	1	2	1	1	1	1	2	1	2
11. Post hoc power calculations or confidence intervals reported for statistically non-significant results?	2	2	2	2	2	2	2	2	2	2	2	2
12. Statistical analyses appropriate?	2	2	2	2	2	2	2	2	2	2	2	2
13. Statistical tests stated?	2	2	2	2	2	2	2	2	2	2	2	2
14. Exact p-values or confidence intervals stated?	2	2	2	2	2	2	2	2	2	2	2	2
15. Attrition of subjects and reason for attrition recorded?	N/A	N/A	N/A	N/A	2	N/A	N/A	N/A	N/A	N/A	N/A	N/A

(*continued on next page*)

Table 3 (*continued*)

Quality assessment	Adler et al. (2018) [26]	do Nascimento et al. (2012) [33]	do Nascimento et al. (2014) [16]	Furuhashi et al. (2022) [31]	Luo et al. (2016) [29]	Marotz et al. (2021) [32]	Katsoulis et al. (2005) [28]	Katsoulis et al. (2005) [14]	Karched et al. (2017) [27]	Vogtmann et al. (2019) [34]	Wilson et al. (1984) [30]	Zhou et al. (2019) [11]
16. Results reported in sufficient detail?	2	2	2	2	2	2	2	2	2	2	2	2
17. Do the results support the conclusions?	2	2	2	2	2	2	2	2	2	2	2	2
Quality score	0.83	0.83	0.89	0.86	0.89	0.78	0.81	0.83	0.83	0.89	0.75	0.89

Abbreviations: N/A, not applicable

3.4.2. Saliva samples

Two saliva-related studies [31,32] reported that storage temperature did not significantly affect microbial profiles. Furuhashi et al. [31] reported no differences between the microbial profiles of saliva samples stored for 2 weeks at − 15 °C and − 80 °C, respectively. Additionally, the microbial profiles were similar even if the samples were not flash-frozen in liquid nitrogen before storage. Marotz et al. [32] also reported that the microbial composition of saliva samples was unaffected by storage time and temperature when 95% ethanol was used as the storage medium. They also found that saliva samples were stable when stored at RT for a week, but they did not investigate long-term storage conditions.

In contrast, two other studies [29,34] reported that storage temperature significantly affected the relative abundance of some phyla and taxa. Luo et al. [29] observed that although the bacterial diversity was unaffected by storage temperature, the relative abundance in some phyla was decreased in the samples stored at RT for 5 and 7 days, respectively, compared with the samples immediately frozen at − 20 °C. Similarly, Vogtmann et al. [34] reported that although the relative abundance was similar in the top 25 genera, some taxa differed when comparing samples stored at RT for 4 days with samples immediately frozen at − 80 °C. The top 25 genera that showed similar relative abundances in their study included *Atopobium, Corynebacterium, Rothia, Capnocytophaga, Porphyromonas, Prevotella, Bulleidia, Catonella, Dialister, Megasphaera, Peptostreptococcus, Selemonas, Veillonella, Fusobacterium, Aggregatibacter, Lautropia,* and *Neisseria* [34]. The intraclass correlation coefficients (ICCs) for these genera showed a high correlation exceeding 0.75. However, according to false discovery rate (FDR) control using the Benjamini–Hochberg procedure to correct for multiple testing in the same study, the relative abundances of *Firmicutes* showed a significant increase, whereas those of Bacteroidetes, Proteobacteria, and Fusobacteria showed a significant decrease after storing for 4 days at RT [34].

3.4.3. Mixed samples of plaque and saliva

In the study where the plaque sample was placed into the saliva sample, none of the three storage temperatures (4 °C, −20 °C, and −80 °C) affected the microbial counts during 6 months of storage compared with the immediately processed sample [16].

4. Discussion

The findings of our scoping review support our assertion that current evidence on the optimal storage temperature and acceptable storage time that minimizes changes in samples of human dental plaque and saliva collected for studies on oral microbiomes is extremely limited and inconsistent. The experimental design of the included studies was quite diverse. The most optimal sample storage temperature was the immediate freezing of fresh samples at − 80 °C (or below) until DNA extraction. The most acceptable storage time was 6 months or less, and storage > 1–2 years was controversial. Regarding the use of specific storage media, dental plaque samples stored in 75 % ethanol or Bead Solution resulted in stable storage for 6 months at − 80 °C [11]. Regarding short-term storage, dental plaque and saliva samples could be stored at RT for 1–2 weeks without significant changes if appropriate transport media or storage media were used, but this varied depending on the microbial taxa.

In the field of microbiome studies, the best-considered approach is the immediate freezing of fresh specimens at − 80 °C or − 20 °C and then batch processing for DNA extraction and sequencing [36–39]. In the case of fecal samples used to study human gut microbiota, transportation to the laboratory within 4 h at RT or within 1–2 days if stored below 4 °C is recommended, followed by immediate storage at − 20 °C or − 80 °C upon arrival [40,41]. When the long-term storage of fecal samples is required, a storage temperature of − 80 °C is preferable to storage at − 20 °C [42]. The long-term storage of fecal samples for 4 and 14 years has been reported to have a negligible impact on microbial community profiles if appropriate storage conditions are met [43,44]. Based on our scoping review, the optimal storage temperature and acceptable storage times of saliva and plaque samples for microbial profile studies do not appear to differ significantly from those used for fecal samples. However, the number of studies performed on oral microbiome samples is significantly limited compared with the number of studies performed on fecal samples. In particular, research on long-term storage periods exceeding 2 years is scarce. When biobanking oral microbiome samples, long-term storage for many years may inevitably be required. Although no studies have investigated storing oral microbiome samples for longer than 2 years, the best-recommended method for biobanking saliva or plaque samples is freezing immediately after collection and storage at − 80 °C or below.

Studies based on checkerboard DNA–DNA hybridization showed inconsistent results regarding the effect of storage at − 20 °C for 6 months on changes in bacterial DNA. do Nascimento et al. [33] reported that the hybridization signal of dental plaque samples stored at − 20 °C for 6 months did not differ from those of immediately processed samples. However, Katsoulis et al. [14] stated that total bacterial DNA and proportional distributions of red complex in dental plaque samples stored at − 20 °C for 6 months differed from those that were immediately processed. The disparity between the two studies may lie in their respective methodologies. The former study analyzed the DNA of 13 microbial strains from subgingival plaque samples obtained from sites with a healthy gingival sulcus of less than 3 mm in healthy subjects. In contrast, the latter study investigated the DNA of 40 microbial strains from subgingival plaque samples collected from sites with a periodontal pocket depth of 6 mm or more in patients with chronic periodontitis. These findings suggest that periodontal pathogenic bacteria may exhibit greater instability compared to microorganisms inhabiting periodontally healthy gingival sulci under the same storage temperature and storage time. However, since the above studies [14,33] did not include a temperature of − 80 °C, the response and stability of microorganisms to this storage condition could not yet be examined, which could be a limitation.

A study based on checkerboard DNA–DNA hybridization that

combined saliva and plaque samples to increase the number of microorganisms revealed that the bacterial counts of samples stored at cold temperatures (i.e., 4 °C, −20 °C, or −80 °C) for up to 6 months were similar to the samples that were immediately processed [16]. Most DNA hybridization-based studies [14,28,33] claimed that a storage time ≥ 1–2 years, regardless of the temperature (i.e., 4 °C, −20 °C, or −80 °C), caused changes in the microbial DNA profiles. These results were consistent with the findings of Moncla et al. [45], which revealed a nucleic acid loss of up to 15% after storing subgingival plaque samples at − 70 °C for ≥ 2 years. One saliva-related study based on UV spectrometry and qPCR analysis revealed that the DNA of most bacterial species could be stored at − 80 °C for at least 6 months without a loss in concentration. However, unlike other studies, the DNA was extracted before storage [27].

Most studies, including RT condition, involved short-term storage periods of up to 1 month rather than studying long-term storage conditions [26,29,32,34]. These studies generally aimed to evaluate the usefulness of transport media or storage media, usually a commercial buffer or kit, for stabilizing samples at RT [26,29,32,34]. Adler et al. [26] used 16 S rRNA gene sequencing to analyze differences in recovered microbial DNA and found no significant difference when VMG II was used as both the transport and storage medium for samples stored at RT for 2 weeks and then frozen at − 20 °C compared with samples that were immediately frozen at − 20 °C. The bacterial community and composition were also reported as stable at RT for at least 1 week when OMNIgene was used as the transport medium [29] or 95 % ethanol was used as the storage medium [32]. However, Vogtmann et al. [34] reported that saliva samples stored in Scope mouthwash at RT for 4 days showed a significant difference in the relative abundance of some taxa compared with the samples immediately frozen at − 80 °C, although the relative abundance of the top 25 genera was similar. The reason for these discrepant results might be that, as in plaque-related studies, the response and stability of microorganisms in saliva can vary considerably across individual taxa even under the same storage conditions. Therefore, it may be desirable to record the conditions under which the primary variable of interest may be correlated (e.g., storage time, storage temperature and etc.) when conducting research using the stored microbiomes so that it can be adjusted in the statistical analysis. From the results of the above studies, it can be inferred that storage at RT for 1–2 weeks may be possible without causing significant changes in general microbial profiles if appropriate transport or storage media are used. However, although the use of transport or storage media aids sample stabilization, the entire media volume must be eliminated for DNA extraction following storage. Since the influence of this process on DNA extraction is still unclear and it might have a direct impact on microbial composition, this should be considered when using transport media or storage media [26,46].

There are several limitations within this review. While our scoping review primarily focused on assessing the impact of storage time and temperature on microbiomes, factors such as the use of transport or storage media, timing and method of DNA extraction, as well as the tools employed for microbial analysis, might have significantly influenced microbial profiles. Moreover, the studies encompassed in this review exhibited notably diverse experimental designs. For these reasons, it was difficult to derive substantive and high-level evidence-based conclusions. In addition, the number of studies was insufficient, and in some studies, important information was not documented. Potential publication bias—manifested in the reluctance to publish negative outcomes—along with dilution errors and measurement inaccuracies during aliquot preparation, might have also influenced the outcomes of the studies included in our scoping review. Therefore, well-designed randomized studies, controlling factors that may influence microbial profiles, are essential for determining acceptable short- and long-term storage times and appropriate temperatures to minimize changes in recovered microbial profiles in the future.

5. Conclusions

Current evidence on the optimal storage temperature and acceptable storage time required to minimize changes in the microbial composition of human dental plaque and saliva samples for use in oral microbiome studies is extremely limited, and the findings are inconsistent. Within the limitations of this study, a suggested approach for storing samples containing oral microorganisms is the immediate freezing of fresh specimens at − 80 °C (or below) until DNA extraction. It can be advisable to avoid storage times longer than 1–2 years, as this may potentially result in alterations in the microbial DNA profiles of dental plaque and saliva samples, regardless of the temperature (i.e., 4 °C, −20 °C, or −80 °C). According to the studies based on checkerboard DNA–DNA hybridization, dental plaque samples could be stored at − 20 °C for 6 months without significant changes. However, the impact of this storage period on microbial DNA profiles could vary depending on the microbial strains. Therefore, a storage period shorter than 6 months can be suggested considering the vulnerable strains. According to the studies utilizing 16 S rRNA gene sequencing, dental plaque samples could be stored at − 80 °C for 6 months within 75 % ethanol or Bead Solution without significant changes in microbial profiles. Notably, some evidences suggested that the storage of dental plaque or saliva samples at RT for 1–2 weeks might be feasible, provided appropriate transport or storage media are used. It can be advisable to keep good records of storage information so that relevant variables can be adjusted during future statistical analyses since the impact may vary depending on the microbial taxa. Further well-designed randomized controlled studies are necessary in the future.

Conflict of interests

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jdsr.2024.05.001.

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