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Microbes associated with spontaneous cacao fermentations - A systematic review and meta-analysis

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ABSTRACT

Chocolate is a product of the fermentation of cacao beans. Performed on-farm or at local cooperatives, these are spontaneous cacao fermentations (SCFs). To better understand SCFs, this study sought to identify SCF microbes, their interrelationships, and other key parameters that influence fermentation. This is important because differences in fermentation can have an impact on final product quality. In this study, a systematic data extraction was performed, searching for literature that identified microbes from SCFs. Each unique microbe, whether by location or by fermentation material, was extracted from the articles, along with parameters associated with fermentation. Data were collected and analyzed for three interactions: microbe-to-geography, microbe-to-fermentation method, and microbe-to-microbe. The goal was to attribute microbes to geographical locations, fermentation materials, or to other microbes. Statistically significant relationships will reveal target areas for future research. Over 1700 microbes (440 unique species) were identified across 60 articles. The top three countries represented are Brazil (22 articles, n = 612 microbes), the Ivory Coast (14 articles, n = 237), and Ghana (10 articles, n = 257). Several countries were far less, or never represented, and should be considered for future research. No specific relationship was identified with microbes to either geographical location or fermentation method. Using a Presence-Absence chart, 127 microbe-to-microbe interactions were identified as statistically significant. Data extraction into SCF research has revealed major gaps of knowledge for the cacao microbiome. By better understanding the cacao microbiome, researchers will be able to identify key microbes and fermentation parameters to better influence the fermentation.

1. Introduction

Chocolate is one of the most well-known delicacies worldwide. Primarily composed of the fruit seeds of *Theobroma cacao* L., also known as cacao, chocolate is desirable due to its complex aroma and flavor profile. Cacao is a domesticated crop that originates from the Amazonian basin, in current-day Southern Ecuador (Cornejo et al., 2018). Cacao pods are oblong, can be multi-colored, and contain, on average, 40 cacao beans and a mucilaginous pulp. To process cacao beans into chocolate, there are several, required post-harvest processing steps. The first post-harvest processing step is the fermentation of cacao beans, which is paramount to cacao quality and the first post-harvest step in developing the flavor profile.

While the flavor profile of cacao is dependent on multiple intrinsic and extrinsic factors pre- and post-harvest (Engeseth and Ac Pangan, 2018), the raw cacao beans are firm, bitter and astringent in flavor after harvest, and must be fermented before becoming chocolate. By initiating a cascade of biochemical reactions, fermentation accomplishes three major tasks: (i) breakdown and removal of the viscous pulp surrounding the beans, (ii) formation of volatile organic compounds (VOCs), lessening the bitter/astringent notes, and (iii) hydrolytic reactions within the cotyledons (Amoa-Awua, 2015; Schwan and Fleet, 2014). Currently, the majority of cacao is still spontaneously fermented on-farm, spanning 5–7 days with optimal weather (Schwan and Fleet, 2014; Schwan and Wheals, 2004). These spontaneous cacao fermentations (SCFs) occur in either wooden boxes, heaps, or in some uncommon materials, such as

Abbreviations: VOCs, Volatile organic compounds; SCFs, Spontaneous cacao fermentations; FF, Filamentous fungi; CI, Culture Independent; CD, Culture Dependent.

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plastic buckets or trays. Typically, heap fermentations are popular for smallholding farmers (≤2 ha) and wooden boxes are more popularly used for plantations (≥5), or at cooperatives (International Cocoa Initiative, 2016; Nair, 2010). However, each farm has its own preferred practices and style, which differ by region, by culture, and over time (Levai et al., 2015). This batch-centric system suggests that the post-harvest processing methods are dependent on the farmer and their resources. Despite this, fermenting cacao remains a successful strategy to enhance product quality.

Based on the inconsistent nature of the cacao microbiome, there is a large array of microbes identified in cacao fermentations. These microbes can be broken down into four, generalizable groups of cacao fermentation: Yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB), and Miscellaneous (Misc). While Misc is not the formal nomenclature, in this article, Misc are microbes that do not fall into any of the other three categories and are not filamentous fungi. Of note, filamentous fungi (FF) will not be discussed in this paper, as they are outside the scope of this study. While the role of FF is still mostly unknown, currently FF are associated with plant diseases, contamination (Fagbohun et al., 2011), and undesirable mycotoxins (Delgado-Ospina et al., 2021; Mounjouenpou et al., 2008) and thus were left out. To obtain a better understanding of the microbes associated with cacao, this study sought to compile current literature on SCFs and accomplish four main objectives. (i) Identify the studied microbes found in spontaneous cacao fermentation, (ii) identify commonly associated microbes, (iii) correlate microbes to specific countries, regions, or geographic locations, and (iv) explore the microbiome differences of various fermentation methods. Accomplishing these objectives will lay the groundwork for target areas in future research, begin unraveling the key microbes in cacao fermentation, and suggest guidelines for SCF research.

2. Materials and methods

2.1. Search strategy

The systematic review protocol described in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement was adopted for this review process (Moher et al., 2009). A keyword search was performed in PubMed, CABDirect, and Web of Science, along with a hand-search, and a complete bibliographic search after full-text review. The keyword search mainly including “cacao” and “fermentation” and all the variations of each word. All three specific search algorithms can be found in Appendix A1 to A3. Titles and abstracts of articles identified through the keyword search were screened against the researchers’ study selection criteria. Articles, in this paper, will mean any piece of literature secured from the database searches. This could include gray literature, such as dissertations, conference publications, theses, or other non-peer reviewed materials. Articles that matched the selection criteria were then reviewed based on full-text inclusion/exclusion criteria. After full-text review, a cited-reference search (backwards search) was conducted on the approved articles. Reference searches were repeated until there were no additional relevant articles found. The initial search was conducted on February 8, 2020, with the backwards search and hand search occurring on March 27, 2020.

2.2. Study eligibility criteria

Articles that were included in the review met all the following criteria: (i) researchers sampled from SCFs, (ii) the country of origin was mentioned for the SCFs, (iii) the microbial identification method/s was described, (iv) to the best of the researchers’ ability, the microbial species were identified, and (v) articles were written in English.

Articles were excluded from review if they met any of the following criteria: (i) the article only mentioned or used starter cultures and not SCFs; (ii) fermentations were not on-farm or mirrored on-farm conditions; (iii) SCFs were used, but microbiome identification was not performed; (iv) no discernible microbial identification method/s used; (v) country of origin was not mentioned; (vi) focus was only on a targeted microbe or microbes (i.e., identifying novel species as the focus); and (vii) articles were not written in English. Dissertations, theses, books or book chapters, and other non-peer reviewed publications were included.

2.3. Data extraction

A standardized data extraction database was formed and used to collect the following data: title, authors, year, country of origin and possible geographic location, identification methods, times when each microbe was sampled and identified, microbial species, type of microbe, fermentation method or material, and persistence indication. Additional variables were eventually included: cacao genetic species, the weight of the fermentation, duration of the ferment, contamination suspects, depth of sample (box method was most commonly reported), frequency of turning the cacao, genetic database accession numbers or hyperlinks (if applicable) and renamed microbes (for outdated species). Each abstract was independently screened. Once approved, each full-text article was read and screened. After full-text screening, data were extracted.

2.4. Data analysis and visualizations

Analyses of import were ones that focused on identifying relationships for: microbe-to-microbe, microbe-to-geography, and microbe-to-fermentation method. For microbe-to-microbe the master dataset (Taylor, 2022) was converted into a presence-absence matrix based on a variation of the original sampling definition. This definition focused on each row being associated with a unique location, whether city, region, state, or country, and each column being a unique microbe. This presence-absence matrix (Taylor, 2022) uses a binary system of presence (1) or absence (0) if the microbe was identified in an article at a unique, singular location. If an article had four unique locations, then there would be four rows for that article. With this new sampling definition, microbes that were found multiple times within a singular article could be readily quantified and categorized than previous.

Using this dataset, Fisher’s exact tests were chosen to examine microbe-to-microbe interactions, microbe-to-geography associations, and microbe-to-fermentation associations. Each of these above analyses were performed in RStudio v1.3.1093 (RStudio & Team, 2010). Multiple testing correction was performed using the Benjamini-Hochberg procedure for false discovery rate (FDR) control. Microbe-to-microbe interactions with FDR <0.05 were visualized using the R package ggraph. Each of the interactions shown are statistically significant interactions. All other figures and tables were either made in Excel for Office 365 or in Tableau Public Desktop v2020.3 (Tableau, 2019).

2.5. Article quality and bias assessment

A study quality assessment was performed, adapted from Littell et al. (2008), ranking each article on 7 criteria (Taylor, 2022). Scores for each criterion ranged from 0 to 2, depending on whether the criterion was not mentioned or met (0), partially met (1), or fully met (2). The total article score is the sum of the scores for each criterion and ranges from 0 to 14. The overall score did not influence inclusion or exclusion and was only used for quality evaluation.

3. Results

3.1. Literature search

In the initial literature search, 1321 articles (Fig. 1) were identified from various databases including CABDirect (98 articles), PubMed (313 articles), and Web of Science (910 articles). After removing duplicates, 1114 articles remained. Article titles and abstracts were reviewed, and

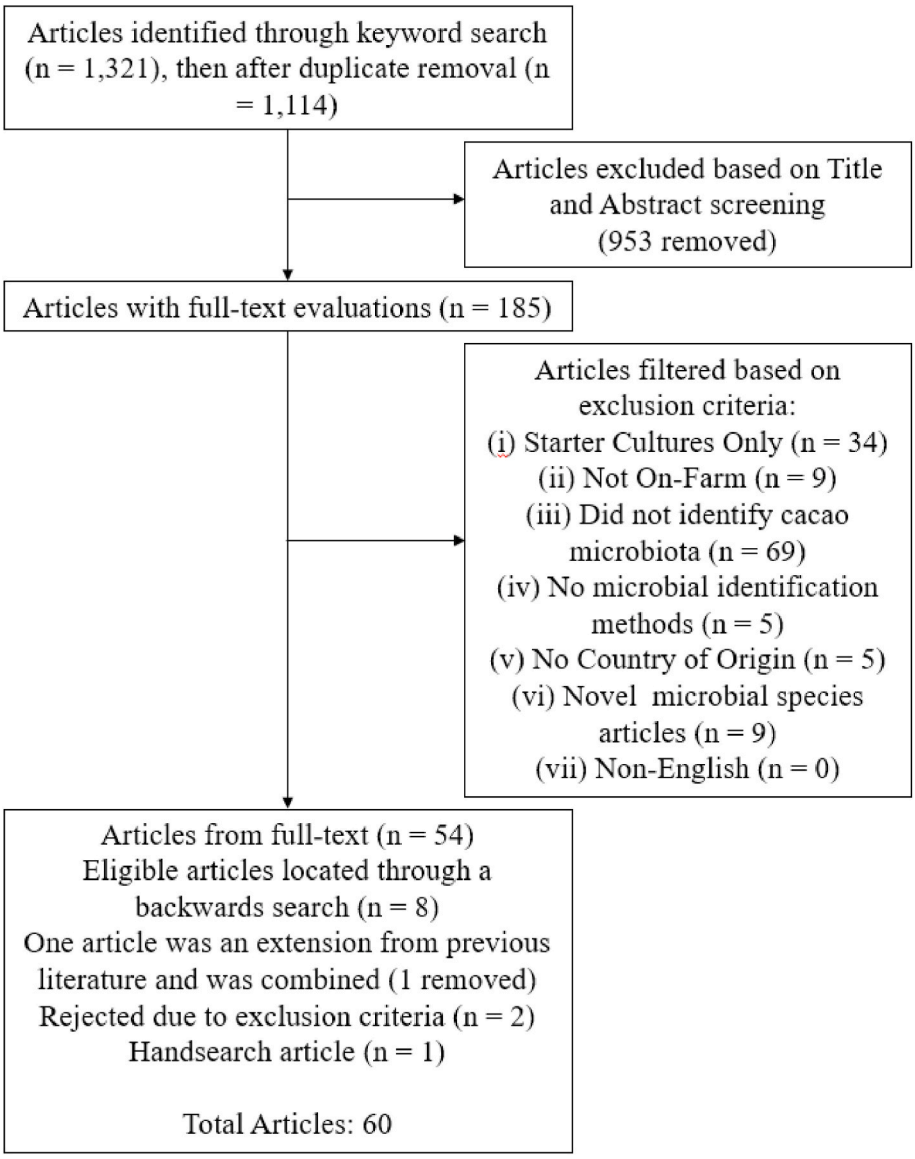

Fig. 1. Flow chart for the collection and exclusion of articles included for review. Articles were first subjected to a title and abstract screening and then a full-text review based on inclusion/exclusion criteria. The bibliography from those articles were scanned for any unique articles in a "backwards search." This was repeated until no new articles were identified.

953 articles were removed, leaving 185 articles. Reviewers conducted full-text reviews, and 131 articles were rejected due to inclusion/exclusion criteria mentioned above. The remaining 54 articles were then data extracted, assessed for study quality, data synthesis, and were backwards searched. From the backwards search, eight new articles were found from the 1016 references. Of those eight, two were rejected due to the inclusion/exclusion criteria. One was an amendment to an already selected article, therefore the two were combined in the data extraction. Therefore, the remaining five were incorporated, bringing the amount, after forwards and backwards searches, to 59 selected articles. Lastly, one article was hand-picked and accepted by the reviewers, due to being published during the timeframe of the backwards search and was not within the three searched databases. In total, 60 articles were selected and are compiled into this review. While some were non-peer reviewed documents, articles will be used for conciseness.

3.2. Study characteristics

The selected 60 articles were compiled for 1757 unique data points. Data points, in this section, will be known as uniquely identified microbial species from a SCF of cacao beans from one location and of one fermentation method. Therefore, there could have been multiples of a microbial species from a single study, depending on the method used and the origin of the beans. In cases where locations and/or fermentation methods were not specified, unique microbial species were only counted once. With each data point the country and, if possible, location of origin, culture-independent (CI) and/or -dependent (CD) methods used, fermentation method, and persistence of the microbes were denoted if known. Persistence, in this article, is to mean one of three ways: 1) Microbes were identified in relatively high counts in at least three continuous time points (as determined by the individual article; e.g., 24, 48, 72 or 12, 24, 36); 2) microbes that were identified in at least three continuous time points, given no count data; or 3) microbes that were sequenced had $\geq$20% of read count data. While this definition is not all encompassing, given the disparity between the articles, it helped

to understand which microbes were more prevalent than others. Both persistent and sporadic microbes were denoted in this data collection of this article. The dataset has been made open-access to all and can be found at the DOI 10.17632/td43wv2jjp.1 (Taylor, 2022).

Basic characteristics of the articles are summarized in Table 1, which include the country of origin, weight of the ferments, cacao cultivars, the duration, and amount of turning of the ferment. Of the 60 articles, multiple countries were represented. The three most prominent countries (Fig. 2) were Brazil (22 articles, n = 612), the Ivory Coast (14 articles, n = 237), and Ghana (10 articles, n = 257). Countries such as Bolivia, Cuba, Dominican Republic, Honduras, Indonesia, Mexico, Nicaragua, Trinidad, and Vietnam all had 1 representative article each (n = 221). Other countries such as Australia (3 articles, n = 86), Cameroon (2 articles, n = 75), Ecuador (3 articles, n = 77), Malaysia (3 articles, n = 84), and Nigeria (2 articles, n = 108) had slightly higher representation.

The weight of the ferments ranged from 0.5 kg to 18,000 kg. As some articles reported the weights in ranges, it is difficult to report an average, median, or a mode for the weight of the ferment. However, the typical range for these articles is between 50 and 100 kg. Moving onto cacao cultivars, ten articles did not mention the weight of the ferment that was sampled. Cacao cultivars varied from "traditional" cultivars (e.g., Criollo, Forastero, and Trinitario) to genetic cultivars (e.g., Amelonado and Nacional) to named or unnamed hybrids and clones. Twelve articles reported either "mixed hybrids" or a hybrid of the aforementioned cultivars. Eighteen articles did not mention the cacao cultivar/s used.

Duration of cacao fermentations typically were between 96 and 144 h, or 4–6 days. Only two articles did not mention the duration of their fermentations. There were some cases where the duration reached up to 8–9 days. There does not appear to be a consistent optimal, singular time across the variables studied in this paper. Since the cutoff point for farmers is indicated by a cut-test, duration does not appear to be associated with any one parameter. Finally, the amount of mixing, which oscillated between either mixing the cacao once a day, once every two days, once a day after some designated time, or never mixing it. Multiple articles did not happen to report the number of mixings (n = 17 articles) or how the fermentation was mixed, if it was. Collecting and standardizing this parameter was difficult as almost every article had a different method of mixing. A few articles that analyzed multiple locations had varying amounts of mixing, based on which location or method was analyzed.

There are a handful of other interesting parameters that could not be easily summarized in Table A. Parameters such as the fermentation methods, microbial identification methods, and types of microbes are the most pertinent. First, the most utilized fermentation method was Wooden Box (38 articles, n = 1042), followed by Heap (24 articles, n = 397). The other methods, such as barrels, plastic containers, platform, steel tank, and tray, were across 14 articles (n = 210). Of note, two articles that identified microbes did not mention the applied fermentation method (n = 108). While some countries tended to use one method over others, no one singular method was unique to a singular country or region.

There is wide variation in the microbial identification methods used in cacao fermentation research. The majority of studies used 16S rRNA and/or 5.8 ITS rRNA sequencing as their CI method of identification. Many articles were not as thorough in their methodology about the primers used, the amplicon library preparation, or data analysis. Some differences are reasonable due to the publication dates of the articles. Without a consistent record of the CI method/s used, the parameters, and how the data was examined, it is difficult to properly confirm or compare the microbial identification methods across the 60 articles. Information for each specific article's CI and CD methods can be found in the Supplemental dataset.

3.3. Microbial characteristics

As aforementioned, there are a total of 1757 unique data points split amongst 17 countries. The specific type of microbe is fairly well spread out (Fig. 3). These data points filter down into 447 unique microbial species. 45 of the 447 are AAB, 85 are LAB, 148 are yeasts, and 170 are Misc. There are some instances (72 of 447) in which the microbe was not identified past the genus level (i.e., *Acetobacter* sp.). There were also some cases (5 of 447) in which two microbial species of the same genus, were not able to be discerned from one another (i.e., *Acetobacter ghanensis/syzygii*). This is likely due to how these microbial species are close relatives to one another but were not yet discernible by CI or CD methods at the time. While the majority of studies looked at all four groupings of microbes, some did not; 11 articles (n = 202) focused on yeasts alone, 7 articles (n = 187) looked at only AAB and LAB together, 4 articles (n = 67) examined only LAB, and 3 articles (n = 36) examined only AAB. Totaling to 492 "stand alone" data points, this adds up to 30.5% of the microbes accounted for in this review.

Meta-analysis results were focused on three key relationships of microbe-to-fermentation method, microbe-to-geography, and microbe-to-microbe. To identify these relationships, the Presence-Absence matrix was analyzed. In the first relationship, it is too hard to discern if microbes can be associated with specific fermentation methods. Nearly half of all the articles, and one-third of the data points, belonged to "Wooden Box" methodologies. With that, most of the Wooden Box articles originated from Brazil. Given that there were too few samples from different countries and from different materials, this relationship was not explored further. For the microbe-to-geography relationship, several microbes were identified that were statistically significantly in the Fisher's exact test. Initially the working hypothesis was that the abundance of particular microbes would be associated with a geographical location (i.e., country). However, there was a heavy bias from Brazil, Ivory Coast, and Ghana and too little from other countries to make a confident claim. Therefore, this relationship cannot be properly explored until other countries have more SCF data.

Before analyzing the last relationship, microbe-to-microbe, only articles that analyzed multiple groups of microbes are able to indicate any connections across groups. While all sixty articles were included in the analysis, some articles only observed individual microbial groups. Therefore, any relationships across the four microbial groups are from a subset and any interconnected relationships are from all articles. Of the sixty articles included in this article, thirty-five observed all four microbial groups. Five articles looked at AAB and LAB, together. All other articles looked at individual groups. The articles are spread over 50 years (Fig. 4). The earliest article within this meta-analysis was 1961; the articles extended to 2020. The majority of articles spiked near the early 2010's, likely due to lower CI costs and increased focus on cacao microbiota research.

Lastly, the microbe-to-microbe interactions were examined. However, as some of the articles only observed particular microbial groups, such as only looking for LAB, there was a reduced number of articles that examined interactions across groups. While all 60 articles were included in the analysis, only two-thirds observed microbe-to-microbe interactions across microbial groups. Despite this reduction, there are 127 statistically significant microbe-to-microbe relationships. These interactions are visualized as a web-network in Fig. 5. The entire network is composed of eight yeast species, eight AAB species, eleven LAB species, and twenty-six Misc species. Within these categories, yeasts only seem to have significant interactions with themselves. *Candida orthopsilosis* and *Pichia kluyveri* are the only yeasts that have more than one interaction. There are two particular AAB species that are well connected, *Gluconacetobacter hansenii* (n = 12 connections) and *Acetobacter sicerae* (n = 4). Whereas there are four major LAB microbes, *Weissella fabaria* (n = 11), *Lactobacillus cacaonum* (n = 9), *Leuconostoc* sp. (n = 9), and *Lactobacillus pontis* (n = 5). Lastly, in the Misc category, these had the most connections. *Halomonas* sp., *Lysinibacillus halotolerans*,

Table 1
A comprised table of condensed information for all of the articles included in this manuscript, their country of origin, microbial identification methods, and cacao fermentation parameters.

Study, year	Country of Origin	Microbial Identification Methods	kg of Ferment	Cacao cultivars[a]	Duration	Frequency of turning
Agyirifo et al., (2019)	Ghana	Shotgun metagenome sequencing	250	C, F	96h	–
Almeida et al., (2019)	Brazil	Culturing & PCR	70	F	144h	Once a day after 48h
Arana-Sanchez et al., (2015)	Mexico	PCR-RFLP, PCR-DGGE	0.5	C, F, T	192–216h	Once every 24h
Ardhana & Fleet (2003)	Indonesia	Culturing	1000	F, T	F - 144h, T - 96h	Once every 24h
Balogu & Onyeagba (2017)	Nigeria	Culturing	15	Amelonado	144h	Once a day after 16h
Bastos et al., (2018)	Brazil	Culturing and PCR	125	TSH565	144h	Once a day after 48h
Batista et al., (2016)	Brazil	PCR	100	PS1319	168h	–
Bortolini et al., (2016)	Ghana, Ivory Coast, Cameroon	PCR	–	–	144–168h	Once after 48h or turned twice overall
Camu et al., (2007)	Ghana	Culturing and PCR	250–1000	C and F	144h	Never
Camu et al., (2008)	Ghana	Culturing and PCR	150	C and F	144h	Either never turned or twice at 48h & 96h
Crafack et al., (2013)	Ghana	Culturing and PCR	20	–	120h	–
da Cruz Miguel et al., (2017)	Brazil	Culturing and Mass Spectrometry	100	PH16	156h	–
da Veiga Moreira et al., (2013)	Brazil	Culturing and PCR	60	PH 9, 15, & 16	144h	–
da Veiga Moreira et al., (2016)	Brazil	Culturing and PCR	60	PH 9, 15, & 16	144h	–
da Veiga Moreira et al., (2017)()	Brazil	PCR	100	PH 15	144h	–
Daniel et al., (2009)	Ghana	Culturing and PCR	250–1000	C and F	144h	Never
de Camargo et al., (1963)	Brazil	Culturing	–	–	120–198h	–
de Melo Pereira et al., (2012)	Brazil	Culturing and PCR	20	–	144h	Once every 24h
de Melo Pereira et al., (2012)	Brazil	Culturing and PCR	600 and 40	–	144h	Once every 24h
Dircks (2009)	Australia	Culturing and PCR	75 (heap), 75 (box), 75 (barrel)	T	120h	Heap (twice after 48h), Box and Barrel (once every 24h)
Fernández Maura et al., (2016)	Cuba	Culturing and PCR	10 kg, 20 kg, and 12 & 18 metric tons	T, UF clones, n.a.	168h	Once a day after 72h
Galvez et al., (2007)	Dominican Republic	Culturing	100	T or T hybrids	144h	–
Garcia-Armisen et al., (2010)	Ghana & Brazil	PCR	70	F	144h	–
Hamdouche et al., (2015)	Ivory Coast	PCR	80	–	144h	Once every 48h
Hamdouche et al., (2019)	Ivory Coast	PCR	30	–	168h	Either turned never or twice over at 48h and 96h
Ho, Zhao and Fleet, (2014)	Australia	Culturing and PCR	5	T	144h	Once every 48h
Ho, Zhao and Fleet, (2015)	Australia	Culturing and PCR	5	T	144h	Only once
Illeghems et al., (2012)	Brazil	Pyrosequencing	1500 and 1850	C & F hybrids	120h	Once per 24, after 48h
Koné et al., (2016)	Ivory Coast	Culturing & PCR	30	Mixed varieties	168h	Once at 24h & at 48h
Kostinek et al., (2008)	Nigeria	Culturing	–	–	144h	–
Lee et al., (2019)	Ecuador	Culturing & PCR	1.2	C	144h	–
Lefeber et al., (2011)	Ivory Coast	Culturing & PCR	20	–	144h	–
Martelli & Dittmar (1961)	Brazil	Culturing	–	F	–	–
Meersman et al., (2013)	Malaysia	Culturing & PCR	544, 640 (box), 292, 590 (heap)	–	144h	B1 & H1: Once at 48h B2 & H2: Once at 48 & at 96h
(Miescher Schwenninger et al., (2016))	Bolivia, Brazil	Culturing & MS	1500 & 850, 3 & 12 (Brazil)	F	240h	Once every 24h
Mota-Gutierrez et al., (2018)	Cameroon	Culturing & PCR	200	F hybrid	120h	Once at 48h & at 96h
Nielsen et al., (2005)	Ghana	Culturing & PCR	200-1000 (heaps), 100 (tray)	Mixed hybrids	144h	Mampong - 48 & 96h; New Tafo - Never
Nielsen et al., (2007)	Ghana	Culturing & PCR	50-750 (heap), 100 (tray)	Mixed hybrids	96h (tray), 144h (heap)	Small heap, Never; large heap, 48h & 96h
Nielsen et al., (2008)	Ghana	PCR		Mixed hybrids		

(*continued on next page*)

Table 1 (*continued*)

Study, year	Country of Origin	Microbial Identification Methods	kg of Ferment	Cacao cultivars[a]	Duration	Frequency of turning
			50-750 (heap), 100 (tray)		96h (tray), 144h (heap)	Small heap, never; large heap, 48h & 96h
Ostovar & Keeney (1973)	Trinidad	Culturing	–	–	168h	Once at 72h & at 120h
Ouattara et al., (2017)	Ivory Coast	Culturing & PCR	50	–	144h	–
Ouattara et al., (2019)	Ivory Coast	Culturing & PCR	50	–	144h	–
Papalexandratou and De Vuyst, (2011)	Ivory Coast, Brazil, Ecuador, & Malaysia	Culturing & PCR	–	–	Brazil (144), Ivory Coast (150), Ecuador (96), Malaysia (120)	Ivory Coast box (24, 48h); Brazil box (54, 76, 96, 120h), Brazil box 2 (48, 72, 96, 120h); Ecuador platform (50, 72h), Ecuador box (24, 72h), Ecuador platform 2 (54, 72h); Malaysia box (48, 96h)
(Papalexandratou et al., 2011a)	Ivory Coast & Brazil	PCR	150 (Heap), 1200 for Box	C & F hybrids	150h (Ivory Coast) & 144h in Brazil	–
(Papalexandratou et al., 2011b)	Ecuador	Culturing & PCR	100 (Platform), 100 (box)	Nacional x T hybrids	96h	–
Papalexandratou et al., (2011)	Brazil	Culturing & PCR	1500 & 1850	C & F hybrids	120h	1500: Once a day after 48h 1850: Once a day after 48h
Papalexandratou et al., (2013)	Malaysia	Culturing & PCR	500	Mixed hybrids	120h	Once at 48h & at 86h
Papalexandratou et al., (2019)	Nicaragua	Culturing & HTS	Varies by the variety of cacao	Chuno, Rugoso, Nicalizo, Johe, & Nugu	120–140h	Once every 24h
Passos et al., (1984)	Brazil	Culturing	–	–	144h	24, 48, 96 & 144h
Pereira et al., (2013)	Brazil	PCR & EM	600	Mixed hybrids	144h	Once every 24h
Ravelomanana et al., (1985)	Ivory Coast	Culturing	–	–	–	–
Romanens et al., (2018)	Honduras	Culturing & MS	1 kg (LS) & ~300 kg (OF)	Hybrids (IMC-67, UF-29, UF-668)	120h	Once every 24h
Samagaci et al., (2016)	Ivory Coast	PCR	50	F, C, & T	144h	–
Schwan, (1998)	Brazil	Culturing	200	Comum	168h	Once every 24h
Serra et al., (2019)	Brazil	Culturing & HTS	–	–	144h	Once a day after 48h
Soumahoro et al., (2015)	Ivory Coast	Culturing	100	F, T, & C mixed	144h	–
Soumahoro et al., (2020)	Ivory Coast	Culturing & PCR	50	–	144h	–
Tavares Menezes et al., (2016)	Brazil	PCR	100	CCN51, PS1030, FA13, CEPEC 2004	168h	Once a day after 48h
Thanh Binh et al., (2017)	Vietnam	Culturing & PCR	–	F & T	144h	Twice daily
Visintin et al., (2016)	Ivory Coast	Culturing & PCR	50 (Heap) & 1600 (box)	F hybrid	144h	Once at 48h & at 96h

a. C, F, and T are abbreviations for Criollo, Forastero, and Trinitario.

Mycobacterium sp., and *Paenibacillus pabuli* all had 17 connections. While the Misc microbes dominated the network, several of the connections are primarily interconnected within the Misc group. In particular, the four main Misc microbes aforementioned are these interconnections. Supplemental Figure B1, B.2, and B.3 show the interconnections within the three microbial groups. All of the significant microbial connections and their p-values are included in the accompanied dataset.

4. Discussion

This systematic review and meta-analysis of SCFs revealed key insights into the cacao fermentation microbiome. While this is by no means a complete identification of the cacao microbiome, this is a key step in identifying the microbes associated with SCFs and their relationship. This systematic review has helped identify connections between microbes seen in SCFs. However, before discussing the importance of this work, there are some biases that must be recognized. First, the majority of articles and data came from Brazil. Likely, as Brazil increased its cacao farming and a handful of research groups were able to experiment with this rising cacao industry. While these articles are able to contribute to identifying microbes associated with SCFs, it is difficult to test the hypothesis of different countries having different microbiomes. Second, there is an unfortunate lack of data from many cacao-producing countries. Colombia, Peru, Papua New Guinea, Uganda, Venezuela, Togo, India, Sierra Leone, Haiti, Guatemala, and Madagascar are all in the top twenty cacao-producing countries (Food and Agriculture Organization Statistics, 2017; International Cocoa, 2018; World Population, 2019), yet do not have a single article that meets the inclusion criteria of this review. Third, the authors recognize that the definition used in this article for an identified microbes may not fit a traditional microbial ecology definition, due to the variance in identification methods. In any case, this definition was optimal to connect all 60 articles.

4.1. Fermentation parameters

Another facet of SCF research, found by this review, is the inconsistent reporting of fermentation parameters. There were several parameters that the authors considered important based on previous literature, such as turning frequency (N. Camu et al., 2008; Guehi et al., 2010a), fermentation method material (Figueroa-Hernandez et al., 2019), weight of the ferment (Hernandez-Hernandez et al., 2016), and location of origin. In several cases, these parameters were not provided. Even if the researchers are unable to accurately report these parameters, mentioning that they are unknown is useful information for transparency and future research. For example, several articles reported

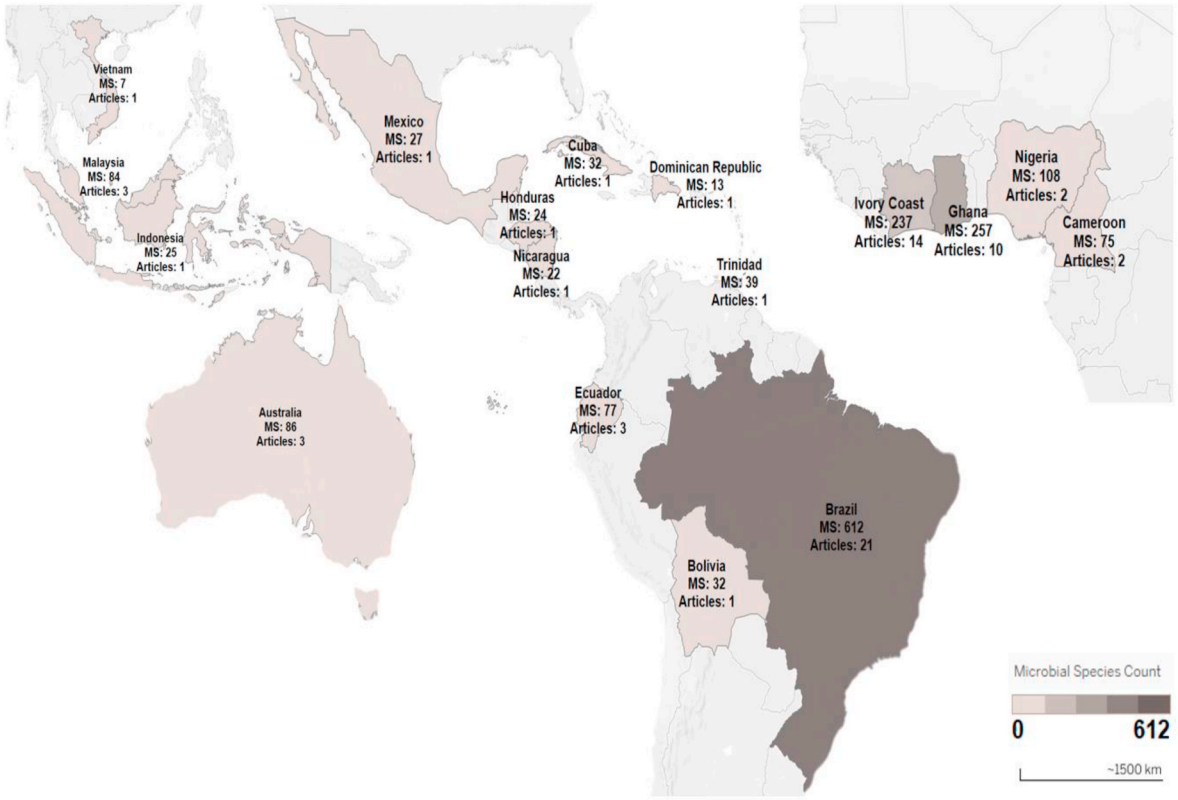

Fig. 2. Heatmap of the countries identified by the sixty collected articles in this systematic review. No color means that the country was no representation in the sixty articles. The heatmap is number of microbial species (MS) identified from the papers of those countries. Under the MS are the number of articles that sampled from SCFs in those countries.

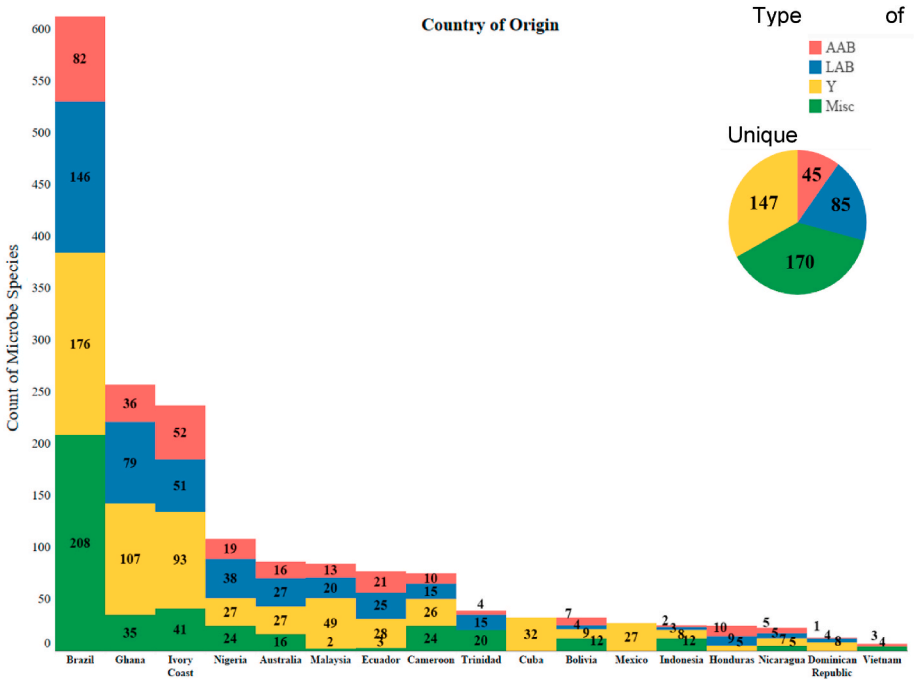

Fig. 3. A histogram of the number of microbial species that has been split into one of the four groupings: Acetic acid bacteria (Red), yeasts (Yellow), miscellaneous (Blue), and Lactic acid bacteria (Teal). The number corresponds to the total identified microbial species. The pie chart is of the unique microbial species found across all sixty articles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

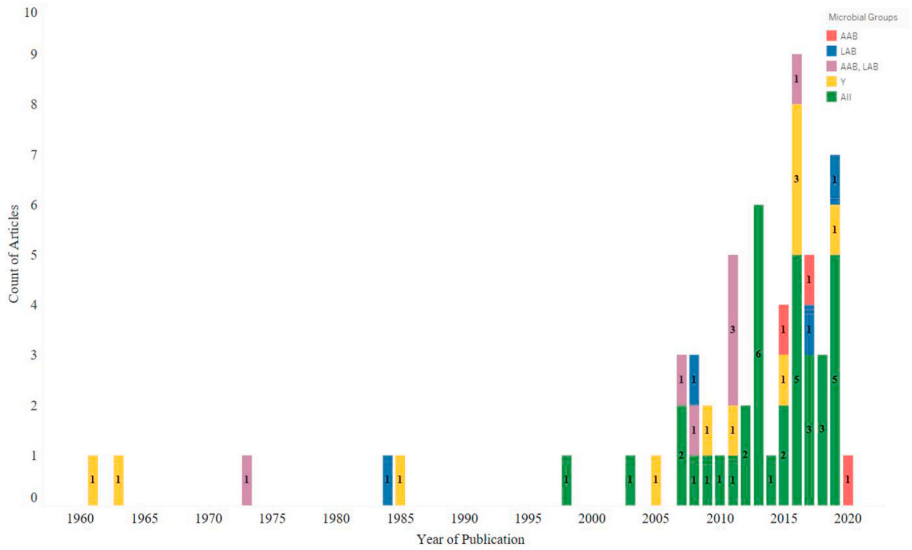

Fig. 4. Stacked bar chart that displays the timeline range for the articles included in this systematic review. The colors correspond to what type of microbes the article observed. Yeast only (Yellow), acetic acid only (Red), lactic acid bacteria (Blue), acetic acid and lactic acid bacteria only (Purple), and all four groups (Green). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

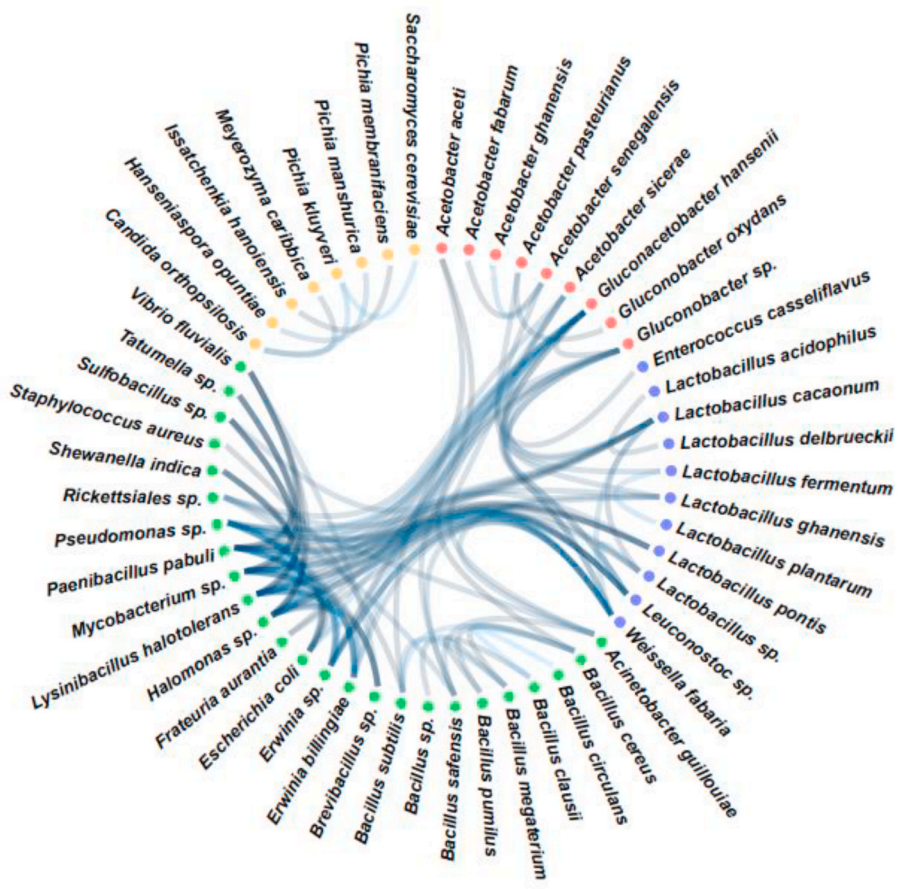

Fig. 5. A microbial web network that shows statistically significant relationships between the two connecting microbes. These relationships are based on a multiple testing correction performed using the Benjamini-Hochberg procedure for false discovery rate (FDR) control, with FDR value < 0.05. The colors indicate the type of microbe, acetic acid bacteria (Red), lactic acid bacteria (Blue), yeasts (Yellow), and miscellaneous (Green). The darker the line is, the lower the p-value is under 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

numerous sampled locations, but would combine all of the samples or report as a conglomerate of their locations. The authors of this article would like to recommend the following parameters and possible ways to report them, to be consistent and transparent.

4.2. Identification methods

Microbial identification methods are difficult to standardize, due to availability of resources (Agyirifo et al., 2019; Z. Papalexandratou, G. Falony, et al., 2011). Thus, the authors cannot advocate for any one particular method of CD or CI method. However, there was a noticeable

issue with CI methodologies: A lack of publicly available genomic datasets and unreported methodologies for data cleaning steps, primers or primer sequences used, and/or the platform or devices used for DNA/RNA analysis. While CI methods have inherent biases associated, they were rarely mentioned or acknowledged. The dataset gathered for this review and meta-analysis would be difficult to analyze or re-confirm due to these inconsistencies, especially for microbes only identified at the genus level only. As genomic tools become more economically available, researchers will be seeking for previous methodologies. By setting the foundation now, there will be better results in the future.

4.3. Cacao species

Accurately reporting from the vast number of cacao varieties is an extremely difficult, yet vital task. The genetic variety of cacao can determine key factors like yield, fat content, fatty acid composition (Mustiga et al., 2019), pulp amount (Afoakwa et al., 2011), resistance to diseases (DuVal et al., 2017), and the "fine flavor" (Aprotosoaie et al., 2016; Castro-Alayo et al., 2019). However, with how easily cacao can hybridize, there are a large number of unknown varieties or farms without access to the genetic background of their cacao crops. There is also the matter of the nomenclature not being standardized. With three different nomenclatures appearing in literature, Traditional, Genetic, and Clonal, identifying the cacao cultivar quickly becomes complicated. In any capacity, as SCF research continues forward, careful consideration must be given to reporting the genetic variety of the cacao.

4.4. Fermentation methodology

One objective of this meta-analysis was to identify if various fermentation materials support or prevent specific microbial communities. This does not seem to be the case with heap or wooden box fermentations. These two fermentation methods were the most popular, but microbes did not have a connection to either fermentation method. This may be due to several different microbial species or because they are SCFs. Fermentation methods do seem to have a direct comparison to quality. Some researchers reported that cacao fermented in wooden boxes and turned, yield more fully-fermented, brown beans (Ale et al., 2018; Guehi et al., 2010a). Whereas other articles claim that there may not be a difference between material types (Hernandez-Hernandez et al., 2016; Mbonomo et al., 2016). The box method may affect pH values and the tannin and sugar levels; whereas the heap method may have a more homogenous mixture (Guehi et al., 2010b). It is unclear, currently, what influence the fermentation material plays in SCF, other than being a vessel (de Melo Pereira, Magalhães, de Almeida, da Silva Coelho, & Schwan, 2012a; de Melo Pereira, Miguel, Ramos and Schwan, 2012b; Ganeswari et al., 2015; Hernandez-Hernandez et al., 2016; Visintin et al., 2016). With the types of vessels, there is also the weight of the ferments. There has been previous discussion on the weights influencing quality or the microbial composition (de Melo Pereira, Magalhães, et al., 2012; Hernandez-Hernandez et al., 2016; Owusu et al., 2013). The papers collected in this systematic review further this debacle. However, none of the 60 articles specifically looked at evaluating quality, microbial composition, and the corresponding ferment weights.

One important point is that mixing promotes growth of LAB and AAB and lends a notably acidic profile to the cacao (N. Camu et al., 2008; De Vuyst and Weckx, 2016; Y. Hamdouche et al., 2019). However, the reasons why some farmers choose to not turn are unknown. Some articles have suggested designed fermenters for controlling the turning process (de Melo Pereira, Magalhães, et al., 2012; Hatmi et al., 2015; Koffi et al., 2017). These fermenters could help aerate and better control LAB and/or AAB. Given how diverse processing cacao is, surveying and working with farmers can help to better understand their particular methodologies and motivations.

4.5. Microbe-to-microbe interactions

Microbial interactions can have several important implications for cacao quality. Before discussing implications, the authors recognize some key factors. As aforementioned, there was a reduction in the number of articles included for this section of the analysis. As a result, there were no significant interactions between yeasts and other microbial groups seen in this study. While current literature has shown how yeasts can influence the subsequent microbial succession (Batista et al., 2016; ; Mota-Gutierrez et al., 2018; Pacheco-Montealegre et al., 2020), it may be that certain yeast species promote growth with each other. Original expectations were that there would be a greater number of interactions between yeasts and some LABs, as the first microbial stage and their simultaneous growth during the anaerobic phase (De Vuyst and Leroy, 2020). It is also possible that there are other non-statistically significant relationships between yeasts and other microbial groups. The authors do recognize that yeasts do have an impact on microbial succession, but the data from this meta-analysis suggests that there is a significant relationship within yeasts mainly.

Another limitation of this paper is a lack of quantitative microbial count data. As these interactions are based on presence-absence, it would be pertinent to overlay these results with microbial load data. This requires extremely accurate data from CI and CD methods (Mota-Gutierrez et al., 2018), and even then, it may still not fully depict the interactions. A possible way to truly identify if these microbes influence one another would be with starter cultures. While there is an abundance of starter culture research in cacao (Figueroa-Hernandez et al., 2019), the data presented here may help influence which microbes to choose, based on their relationships or even on the lack of one. In either situation, the usage of starter cultures can help answer microbial load dynamics and interactions, throughout the entire fermentation.

Another type of data, that would be relatively important, are definitive chocolate sensory qualities. Understandably, many researchers do not pursue sensory data after fermentations, due to the complexity of cacao flavor and aroma (Aprotosoaie et al., 2016; Engeseth and Ac Pangan, 2018; Z. Papalexandratou et al., 2019). However, connecting specific microbes or microbial groups and sensory properties may indicate how certain microbes impact the sensory profiles of the resulting chocolate. In particular, identifying and reporting strain-specific microbes is the key to linking microbes and VOCs or flavor/aroma profiles (T. Lefeber, Papalexandratou, Gobert, Camu and De Vuyst, 2012; Z. Papalexandratou et al., 2019). Recently, some farmers have actually been avoiding using any sort of fermentation at all.

As of this publication, there is not a significant amount of literature on unfermented cacao beans. Unfermented, dried cacao beans are storable, transportable, and can be fermented by rehydrating them with a simulated pulp media (Lee et al., 2019; Racine et al., 2019; Schlüter et al., 2020). This allows for more research to be conducted at lab- or pilot-scale. However, microbes that survive the drying process may not directly mirror those found on-farm or cooperative. Some research has shown that unfermented cacao does not contain the same aroma precursors and flavor profiles as fermented cacao (Fang et al., 2020; Juan Manuel Cevallos-Cevallos and María José, 2018). In either case, to better understand cacao fermentations, more research is needed at both the on-farm and lab-scale levels.

5. Conclusion

The number of microorganisms associated with spontaneous cacao fermentations is quite large. By identifying and compiling these microorganisms with key fermentation parameters, researchers can start to understand how impactful cacao fermentation is on cacao quality and flavor profile. In this article, the authors set out to identify if there were possible relationships between the microbes of SCF and specific locations, SCF microbes and fermentation materials, or between SCF

microbes. While the first two relationships are not obtainable right now, there are statistically strong relationships between some SCF microbes. Overall, research on SCFs needs to accurately report fermentation parameters and the identified microbes. Future research should also expand into regions and nations other than Brazil, Ivory Coast, and Ghana. Lastly, the connection between SCFs and sensory aspects of cocoa liquor or chocolate should be explored to test the idea that different microbes can make different flavor profiles.

CRediT authorship contribution statement

Alexander J. Taylor: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization, and, Reviewing. **Eduardo Cardenas-Torres:** Formal analysis, Software, Data curation, and, Visualization. **Michael J. Miller:** Writing – review & editing, Supervision, and, Resources. **Sihai Dave Zhao:** Formal analysis, Software, Data curation, Visualization, and, Supervision. **Nicki J. Engeseth:** Writing – review & editing, Supervision, Project administration, and, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix

A. Systematic Review Formulae

A.1 CABDirect.
((cocoa) OR (cacao) OR (cocoa bean*) OR (cacao bean*) AND (fermentation))

A.2 PubMed

((((“cacao"[MeSH Terms] OR “cacao"[All Fields]) OR (“chocolate"[MeSH Terms] OR “chocolate"[All Fields] OR “cocoa"[All Fields] OR “cacao"[MeSH Terms] OR “cacao"[All Fields])) OR (cocoa bean[All Fields] OR cocoa beans[All Fields])) OR (cacao bean[All Fields] OR cacao beans[All Fields])) AND (“fermentation"[MeSH Terms] OR “fermentation"[All Fields])

A.3 Web of Science

TS = (cacao bean* OR cocoa bean* AND fermentation), English Only.
B. Figures B1 to B.3.

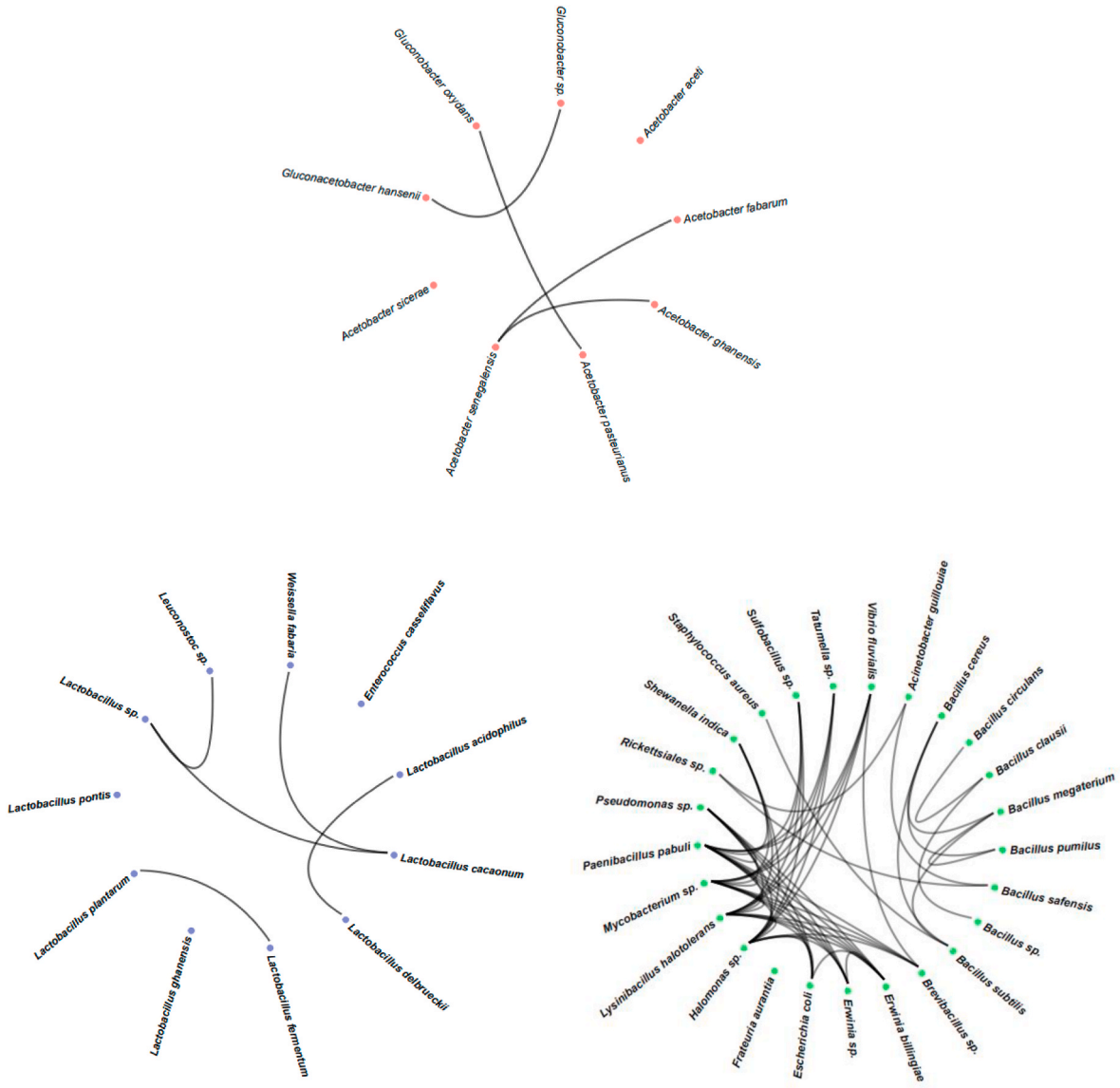

Fig. B.1. to B.3. An expansion of Fig. 5 that has been separated to clearly see the intrarelated relationships within each microbial group.

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