Fresh Versus Frozen Versus Lyophilized Fecal Microbiota Transplant for Recurrent Clostridium Difficile Infection

A Systematic Review and Network Meta-analysis

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Introduction: *Clostridium difficile* Infection is a significant source of morbidity and mortality, which is on the rise. Fecal Microbiota Transplantation (FMT) is an alternative therapy to antibiotics with a high success rate and low relapse rate. Current data regarding the efficacy of the types of FMT used, namely fresh, frozen, and lyophilized is conflicting. Our review attempts to consolidate this data and highlight the most efficacious treatment currently available.

Methodology: MEDLINE, Embase, Web of Science Core Collection, Cochrane Central Register of Controlled Trials, SciELO, the Korean Citation Index, and Global Index Medicus were systematically searched from inception through May 3, 2022. Studies in which patients are undergoing any form of FMT who had failed antibiotic treatment previously were included. Both pairwise (direct) and network (direct + indirect) meta-analysis were performed using a random effects model and DerSimonian-Laird approach. A frequentist approach was used for network meta-analysis. Risk differences with (RD) with 95% confidence interval (CI) were calculated.

Results: A total of 8 studies, including 4 RCTs and 4 cohort studies, were included with a total of 616 patients. Fresh FMT was determined to be most successful with 93% efficacy 95% CI (0.913 to 0.999) followed by frozen with 88% efficacy 95% CI (0.857 to 0.947) and lyophilized with 83% efficacy 95% CI (0.745 to 0.910). The direct meta-analysis showed no statistically significant difference between fresh and frozen group. (RD −0.051 95% CI −0.116 to 0.014 $P=0.178$). No significant differences were noted in frozen versus lyophilized groups with an overall trend towards Fresh FM (RD −0.061 95% CI −0.038 to 0.160 $P=0.617$). On network meta-analysis, when compared with fresh group, a lower recovery rate was noted with both frozen group (RD −0.06 95% CI −0.11 to 0.00 $P=0.05$) and lyophilized group (RD −0.16 95% CI −0.27 to −0.05 $P=0.01$).

Conclusion: We conclude the efficacy of Frozen and Lyophilized preparations is high with no difference in direct comparison, and the relative efficacy reduction based on network analysis is outweighed by the safety, accessibility, and practicality of Frozen or Lyophilized preparations.

Key Words: clostridium difficile, fresh FMT, frozen FMT, lyophilized FMT

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Clostridium difficile Infection (CDI) is the leading cause of hospital-acquired infection, accounting for 15% of all cases in the US.[1] Antibiotic use predisposes the development of CDI since they lead to a disruption in the protective barrier created by native gut microbiota.[2] With time CDIs have been on the rise and are associated with significant morbidity and mortality. Conversely, antibiotics also play a role in the treatment of CDI however, they have a high chance of reinfection. Roughly 15% to 30% of patients initially treated with antimicrobial therapy experience recurrence, and the risk of subsequent recurrence increases to 40% after the first relapse and to 45% to 65% risk thereafter.[2] Current American College of Gastroenterology guidelines recommend treatment with FMT to prevent recurrences after patients experience their second or subsequent recurrence of CDI or consider FMT for patients with severe and fulminant CDI refractory to antibiotic therapy.[3]

Fecal Microbiota Transplantation (FMT) is an alternative treatment used to treat CDIs, which has recently increased in popularity due to its high cure rate (90%) and effectiveness in treating severe and recurrent CDI. FMT works by transplanting beneficial gut flora from a healthy donor to the patient. Currently, there are 3 main standards of FMT preparation that is, fresh, frozen, and lyophilized donor stool.[4] Although each individual FMT preparation has its logistical advantages, the effectiveness and difference have not been comprehensively compared. Multiple studies have shown variable efficacy results when comparing each FMT method. Our review attempts to consolidate the current data available and determine the most effective FMT modality.

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METHODOLOGY

Search Strategy

A detailed and comprehensive search of the following databases was conducted from inception through May 3, 2022: MEDLINE (OVID platform), Embase (Embase.com, Elsevier), Web of Science Core Collection, Korean Citation Index, and SciELO (Clarivate), Global Index Medicus (World Health Organization) and Cochrane Central Register of Controlled Trials (Cochrane Library, Wiley). A further hand search was also performed. The keywords and subject terms for the concepts of "Fecal Microbiota Transplantation", "Frozen", and "Lyophilized" were developed for Embase and translated into the vocabulary of other databases. The search strategy was created by an experienced librarian (W.L.S.) and reviewed by another investigator (M.A). A detailed search strategy for Embase is provided in Supplementary table 1, Supplemental Digital Content 1, http://links.lww.com/JCG/A899. All results were exported to EndNote 20 citation management software (Clarivate), and duplicates were removed by successive iterations of EndNote's duplicate detection algorithms and manual inspection. Screening of the articles was performed by 2 independent reviewers (A.A. and F.P.) and discrepancy was resolved through mutual discussion. Preliminary screening was done using titles and abstracts, and full texts of relevant articles were obtained. The bibliographies of the included articles were also checked to see if any additional articles fulfilled our study criteria. We adhered to Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines, and no restriction to language was applied.[5] The review was not registered, and the protocol was not prepared.

Study Definitions

Efficacy was defined as the resolution of CDIs based on the criteria set by each individual study. FMT was categorized as either fresh, frozen, or lyophilized stool administered through oral route, by enema or colonoscopically.

Inclusion and Exclusion Criteria

We included studies that met the following criteria: Population: Patients undergoing FMT transplantation following the failure of antibiotic therapy to treat CDI. Intervention: FMT either fresh versus frozen versus lyophilized. Only studies with a head-to-head comparison of 2 out of 3 FMTs or all 3 FMTs were included. Outcomes: resolution of symptoms. Case reports, case series, review articles, guidelines, and studies with less than 20 sample sizes were excluded.

Data Extraction and Study Outcomes

Data from finalized studies included baseline demographics including age, gender, type of FMT (frozen, fresh, and lyophilized), CDI type (recurrent, refractory, or both), previous antibiotic therapy, donor (related, unrelated donors, or both), and delivery method were collected and tabulated on Microsoft Excel (Microsoft) by 2 independent reviewers (F.P. and A.A.). Any discrepancy in data collection was resolved through mutual discussion and a third reviewer (M.A).

Data Synthesis and Statistical Analysis

We performed a direct meta-analysis to generate direct evidence for studies making a head-to-head comparison between intervention groups. We also performed a network meta-analysis to generate direct and indirect evidence comparing all groups simultaneously. DerSimonian-Laird method and random effects model (https://handbook-5-1.cochrane.org/chapter_9/9_4_3_a_generic_inverse_variance_approach_to_meta_analysis.htm) was used to perform a direct meta-analysis on Open Meta Analyst (CEBM). Network meta-analysis was conducted using a random effects model on "R" package "Netmeta" (Bell labs). A pooled proportion meta-analysis using the transformation method was performed to determine efficacy. Risk Difference (RD) with 95% confidence interval (CI) for each proportional outcome was calculated. A P value of <0.05 was considered statistically significant. The "frequentist method"[6] was used to rank the intervention, and a P score was generated. A higher P score (closer to 1.00) corresponds to a higher recovery rate for the respective intervention group. Study heterogeneity was assessed using the I2 statistic defined by the Cochrane Handbook for systematic reviews, and value $>50\%$ was considered substantial heterogeneity (https://handbook-5-1.cochrane.org/chapter_9/9_5_2_identifying_and_measuring_heterogeneity.htm). Disagreement between direct and indirect evidence was assessed using the node-splitting technique (https://training.cochrane.org/handbook/current/chapter-11#section-11-4-4). The Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach was utilized for assessing the certainty of evidence and rating of VERY LOW, LOW, MODERATE, and HIGH was determined (https://gdt.gradepro.org/app/handbook/handbook.html).

Bias Assessment

The bias assessment for included studies was evaluated using Newcastle-Ottawa scale for observational studies and Cochrane Risk of tool bias for RCTs (https://handbook-5-1.cochrane.org/chapter_13/13_5_2_3_tools_for_assessing_methodological_quality_or_risk_of.html).[7,8] Publication bias was visually assessed using funnel plots and quantitatively assessed using Egger's regression analysis. A P value <0.05 was indicative of substantial publication bias.

RESULTS

Using the search strategy above, a total of 424 studies were screened, duplicates were removed and 8 studies,[9–16] including 4 RCTs and 4 observational studies (Tables 1 and 2) were included after rigorous screening. Among the 8 studies, each assessed fresh FMT, along with 6 also assessing Frozen FMT while 3 studies assessed Lyophilized FMT. A total population of 616 patients was included. Fresh FMT was determined to be most successful with 93% efficacy 95% CI (0.913 to 0.999) followed by frozen with 88% efficacy 0.902 95% CI (0.857 to 0.947) and lyophilized with 83% efficacy 0.828 95% CI (0.745 to 0.910) by using performing a pooled proportion meta-analysis.

Baseline Demographics

The mean age of included participants in the study was 66.1 years, with 384(62.3%) being female. There were a total of 604 patients, with 237 in the Fresh group, 287 in the Frozen group, and 80 in the Lyophilized group. Table 1 consists of the demographic details of each study.

Direct Meta-analysis

The direct meta-analysis showed no statistically significant difference between the fresh versus frozen group (6 studies) as shown in Figures 1 and 2A (RD -0.051 95% CI -0.116 to 0.014 $P=0.178$) with I2 of 34.4% (0.0%; 75.1%). No

significant differences were noted in the frozen versus lyophilized group (3 studies), as shown in Figure 2B (RD −0.061 95% CI −0.038 to 0.160 $P=0.617$) with I2 = 0% (0.0%; 89.6%).

A Forest Plot comparing the RD of Fresh, Frozen, and Lyophilized FMT is shown in Figure 2.

Network Meta-analysis

The results of the network meta-analysis are summarized in Table 3 and subsequent network plots are demonstrated. The net diagram is subsequently shown in supplementary figure 2, Supplemental Digital Content 1, http://links.lww.com/JCG/A899. When compared with the Fresh group, a lower recovery rate was noted with both the Frozen group (RD −0.06 95% CI −0.11 to 0.00 $P=0.05$) and the Lyophilized group (RD −0.16 95% CI −0.27 to 0.05 $P=0.01$). The heterogeneity noted for this model was 20.1% with CI (0.0%; 62.5%). To determine any inconsistency in network meta-analysis, node splitting was performed, which showed no difference between direct and indirect evidence, as shown in supplementary table 2, Supplemental Digital Content 1, http://links.lww.com/JCG/A899. Using the Grading of Recommendations Assessment, Development and Evaluation Classification, the certainty of the evidence for our outcome of efficacy was low due to the risk of bias.

Ranking of Interventions

Based on the frequentist approach and P score, the fresh group was ranked highest (0.99), followed by Frozen (0.50), and Lyophilized (0.02). The higher P score signifies a higher recovery rate in respective interventions (Supplementary Figure 1, Supplemental Digital Content 1, http://links.lww.com/JCG/A899).

Risk of Bias

Publication bias could not be assessed due to the total number of studies being less than 10 studies. Three studies were assessed using the Newcastle-Ottawa scale (Supplementary Table 3, Supplemental Digital Content 1, http://links.lww.com/JCG/A899). The score ranged from 6 to 7. Three RCTs were evaluated for bias using the Cochrane risk of bias tool for randomized trials (Supplementary Table 4, Supplemental Digital Content 1, http://links.lww.com/JCG/A899), and nonRCTs were evaluated using ROBINS-I tool (Supplementary Table 5, Supplemental Digital Content 1, http://links.lww.com/JCG/A899). The risk of bias was high for each study. Two studies could not be evaluated for bias since they were abstracts.

DISCUSSION

Our meta-analysis shows no statistically significant differences in efficacy rates between different FMT formulations upon direct comparison, although pooled recovery rates and network analysis show a trend towards increased efficacy of Fresh FMT relative to Frozen and Lyophilized FMT.

Fecal microbiota transplantation is now the recommended treatment for recurrent CDI. Recent studies have reported procedures using either frozen or freeze-dried stool, allowing oral administration in both in-patient and outpatient settings. However, a central question remains on the viability, engraftment, and efficacy of the microbiome over time, especially during storage. Fresh FMT is stool readily prepared from a donor and immediately transferred to a patient to preserve the microbial load and diversity.[8] Although it has a high cure and low relapse rate, the logistics of having a donor available and providing immediate transplantation make it a challenging method of FMT. Frozen

TABLE 1. Baseline Study Characteristics and Patient Demographics

References	Study Type	Country	Total Sample Size	Age	Female Gender n (%)	Type of FMT (Frozen, Fresh, Lyophilized)	Previous Antibiotic Therapy	Donor Relation (Related, Unrelated)	Mode of Delivery
Agarwal et al[7]	Full text cohort study	US	100	66.3	72 (72)	Frozen and fresh	Normal standard therapy	Both	Enema/ Colonoscopy
Jiang et al[8]	Full text (RCT)	US	65	65	46 (71)	Frozen and lyophilized	Received one course of anti-CDI antibiotics	Not reported	Enema
Jiang et al[9]	Full text RCT	US	72	66.1	52 (72)	All	2 wk of vancomycin	Not reported	Colonoscopy
Dupont[10]	Abstract RCT	US	54	71	37 (69)	Frozen and lyophilized	X	X	Enema
Lee et al[11]	Full text RCT	Canada	178	72.5	112 (63)	Fresh and frozen	In the recurrent group vancomycin was administered for at least 10 d vs. at least 5 d in the refractory group.	Unrelated donors	Enema
Satokari et al[13]	Full text cohort study	Finland	49	56.2	34 (69)	Fresh and frozen	Vancomycin treatment	Both	Colonoscopy
Arkilla et al[13]	Abstract cohort	Finland	44	56	X	Fresh and frozen	2 Antibiotic treatment regiments before transplantation	Both	Colonoscopy
Hamilton et al[14]	Full text cohort study	US	43	58.3	31 (72)	Fresh and frozen	Vancomycin 6wk course or vancomycin + Fidaxomicin	Both	Colonoscopy

TABLE 2. Efficacy Outcomes for Each Individual Study

References	Subgroup Type of FMT (Frozen, Fresh, Lyophilized)	Total Sample Size	Subgroup Sample	Age (Mean ± SD/ Range)	Subgroup Age	Total Female/ Male	Subgroup Female/ Male	Recovery Rate/ Subgroup	Definition of Recovery
RCT Studies									
Jiang et al[8]	Frozen	65	34	65	63	46/19	25/9	30/34	Clinical cure was defined as no episodes of CDI during the 60 d after FMT treatment.
	Lyophilized	—	31	—	67	—	21/10	26/31	
Jiang et al[9]	Fresh	72	25	66.1	75	52/20	21/4	25/25	Primary outcome was recurrence of symptomatic, laboratory-confirmed CDI within 120 d of the intervention
	Frozen	—	24	—	62.5	—	18/6	20/24	
	Lyophilized	—	23	—	63	—	13/10	18/23	
Lee et al[11]	Frozen	178	91	72.5	72.2	112 (63%)	58/33	76/91	The primary end points were no recurrence of CDI-related diarrhea at 13 wk after receiving up to 2 FMTs without the need for antibiotics
	Fresh	—	87	—	72.9	—	54/33	74/87	
Cohort Studies									
Agarwal et al[7]	Frozen	100	50	66.25	64.2	72/28	34/16	43/50	The primary outcome was clinical success, defined as resolution of clinical symptoms and no recurrence within 8 wk of FMT. If an episode of CDI occurred after 8 wk of FMT, this was considered a separate infection and not a failure of FMT.
	Fresh	—	50	—	68.3	—	38/12	50/50	
Arkkila et al[13]	Fresh	55	27	56	53	X	X	26/27	The result of FMT was tested by control Clostridium difficile culture and toxins after one month, and by symptom follow-up up to one year.
	Frozen	—	17	—	61	—	X	16/17	
Dupont et al[10]	Lyophilized	54	26	71	X	37/17	X	22/26	The clinical outcome was absence of CDI during the 60 d after FMT. The subjects were followed for 6 mo for safety.
	Frozen	—	27	—	X	—	X	26/27	
Satokari et al[13]	Fresh	49	26	56.2	52	34/15	20/6	25/26	Treatment failure at 12 weeks or 1 year was defined as persisting diarrhea with a positive C. difficile toxin stool test, and a need for new therapy.
	Frozen	—	23	—	61	—	14/9	22/23	
Hamilton et al[14]	Fresh	43	22	58.3	57.7	31/12	17/5	18/22	Defined as resolution of diarrhea and negative C Dif stool test at 2 months
	Frozen	—	21	—	59	—	14/7	19/21	

RD indicates risk difference for recovery rates.

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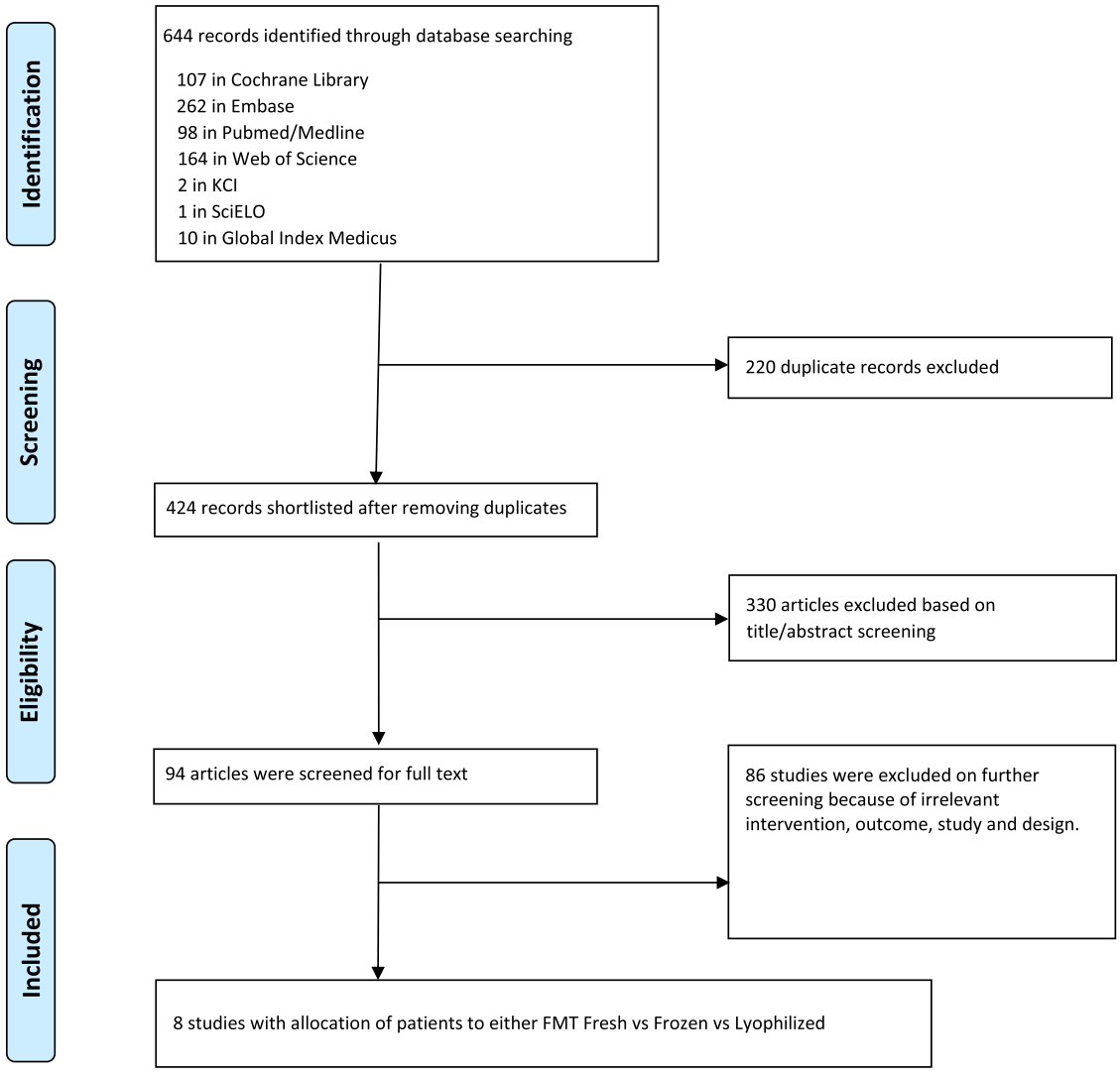

FIGURE 1. PRISMA Flow Diagram for 6 studies included in the review.

FMT is more practical as it is conveniently stored and transported; however, there is a compromise on the efficacy of the transplant, particularly if proper preservation techniques (addition of cryoprecipitant and glycerol) are not utilized during storage. In addition, optimal conditions, including temperature of storage, time stored before use, and thawing techniques, are inconsistent and may add further variability in efficacy. Finally, lyophilized FMT is a form of freeze-dried FMT that can conveniently be incorporated into capsules and requires no invasive procedures to administer. Both frozen and fresh FMT are usually delivered by colonoscopy, enema, or a nasogastric tube. Given the logistical issues in the different types of FMTs, it is imperative to assess the efficacies so a well-rounded clinical decision can be made.[11,17] Multiple studies have evaluated the efficacy of each FMT type, but the results have not been consistent. This is because there is considerable variability in treatment protocols, study designs, and patient populations among studies.

Among the 6 studies included in our analysis, only 1 study found frozen FMT to be more efficacious than fresh FMT.[13] Patients included in this study were repeatedly given FMT infusions until symptom resolution, and hence most patients recovered due to multiple treatments. Interestingly on the first FMT infusion, Fresh FMT was found to be more efficacious than Frozen FMT; however, upon repeated infusions, the efficacy of Frozen FMT became comparable. This initial discrepancy can be explained due to a higher number of viable bacteria and diversity of organisms in fresh FMT compared with frozen FMT, although the difference is slight.[11] Lyophilized FMT has the least amount of bacterial viability and diversity among the group. Since the purpose of FMT after *C. difficile* colonization is to recolonize the gut with healthy bacteria, a higher number of infusions are required from lyophilized FMT to be as efficacious as fresh and frozen FMT. This was demonstrated by Jiang et al[16] when the efficacies of each FMT group started levelling out after repeated infusions. Hence there might be a possibility that despite the logistical advantage of frozen FMT, a higher volume may be required to be comparable in efficacy with fresh FMT.

Mode of transmission may also play a role in the efficacy of FMT. A previous review comparing the efficacy of FMT found Lower GI delivery to be significantly more

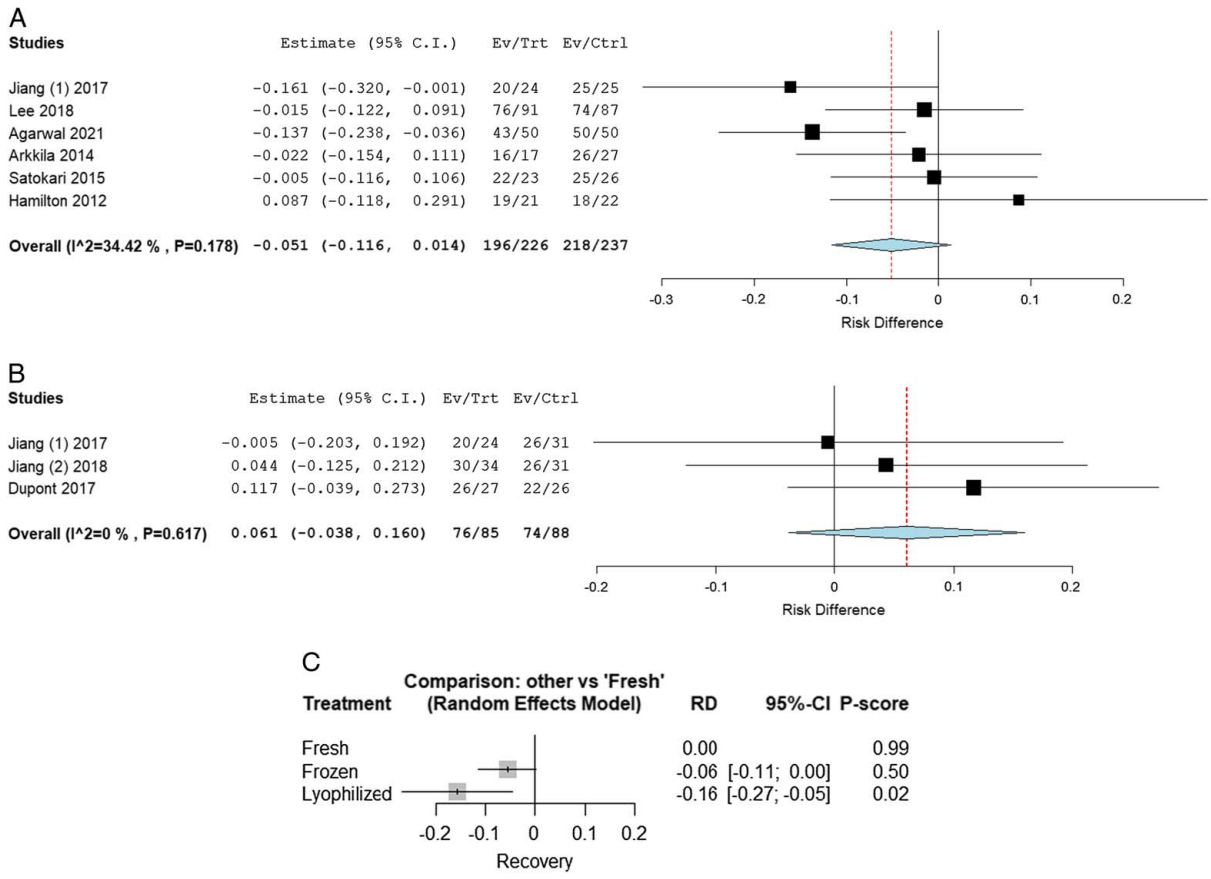

FIGURE 2. A, Forest plot showing a direct comparison between Frozen Versus Fresh. B, Forest plot showing direct comparison between Frozen Versus Lyophilized. C, Forest Plot comparing Frozen and Lyophilized FMT with Fresh using Network meta-analysis (direct + indirect).

efficacious than Upper GI delivery (95% vs. 88%, respectively, $P = 0.02$).[18] Similarly, Hui et al found fresh FMT to have reduced efficacy compared with frozen, when given through enema.[19] Among the studies within our review, almost half of the patients had FMT delivered by enema, while the other half had FMT delivered by colonoscopy. Although this could have an effect on the efficacy rates, no study utilized different modes of delivery when comparing the different FMT groups.

There was also a wide range of FMT dosages in each study we evaluated. Among 5 studies in our review, 3 studies had the same dose in their comparison groups; however, the total amount varied considerably among all groups. Amounts of FMT usually exceeded >50gm. Hypothetically higher doses can obscure a difference in efficacy as higher biomass of fecal bacteria is transmitted (Supplementary Table 6, Supplemental Digital Content 1, http://links.lww.com/JCG/A899).

A previously conducted meta-analysis comparing the efficacy of fresh Versus frozen FMT found no significant difference in the efficacy of fresh versus frozen.[20] This difference in our conclusion can be attributed to a variety of factors. We evaluated a total of 8 studies in our review, all of which were direct head-to-head comparisons of each FMT group and did not include studies that included standard antibiotic treatment as a control group. Furthermore, we conducted a network meta-analysis in addition to a direct meta-analysis and included 3 recent studies (2RCTs and 1 Cohort Series) in our dataset that were published after the previous review was conducted. Lastly, we also included lyophilized FMT as part of the review, which had not been previously discussed.

Our review has a few limitations, primarily due to the lack of standardization of FMT. We evaluated patients with recurrent or refractory CDIs however, there needs to be further stratification of patients based on age, gender, donor

TABLE 3. Outcomes Based on Network Meta-Analysis Results

	Fresh	Frozen	Lyophilized
Fresh	—	0.06 (0.00 to 0.11) $P = 0.05$	0.16 (0.05 to 0.27) $P = 0.01$
Frozen	−0.06 (−0.11 to 0.00) $P = 0.05$	—	0.10 (0.00 to 0.21) $P = 0.06$
Lysophilized	−0.16 (−0.27 to −0.05) $P = 0.01$	−0.10 (−0.21 to 0.00) $P = 0.06$	—

Results of Network Meta-Analysis.

status, and a previous/current history of IBD. All these factors may possibly contribute to the prognosis of the disease. We suggest future studies to evaluate the efficacy of FMT by ensuring the above possible confounders be addressed not only by the type of FMT but also by the route of delivery, including nasogastric tube, colonoscopy, enema, or oral delivery methods. However, within our review, there were similar demographic details within each group, and the mode of delivery did not have considerable variability among studies. Among the different studies in our review, the low heterogeneity in the data can be explained by slight variations in demographic details, treatment protocols, and sample sizes; however, for the most part, the data was generally uniform.

To truly assess the clinical utility of each type of FMT, there needs to be a separate review of their adverse effects. Based on this, a holistic decision can be made relying on each types' efficacy, adverse effects, and logistics (dosing, transportation, storage, and patient preference). Although fresh FMT has a higher number of viable bacteria, it cannot be stored or transported and must be administered immediately through invasive processes as it has no quarantine period, while serology window periods to close. Frozen and lyophilized FMT can be given as an encapsulated oral capsule or given through an enema or colonoscopy. Both have a longer shelf life than fresh FMT; however, still require to be frozen at -80 degrees and are difficult to transport and store, which is only likely possible at larger centers. Typically, stool banks release Frozen or lyophilized products after 8-12 week quarantine to allow adequate screening to minimize the risk of false negative results. Hence clinical decisions require considering multiple factors when administering FMT. With the advancement of cryopreservation techniques designed to preserve bacterial viability and diversity, Frozen and lyophilized FMT techniques have significantly improved.

Finally, our review included studies with small sample sizes, and although using only comparator studies increases homogeneity and strength of analysis, it reduced the number of studies that could be included in the study. However, 1 concern that may arise from our paper would be if the study was underpowered to begin with, as this essentially is a negative study. Previous studies (RCTs and retrospective studies) included a low number of study participants, and while this concern may have been valid, our study is the most robust with less than 600 patients. We would also like to point out that apart from the direct analysis between frozen Versus lyophilized, all other outcomes had narrow intervals suggesting adequate statistical power. As such, further increasing the size of the study population will not significantly influence the study outcomes.

In conclusion, the efficacy of Frozen and Lyophilized FMT is high, and a reduction in the relative overall efficacy trend, based on our network analysis, is very small. Any minor reduction is outweighed by the safety, accessibility, and practicality of Frozen or Lyophilized preparations. Additional studies evaluating feasibility, accessibility, and cost analysis should be performed. There should also be a focus on continuous improvement in processing Frozen and Lyophilized FMT and potentially evaluating the role of using Fresh FMT after Frozen FMT failure.

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