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Article

Improvement, Standardization, and Validation of A Novel Genomic DNA Extraction Method for Human Breastmilk

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Abstract: Recent advances in DNA sequencing technology have shown that the human milk microbiota of healthy women varies substantially. The gDNA extraction method may influence the observed variation, biasing the microbiological reconstruction after all. In this study, a genomic DNA extraction method for DNA isolation from human milk samples was standardized and compared with commercial and standard hose make methods. Spectrophotometric measurements, gel electrophoresis, and PCR amplifications were used as criteria for evaluating the quantity, quality, and functionality of the extracted DNA. Furthermore, the standardized method of extracting gDNA from human milk was evaluated for its ability to isolate functional DNA from gram-positive, and gram-negative bacteria and fungi, to improve the reconstruction of microbiological profiles. The novel DNA extraction method increased the quantity and quality of the gDNA extracted compared with commercial and standard house-make protocols. This method even allowed PCR amplification of the V3-V4 regions of the 16S ribosomal gene in all samples, and the ITS-1 region of the fungal 18S ribosomal gene in 95 % of the samples as well. It is concluded that the proposed method provides better performance for the extraction of gDNA from complex samples such as human milk.

Keywords: human milk; DNA extraction; DNA quality, microbiota

1. Introduction

In metagenomics studies the objective is to understand the ecological structure of the communities present in a given habitat, therefore it is important to consider the characteristics of the sample since the high variability of conditions will require methodological considerations to ensure that the sampling, processing and DNA extraction will accurately reconstruct the identity and abundance of the microorganisms present in the sample, which will reflect the biotic and abiotic interactions of the habitat [1].

Human milk (HM) is the first source of nutrients, bioactive factors, enzymes, and antibodies necessary for the newborn's survival during the first six months of life [2]. Previously this secretion was considered sterile [3], however recent research with massive sequencing tools evidences a complex community of bacteria [4–7], fungi [8–11] and archeobacteria [12], either commensalistic and/or mutualistic, whose presence [13] and interaction [14] are related to the healthy establishment of the microbiota in infants [15].This set of microbial communities is referred as the human milk microbiota (HMM). The composition of the HMM of healthy women reported in sequencing-based studies varies substantially [14,16–24], this may be related to inherent characteristics of the sample type, but

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it has also been reported that the method of collection, storage [25–27], and processing [28] may influence the reconstruction of taxonomic profiles. It has also been reported that the method of DNA extraction influences the taxonomic results [26,28,29].

In addition to the complexity of the matrix (highly rich in proteins and lipids), it is also challenging to consider the low amount of biomass [30] and the high diversity of HMM (gram positive and gram-negative bacteria, fungi, and archaea), whose differences in the composition of the cell wall makes some species more difficult to lyse than others [31]. Inadequate lysis of these groups may result in a biased representation of the microbial community present in the HM samples [25,31,32]. Certainly, these characteristics can interfere with the gDNA isolation process, making human milk a relatively difficult media for high-quality DNA extraction. The methods usually used to extract DNA from HM have low efficiency in terms of quantity and quality [25,29,31] which impact directly in PCR down-stream applications, such as high-quality sequencing, showing the need for optimized microorganism DNA extraction protocols for human milk.

Hence, the aim of this study was to standardize a gDNA extraction method for HM samples and compare its efficiency over other metagenomic DNA extraction methods by comparing the quantity and quality of the isolated DNA, as well as compatibility with downstream applications such as PCR. Our study also evaluates the ability of the HM standardized method to extract bacterial and fungal gDNA from HM samples, culture dependent microorganisms, and effects of processing sample over gDNA quantity and quality as well.

2. Materials and Methods

2.1 Human milk sampling and sample processing

The collection was carried out in the obstetrics service in the prenatal and puerperium care office civil hospital "Fray Antonio Alcalde" (HCFAA) of the University of Guadalajara in Jalisco, Mexico. Human milk (HM) samples were collected from healthy lactating women residing in the Metropolitan Area of Guadalajara Jalisco Mexico, after their informed consent was obtained. The sample collection was performed as follows, the women were asked to wash their hands and put on sterile gloves, disinfect with a 5 % chlorhexidine solution the aureole and nipple, then discard manually by hand expression the first few drops (0. 5-1 mL). After a second cleaning with a 5 % chlorhexidine solution, 20 to 50 mL of HM were collected with an electric breast pump, stored in pre-sterilized collection bags (Lansinoh®), labeled, and registered. After collection, the samples were transported on ice and aliquoted with a total of 5 mL per sample. HM aliquots were pelleted by centrifuging at 4,500 rpm, 4 °C, 20 min. A layer of fat was removed along with supernatant after cell pelleting. The cell pellet was washed with TE (10 mM tris-HCl:1 mM EDTA, pH 8) and concentrated again at 13,000 rpm, finally the cell pellet was resuspended in 300 µL TE and processed (fresh) or stored at −20 °C until further processing (freezing/thaw cycle). The women who participated in the study were informed and the guidelines for informed consent and confidentiality were followed in accordance with the provisions of the Federal Health Law on Health Research [33], title 2, chapter I, article 17, section I, minimal risk research, always taking care of the integrity of the donors, and taking into account the ethical criteria established by the research ethics committee of the HCFAA of Guadalajara, as well as the safeguarding of the personal information of the donors, as stipulated by the Federal Law for the Protection of Personal Data [34], through official letter No. HCG/CEI-0907/22 with Record 141/22 dated 8th June, 2022.

2.2 Bacteria and yeast growth conditions

Twenty-three type strains were chosen in this study. Ten of them are gram-negative, nine are gram-positive and four are yeasts, so different types of cell wall architecture were represented, emulating a high diversity of microorganisms as found in MH samples. The species and culture conditions used are shown in Table S1. Bacteria were plated on the

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corresponding culture medium with agar, after confirming gram staining, isolated colonies were grown aerobically or anaerobically at 37 °C for 16-18 h in 20 mL of culture broth. For yeast strains cells were plated on PDA and grown aerobically at 30 °C for 48 h. Broth cultures were harvested at the end of the exponential growth phase by centrifugation at 4500 rpm for 20 min. while yeast cells were collected from plates. All cell pellets were resuspended in TE buffer and stored at −20 °C until further processing.

2.3 Cell counting and preparation of the mock community

The cells of type strains that readily cultivated in liquid medium were collected by centrifugation and then re-suspended TE buffer on ice. All yeast cells were collected from plates and re-suspended in TE buffer on ice. The cell density of each type of strain was determined by spectrophotometry at 600 nm (spectrophotometer Thermo Fisher Scientific Waltham, MA, USA). We adjusted 1 mL of the cell density (O.D) of each type of strain to 0.474 ± 0.015(~3.198 cells) by diluting with TE buffer. Aliquots were pelleted by centrifuging at 13,000 rpm, 4 °C, 10 min, supernatant was removed after cell pelleting and concentrated in 300 µL TE buffer. Finally, cell suspensions were placed in microcentrifuge tubes and processed (fresh) or stored at −20 °C until further processing (freezing/thaw cycle).

2.4 DNA extraction methods

Four different DNA extraction methods were evaluated using cell pellets described before, obtained from individual milk samples. Previously, these methods and/or modifications have been used for gDNA extraction in metagenomic studies of human milk [10,20,35,36]. Table S2 briefly illustrates the main differences among extraction methods.

2.4.1 Zymo-method (ZYMO)

Human milk DNA was extracted from the previous pellets using the commercially available kit Quick-DNA™ Fecal/Soil Microbe Kit from ZYMO research (Zymo Research Corp., Irvine, CA, USA), subsequently called ZYMO, following the manufacturer's instructions with no modifications. The milk pellets are added directly to a ZR Bashing-Bead™ Lysis Tube (0.1 & 0.5 mm) and lysed by bead beating. Zymo-Spin™ Technology is then used to isolate the DNA in the presence of high salt concentrations of chaotropic salt, which is subsequently filtered to remove humic acids/polyphenols that inhibit PCR. Isolated DNA was then stored at -20 °C until further processing.

2.4.2 Guanidinium-thiocyanate method (GTC)

It was an in-house developed protocol based on the lysing and nuclease-inactivating properties of a chaotropic agent, guanidinium thiocyanate (GTC). Briefly, human milk cell pellets were resuspended in lysis buffer(4 mL of 4 M guanidine thiocyanate-Tris 0.1 M [pH 7.5]) and 600 mL of 10 % N-lauroyl sarcosine). Grind with liquid nitrogen and transfer 250 µL of the material to a micro centrifuge tube. 500 µL of 5 % N-lauroyl sarcosine 0.1 M phosphate buffer (pH 8) were added and incubated at 70 °C for 1h. A 750 µL of 0.1 mm diameter silica beads previously sterilized and 15 mg of polyvinylpyrrolidone were added to the tube and it was vortexed for 10 min, then it was centrifuged for 3 min at 12,000 rpm at room temperature. Supernatant was recovered and the sediment was washed with 500 µL of TENP (50 mM Tris [pH 8], 20 mM EDTA [pH 8], 100 mM NaCl, 1 % polyvinylpyrrolidone), centrifuged for 3 min at 12,000 rpm and the new supernatant was added to the previous one. The washing step was repeated 3 times. Pooled supernatants were centrifuged briefly to remove particles and divided into two 1.5 mL tubes. Nucleic acids were precipitated by adding 1 volume of isopropanol for 10 min at room temperature and centrifugated for 15 min at 13,000 rpm at 4 °C. Pellets were resuspended and pooled in 450 µL of 100 mM phosphate buffer pH 8 and 50 µL of 5 M potassium acetate, placed on ice for 90 min and centrifugated at 13,000 rpm for 30 min at 4 °C. The supernatants were transferred to new tubes with 3 µL of RNAase-free DNAase (Promega) per reaction (10 U/µL) and were incubated at 37 °C for 30 min. 50 µL of 3 M sodium acetate and 1 mL of

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absolute ethanol were added and incubated for 10 min at room temperature. Then they were centrifugated at 13,000 rpm for 15 min at room temperature. The DNA pellets were washed with cold 70 % ethanol and centrifugated at 13,000 rpm for 5 min at room temperature, dry and resuspended in 40 µL of TE buffer. Isolated DNA was then stored at -20 °C until further processing.

2.4.3 CTAB-based method with doubled phenol extraction (CTAB-DPH)

Genomic DNA was extracted from 5 mL of human milk of each sample by CTAB-based method according to William et al., [37] with slight modification. Human milk pellets were thawed and mixed with 100 µL of TE buffer, plus 20 µL of lysozyme (4 U/µL), and incubated for 30 min at 37 °C. Then 40 µL of 10% Sodium Dodecyl Sulfate (SDS) and 8 µL Aspergillus oryzae proteinase (1 U/µL) were added. The samples were incubated for 3 hours at 56 °C. Then, 100 µL of NaCl 5 M and 100 µL of Cetyltrimethylammonium bromide buffer (CTAB/NaCl: 100g CTAB/L, 0.7 M NaCl) and incubate at 65 °C for 10 min. The samples were extracted twice with 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifugated to 13,000 rpm for 10 min at room temperature. The upper phase was transferred to a new tube and 500 µL chloroform:isoamyl alcohol (24:1) was added and centrifugated to 13,000 rpm for 10 min at room temperature. The aqueous phase was transfer in a new tube and 0.6 vol of cold isopropanol was added. Samples were incubated overnight (16 h) and centrifugated at max speed for 15 min at 4 °C. Pellets were washed with cold ethanol (70 % v/v)and spin at max speed for 5 min. The supernatant was discarded, and the pellets were dry at room temperature. Pellets were eluted in 40 µl of DNAase-free water. RNAase treatment was performed as follow: DNA resuspended was mixed with 4 µL RNAase-free DNAase (Promega) per reaction (10 U/µL). Then the mixture was incubated at 37 °C for 1 hr. The inactivation enzyme was at 70 °C for 15 min and cool down on ice. The precipitation was performed adding sodium acetate (1/10 volume 3 M) and 2.5 volumes of absolute ethanol to each sample. Samples were mixed gently and placed at -20 °C for 2 hrs. Samples were centrifugated to 13,000 rpm at 4 °C for 20 min to pellet DNA. Carefully, pour off supernatant and wash the pellets with cold ethanol (70 % v/v). Spin samples at 4 °C for 5 min and the ethanol was removed with a pipet tip, dried, and resuspended in 40 µl of TE buffer. Isolated DNA was then stored at -20 °C until further processing.

2.4.4 CTAB-standardized for human milk samples (CTAB-STD)

Genomic DNA was extracted from 5 mL of human milk from each sample using the CTAB method coupled with phenol-chloroform extraction [37]. A standardization process was carried out to achieve maximum DNA extraction at the highest possible purity. The protocol is as follows: Thaw the cell pellet stored in TE and ground it into a fine powder using liquid nitrogen, add 200 µL of TE at 55 °C, once the powder is dissolved add 40 µL lysozyme (4 U/µL) and mix by inversion, incubate at 37 °C for 30 minutes at 150 rpm. Add 30 µL of SDS 10 % and mix by inversion carefully, add 16 µL of proteinase from Aspergillus oryzae (1 U/µL), mix by inversion and incubate at 56 °C for 2 hours (shake the samples approximately every 30 min, or if it is possible incubate shaking at 200 rpm). Add 100 µL of 5 M NaCl and 100 µL of 10 % CTAB pre-warmed to 55 °C and mixed by inversion carefully, incubated at 65 °C for 10 minutes and cooled for 5 minutes at room temperature and 5 minutes at 4 °C. After incubation, the tubes with solution were centrifuged at 13,000 rpm for 10 minutes, the supernatant was transferred to a new tube and a series of washes were performed with 1 volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 13,000 rpm for 10 minutes, then 4 µL of RNAase (10 U/µL) was added with incubation at 37 °C for 1 hour, followed by an extraction with 1 volume of phenol:chloroform-isoamyl alcohol (25:24:1) and centrifuged at maximum speed, again a wash with chloroform:isoamyl alcohol (24:1) was performed. For DNA precipitation, v/v of isopropanol were used and incubated at -20 °C for two hours and after centrifugation (13,000 rpm for 15 min), the solution was removed. The generated cell pellet was washed twice with ethanol (70%),

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allowed to dry, and resuspended in 40 µL of TE buffer. When necessary, the elution was heated at 40 °C for 5 min to improve solubility without physical mixing. Once the sample was tempered, it was properly labeled and stored at -20 °C for later use.

2.5 Spectrophotometric analyses of DNA

The concentration and purity (A260/A280 and A260/A230 ratio) of the extracted DNA were measured with a NanoDrop™ ND-2000 UV Spectrophotometer (Thermo Fisher Scientific Waltham, MA, USA) using 2 µL of each sample.

2.6 PCR amplification

The internal transcribed spacer 1 (ITS1) of nuclear ribosomal DNA from fungi and V3-V4 fragment of 16S rDNA from bacteria were used to verify the functionality of gDNA. For polymerase chain reaction (PCR) analysis, each DNA sample was diluted to a working concentration. Amplifications were performed in a Thermal Cycler (AXYGEN, Maxygene) selected samples were amplified with forward primers ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ ′ and reverse ITS2: 5′-GCTGCGTTCTTCATCGATGC-3′for ITS-1 region [38] and forward primers 341F:5′-CCTACGGGNGGCWGCAG-3′and reverse 785R: 5′-GGACTACHVGGGTATCTAATCC-′3 for V3-V4 region [39]. The amplification product should be ~200 and ~400 bp, respectively. The oligonucleotides were synthesized and purified by T4OLIGO®. PCR reaction was carried out in a final volume of 25 µL containing 1 µL of DNA-template at working concentration of 10 ng/µL, 0.5 µL dNTPs (2.5 mM), 2.5 µL 10X buffer, 0.75 µL MgCl2, 1 µL forward and reverse primer (5 µM), 0.2 µL Taq DNA polymerase (Invitrogen), and 18.55 µL sterile water). PCR thermal cycling conditions were as follow: pre-denaturation at 95 °C for 3 min, followed by 35 cycles of denaturing at 95 °C for 30 seconds, annealing at 53 °C for 40 seconds for ITS1 and 55 °C for 40 seconds for V3-V4 amplicons, extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. PCR products were stored at -20 °C until further analysis.

2.7 Agarose gel electrophoresis

The assessment of the integrity of the DNA was analyzed by agarose gel electrophoresis using 1, and 2% (w/v) agarose gel (thermoscientific TopVision Agarose, LT-02241) gels for genomic and amplified DNA). Electrophoresis was performed using 1× Tris Acetate-EDTA (TAE) buffer containing 1 µg/mL of ethidium bromide (EtBr) and a constant voltage of 100 V for 50 min. The DNA bands were visualized, and images were acquired using Gel Doc XR+ Imaging system (Bio-Rad Laboratories Inc., Germany). A DNA sample is considered to be intact when its profile on agarose gel electrophoresis corresponds to a well-defined "line" of DNA [40]. The level of degradation of a sample is determined by the loss of definition of the predominant band and the accompaniment of a smear along the gel [41]. In order to determine in an objective and standardized way the integrity of each sample, a measurement scale has been defined for the different DNA profiles observed after the electrophoretic run as follow in Table S3.

2.8 Statistical analysis

Differences in yield, purity, integrity, and bacterial amplification were assessed by one-way ANOVA or Welch-ANOVA with Tukey's Honest Significant Difference (HSD) or non-parametric Kruskal-Wallis one-way ANOVA with the Mann-Whitney-Wilcox procedure with Bonferroni significance correction post hoc multiple comparisons test, according to data distribution (Shapiro-Wilk statistic). Effect sizes of dependencies were evaluated using the η2 coefficient and categorized as a small/medium/large effect size according to Cohen's conventions [42]. All analyses and graphs were performed using R [43] and R studio software [44]. The Microsoft Excel suite was also used to visualize and/or process tables when needed.

3. Results

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3.1 DNA quality and quantity assessment of different methods to isolate gDNA from human milk samples

The efficiencies of four DNA extraction methods (A brief protocol description is provided in Table S2) were compared based on yield, purity, and integrity of the extracted gDNA from human breast milk samples. The quality of each extracted DNA sample was verified spectrophotometrically and agarose gel electrophoresis. The yield of genomic DNA (gDNA) extracted was statistically significantly different between the methods compared (Kruskal-Wallis: df = 3, H = 22.405, p-value = 5.371e-05). The CTAB base extraction method with double phenol wash (CTAB-2PH = 41.42 ± 12.93 ng/µL), the CTAB method standardized for human milk samples (CTAB-STD = 37.14 ± 22.57 ng/µL) and the guanidine thiocyanate method (GTC = 29.62 ± 18. 07 ng/µL) presented similar values, however the values of gDNA concentration decreased when the extraction was performed with the commercial package Quick-DNA™ Fecal/Soil Microbe Kit from ZYMO research (ZYMO = 5.39 ± 4.18 ng/µL). The Mann-Whitney-Wilcox procedure with Bonferroni significance correction post hoc multiple comparisons test indicated that the decrease in DNAg concentration was significant in the ZYMO method compared to all other methods ($P_{ZYMO-GTC}$= 0.0089, $P_{ZYMO-CTAB-2PH}$ = 0.0058, $P_{ZYMO-CTAB-STD}$ = <0.0001), but not between them (Figure 1a). In other words, we detected significant differences in the ability of the ZYMO method to extract DNA from human milk samples, perhaps due to the complexity of the matrix being rich in lipids, carbohydrates, and proteins, in addition to using a physical fragmentation method different from the others by bead beatings.

gDNA purity, as assessed by 260/280 nm absorbance ratios, also differed significantly between methods (Kruskal-Wallis: df = 3, H = 34.748, p-value =1.377e-07), with mean ratios achieved with CTAB-STD (1.67± 0.12) significantly higher than those achieved using CTAB-2PH (1.51 ± 0.09), GTC(1.1 ± 0.26) and ZYMO (1.33 ± 0.08) methods as the Mann-Whitney-Wilcox procedure with Bonferroni significance correction post hoc multiple comparisons test showed ($P_{CTAB-STD-CTAB-2PH}$ = 0.01, $P_{CTAB-STD-GTC}$ = 0.0022, $P_{CTAB-STD-ZYMO}$= <0.0001). CTAB-2PH method also achieved significant difference when compared to the ZYMO method ($P_{CTAB-STD-ZYMO}$ = 0.0488)(Figure 1a).

gDNA purity was assessed also using the 260/230 absorbance ratio measure and significant differences were observed between methods (Kruskal-Wallis: df = 3, H = 44.669, p-value = 1.088e-09), with mean ratios achieved with CTAB-STD (0.58 ± 0.26) significantly higher than those achieved using ZYMO (0.29 ± 0.14) and GTC (0.11 ± 0.08) methods as the Mann-Whitney-Wilcox procedure with Bonferroni significance correction post hoc multiple comparisons test showed ($P_{CTAB-STD-GTC}$ = < 0.0001, $P_{CTAB-STD-ZYMO}$ = 0.00226). CTAB-2PH method also achieved significant difference when compared to the ZYMO and TG method($P_{CTAB-STD-ZYMO}$ = 0.00022, $P_{CTAB-STD-ZYMO}$ =0.01818). The ZYMO method was also different when compared to the GTC method ($P_{ZYMO-GTC}$ = 0.01690). There was no significant difference in DNA purity between CTAB-STD and CTAB-2PH. According to our purity results (260/280 and 260/230 nm ratio), the CTAB based methods protocol, which were free from guanidine contamination, along with the phenol-chloroform washes were associated with the highest (most desirable) values (Figure 1a).

Integrity of extracted gDNA was assessed by agarose gel electrophoresis (Figure 1b). All extractions were evaluated using Gel Doc XR+ Imaging system (Bio-Rad Laboratories Inc., Germany). Quantitative evaluation of DNA integrity was measured using a scale from 0 to 3 as described in the methods (Table S3). Means and medians for each extraction method are shown in Table 1. Gel electrophoresis revealed that the CTAB-STD, CTAB-2PH and GTC extracted more intact DNA compared to the ZYMO method (Figure 1b). The introduction of modifications in the CTAB-STD protocol attained statistical significance in performance compare with the other methods (Kruskal-Wallis, df = 3, H = 34.55, p-value = <0.0001), showing the highest value (Figure 1a), as the Mann-Whitney-Wilcox procedure with Bonferroni significance correction post hoc multiple comparisons test showed ($P_{GTC-ZYMO}$ = 0.0014, $P_{CTAB-2PH-ZYMO}$ = 0.0298, $P_{CTAB-STD-ZYMO}$ = <0.0001, $P_{GTC-CTAB-2PH}$ = 0.0092, $P_{CTAB-2PH-CTAB-STD}$ = 0.0012). Lowest quantity and highest degradation of DNA was

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observed when ZYMO protocol was used. Moreover, 4 out 7 samples (0.57 prevalence) extracted by ZYMO method yielded no detectable by gel electrophoresis gDNA (Figure 1b). No statistically significant differences were detected in the integrity of the samples when processed with the GTC method (even visually they presented more defined bands and no smears), however the prevalence was lower compared to the CTAB-STD method, finding samples that did not present bands. Unfortunately, all samples processed with GTC method failed functionality tests, such as amplification of the V3-V4 fragment of the 16S ribosomal, fungal ITS1 and restriction with multiple enzymes (data not shown). Table 1 summarizes the DNA concentration, purity (at D.O. 260/280and 260/230), and integrity obtained for all human milk samples using the four extraction methods. Although the amount of DNA extracted and purity can be influenced by the sample's own characteristics, the samples were subjected randomly to the different extraction methods in such a way as to minimize the matrix effect, so variations in the data can be attributed to differences in the extraction methods. A hundred and five milk samples were analyzed PCR targeting the V3-V4 16S rRNA and ITS1 rRNA regions, one-way-Welch correction significantly showed difference ($F = 814.98$, p-value < 0.001), all comparisons were significant as the Tukey-Kramer multiple comparisons test (HSD) showed ($P < 0.01$) except for CTAB-2PH-ZYMO comparison ($p = 0.2415$). CTAB-STD method showed amplification for V3-V4 16S rRNA region in all samples, while ITS1 amplify for 71 of 74 milk samples (95%) (Figure 1c). All in all, our results suggest that the standardization reported here to improve the quantity and quality of gDNA from HM have a better performance comparing with commercial and house making traditional methods applied previously to similar samples, influencing the functionality and consequently the taxonomic resolution of metagenomes.

Table 1 Summary of the quantity and quality of human milk DNA extraction by DNA extraction method

DNA extraction Method	ZYMO		GTC		CTAB-2PH		CTAB-STD		† p-value
	Mean ± SD	*Median*	*Mean ± SD*	*Median*	*Mean ± SD*	*Median*	*Mean ± SD*	*Median*	
[DNA ng/µL]	5.39 ± 4.18	3.4	29.62 ± 18.07	36.85 $_{f}$	41.42 ± 12.93	31.65 $_{e}$	37.14 ± 22.57	31.19 $_{c}$	5.37E-05
Purity (260/280 nm)	1.33 ± 0.08	1.3	1.1 ± 0.26	1.38	1.51 ± 0.09	1.51 $_{e}$	1.68 ± 0.12	1.67 $_{a,b,c}$	1.38E-07
Purity (260/230 nm)	0.29 ± 0.14 $_{f}$	0.24	0.11 ± 0.08	0.1	0.56 ± 0.22	0.47 $_{d}$	0.58 ± 0.26	0.48 $_{a,b}$	1.09E-09
Integrity	0.61 ± 0.8	0.25	2.68 ± 0.36	2.75	1.9 ± 0.58	2 $_{d}$	2.64 ± 0.54	3 $_{a,c,e,f}$	1.51E-07

*Values are means ± standard deviation (SD)

† P-values for differences between the four methods were calculated using the Kruskal-Wallis statistic. When the difference between regions was significant ($p < 0.05$), all pairwise comparisons were tested for significance using the Mann-Whitney-Wilcox procedure with Bonferroni significance correction.

a The value for the variable in the method with CTAB-STD is significantly different from the value in the method with ZYMO ($p < 0.05$).

$_{b}$ The value for the variable in the method with CTAB-STD is significantly different from the method with GTC ($p < 0.001$).

$_{c}$ The value of the variable in the method with CTAB-STD is significantly different from the method with CTAB-2PH ($p < 0.001$).

$_{d}$ The value of the variable in the method with CTAB-2PH is significantly different from the method with GTC ($p < 0.01$).

$_{e}$ The value of the variable in the method with CTAB-2PH is significantly different from the method with ZYMO ($p < 0.001$).

$_{f}$ The value of the variable in the method with GTC is significantly different from the method with ZYMO ($p < 0.001$).

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ZYMO: Quick-DNA Fecal/Soil Microbe Kit from ZYMO research® ; GTC: Guanidinium-thiocyanate method ; CTAB-2PH: Hexadecyltrimethylammonium bromide - double phenol step; CTAB-STD: Hexadecyltrimethylammonium bromide standardized for human milk; ng: nanograms; µL: microliters; nm: nanometers.

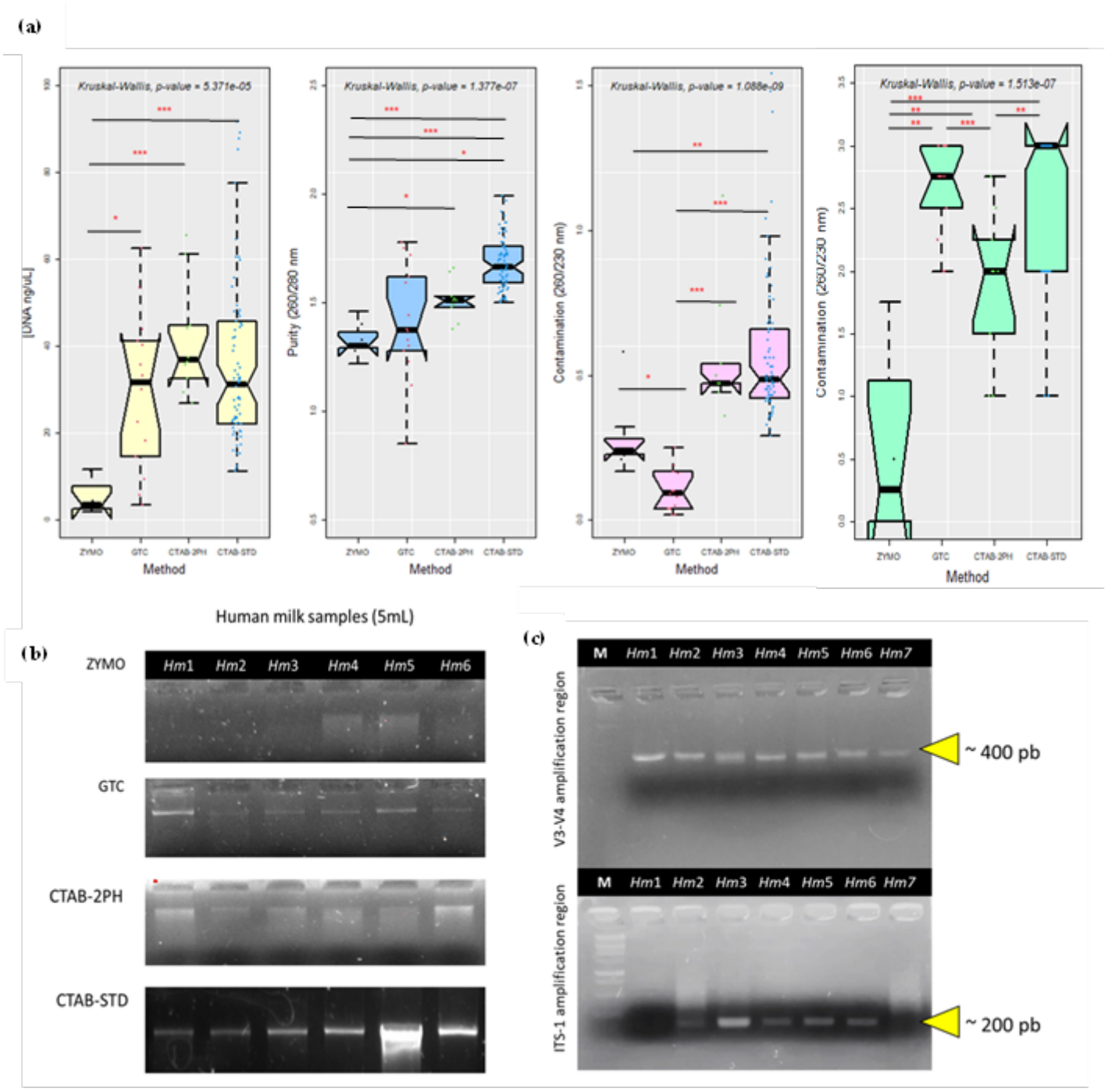

Figure 1. DNA quantity and quality assessment of human milk samples. **(a)** box plots showing the DNA concentration (ng/µL), DNA purity at (260/280 nm), DNA purity at (260/230 nm) and the DNA integrity of the four methods assessed. **(b)** Representative results from gel electrophoresis analysis of gDNA from human milk samples extracted by four different methods. **(c)** Representative results from gel electrophoresis analysis of PCR amplification (V3-V4 and ITS-1 regions) from human milk samples extracted with STD-CTAB method. The box signifies the 75% (upper) and 25% (lower) quartile showing the

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distribution of 50% of the samples. The line inside the box plot represents the median. The whiskers (top and bottom) represent the maximum and minimum values. Outliers, which are beyond 1.5 times the interquartile range above the maximum value and below the minimum value, are shown with the corresponding color dot. P-values for differences between the four methods were calculated using the Kruskal-Wallis statistic. When the difference between regions was significant ($p < 0.05$), all pairwise comparisons were tested for significance using the Mann-Whitney-Wilcox procedure with Bonferroni significance correction.*** represents significancy were $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

3.2 CTAB-STD method is able to extract high quantity and quality of gDNA from Gram-positive, Gram-negative bacteria and fungi

The human milk microbiota is constituted by gram-positive, gram-negative bacteria, fungi and archaeobacteria [45], therefore an efficient DNA extraction method must be able to extract the total amount of DNA present in a complex sample from this wide range of organisms, whose cell wall composition differs considerably. Our CTAB-STD method optimized for human milk samples was able to extract DNA from isolated cultures of Gram-positive, Gram-negative bacteria, and yeasts (Figure 2e), showing a high quantity and quality of gDNA (Figure 2a-d). Table 2 summarizes the DNA concentration, purity (at D.O. 260/280 and 260/230), and integrity obtained for all microorganism samples. DNA concentration showed statistical difference between the cell types ($F = 13.6$, $P = < 0.001$).

The DNA concentration in Gram-positive bacteria (33.74 ± 25.83 ng/µL) was lower than Gram-negative bacteria (80.68 ± 32.89 ng/µL) and yeasts (85.86 ± 34.84 ng/µL). It is most likely that the cell wall composition of gram-positive bacteria is responsible for this difference. These values were statistically significant when compared between Gram-positive and Gram-negative bacteria ($p=0.0205$, $\eta2= 0.61$, large effect size) and Gram-positive and yeast ($p = 0.0394$, $\eta2= 0.39$, medium effect size), as demonstrated by the Tukey-Kramer multiple comparisons test (HSD), but not between Gram-negative and yeast. We did not observe any significant changes in DNA purity, both 260/280 (Kruskal-Wallis: df = 2, H = 3.4454, p-value =0.528) and 260/230 nm ratio (Kruskal-Wallis: df = 2, H = 2.6464, p-value = 0.4454). The average values of all measured samples were in the range 1.5–1.9 (260/280 nm ratio) and 0.6–2.0 (the 260/230 nm ratio) (Figure 2a). The subsequent integrity analysis did not show any changes in DNA quality due to the cell types (Figure 2b). Functionality test didn´t show significant differences (data not shown).

Table 2 Summary of the quantity and quality of DNA extraction by cell type using de CTAB-STD method

Cell type	**[DNA ng/µL]**		**Purity (260/280 nm)**		**Purity (260/230 nm)**		**Integrity**	
	Mean ± SD	*Median*	*Mean ± SD*	*Median*	*Mean ± SD*	*Median*	*Mean ± SD*	*Median*
Gram (+) bacteria	33.74 ± 25.83 [a,b]	28.15	1.76 ± 0.14	1.83	1.35 ± 0.49	1.49	1.86 ± 0.69	2
Gram (-) bacteria	80.68 ± 32.89	60.34	1.83 ± 0.09	1.86	1.6 ± 0.43	1.68	1.67 ± 0.5	2
Yeast	85.86 ± 34.84	97.22	1.75 ± 0.09	1.75	1.48 ± 0.14	1.48	2.25 ± 0.5	2
p-value	0.0133†		0.528¥		0.4454¥		0.2663¥	

*Values are means ± standard deviation (SD)

† P-values for differences between the four response variables were calculated using ANOVA statistic. When the difference between regions was significant ($p < 0.001$), all pairwise comparisons were tested for significance using the Tukey-Kramer Honest Significant Difference (HSD) test

¥ P-values for differences between the four response variables were calculated using the Kruskal-Wallis statistic. When the difference between regions was significant ($p < 0.05$), all pairwise comparisons were tested for significance using the Mann-Whitney-Wilcox procedure with Bonferroni significance correction.

[a] The value for the variable in Gram (+) bacteria is significantly different from the value in Gram (-) bacteria ($p < 0.01$).

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[b] The value for the variable in Gram (+) bacteria is significantly different from the value in yeast ($p < 0.01$).

[c] The value of the variable in Gram (-) bacteria is significantly different from value in yeast ($p < 0.01$).

ng: nanograms; µL: micrograms; nm: nanometers; ns: non-significant

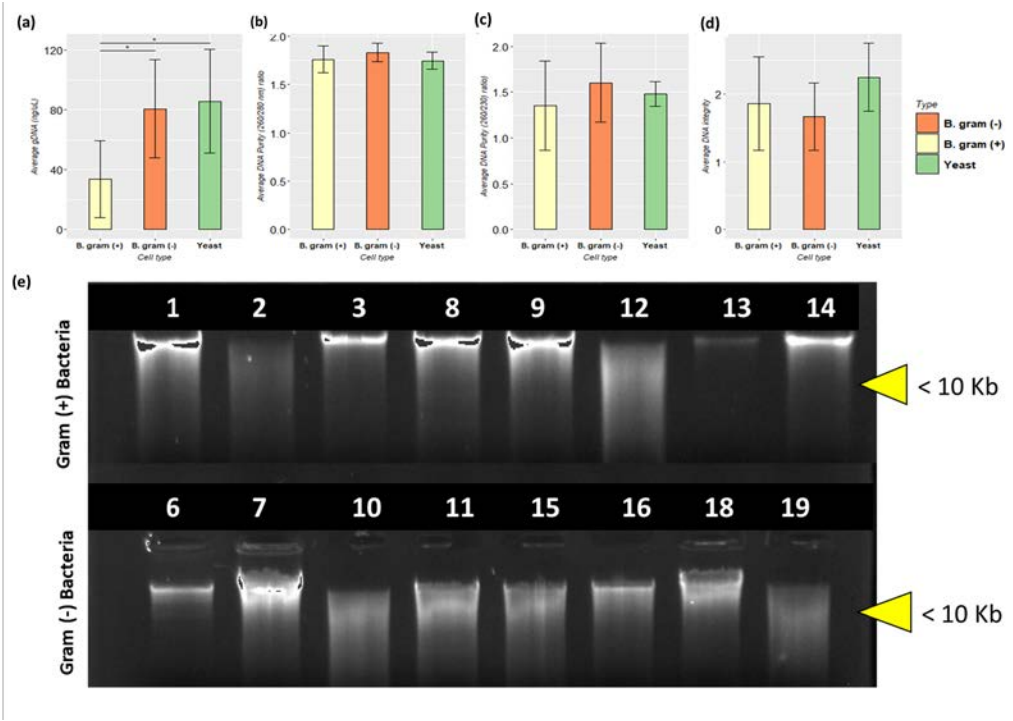

Figure 2. Quantity and quality of DNA from microorganisms isolated using the CTAB-STD method. (a) bar plots showing the DNA concentration (ng/µL), (b) the DNA purity at (260/280 nm), (c) the DNA purity at (260/230 nm) and (d) the DNA integrity of the three cell types assessed. (e) Representative results from gel electrophoresis analysis of gDNA from microorganisms isolated using the CTAB-STD method. The bar signifies the mean. The line inside the bars represents the standard deviation. P-values for differences between the different cell types were calculated using an ANOVA statistic. When the difference between regions was significant ($p < 0.05$), all pairwise comparisons were tested for significance using the Tukey-Kramer multiple comparisons test (HSD). * Represents significancy were $p < 0.05$. Orange represents values for bacteria gram negative, yellow represents values for bacteria gram positive and green represents values for yeast.

3.3 Effect of processing in DNA quality from isolated microorganism samples

Previously our results suggested that CTAB-STD method is able to extract more gDNA from gram-negative bacteria than gram-positive or yeast. To test whether there was a possible trend to extract more gDNA from organisms with different cell wall composition and whether sample processing could affect the quantity and quality of gDNA, we proposed a multifactorial design with repeated measures, using the factors: i) Organism type, with two levels, gram-positive bacteria (B. gram +) and gram-negative bacteria (B. gram -) as focal variable, the second factor was ii) Physical fragmentation, with two levels, macerated with liquid nitrogen (N2 +) and those that were not macerated (N2 -) and the third factor corresponds to the processing of the sample iii) Freezing process, with two levels, those corresponding to a storage time of 5 months and 5 freeze/thaw cycles (FT) and those corresponding to fresh samples with only one FT cycle (fresh), the latter two factors as moderating variables. All samples were standardized to an average O.D. of 0.47 ± 0.013 at 600 nm. Expected concentration was calculated based on the size of each bacterial genome, the weight of a base pair, and the colony forming unit (CFU) of each bacterium.

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The results suggested that there is no statistically significant effect for the three-way interaction for DNA concentration F(1, 3) = 0.787, p = 0.44., but there was a statistically significant simple two-way interaction between Freezing process and Organism type only for N2(+), F(1, 3) = 15.1, p = 0.03, but not for N2(–), F(1,3) = 5.88. p = 0.094. Simple effect of Organism type resulted also significative F(1,3) = 11.9. p = 0.041. For N2(+), this result suggests that the effect of treatment on DNA concentration by CTAB-STD method depends on Organism type. When simple-simple main effect was calculated, there was a statistically significant increase in DNA concentration (190 ± 31.4) for gram (-) bacteria macerated with N2 and subjected to multiple FT cycles, F(1, 92) = 17.3, p < 0.0001), but not for bacteria gram (+) (67.7 ± 25.9), without maceration and fresh, F(1,92) = 2.48, p = 0.118.All simple-simple pairwise comparisons, between the different treatment groups, were run for all organism types, trial with a Bonferroni adjustment applied. Pairwise comparisons showed a tendency to decrease significance within FT cycles ($P_{N2(-)}$ = 0.012 and $P_{N2(+)}$ = 0.036), suggesting a reduction in DNA concentration bias due to organism differences. All these results together suggest that when liquid nitrogen is used to macerate the samples and multiple freeze-thaw cycles, the significant difference decrease in DNA concentration due to cell type, which would allow us to avoid a bias in the reconstruction of microbiological profiles due to the method of DNA extraction (Figure 3a).

Purity at 260/280 nm ratio showed a range average between 1.46 ± 0.10 in all conditions, however no statistically significant effect for the three-way interaction for purity at 260/280 (F(1, 3) = 0.507, p = 0.52) was shown. There was a statistically significant simple two-way interaction between Freezing process and Fragmentation (F(1, 3) = 51.41, p = 0.006) only for gram (–) (F(1,9) = 25, p = <0.0001), suggesting that the variation observed is influenced by the type of organism used in the extraction. Simple-simple interaction were performed only for gram (–) showing significative difference when they were fresh and no macerated with liquid nitrogen (1.36 ± 0.07), so it is inferred that the decrease in purity is due to the lack of effective cell fragmentation. Interestingly, the pairwise comparisons showed no significant difference in any of the combinations when the focus variable (organism type) was contrasted with (Figure 3b).

Purity at 260/230 nm ratio showed no significant differences for the three-way interaction neither simple two-way interaction. Simple -simple interactions were performed resulting in Fragmentation as the only significative variable (F(1, 9) = 26.5, p = <0.001) for gram (–) for both FT cycles (F(1,17)= 18.2, p = <0.001) and fresh (F(1,9)= 43.2, p = <0.001) (Figure 3c).

Integrity also showed no significant difference in the three-way interaction, again the two-way interaction between fragmentation and freeze-thaw cycles showed a significant difference only for gram (+) (F(1,15) = 35.6, p = < 0.001), increasing integrity in all cases when compared to gram negatives. Pairwise comparisons suggest that there is significant difference between gram (+) and gram (-) when macerated or not (p = 0.07), decreasing integrity if liquid nitrogen is used and multiple FT cycles are present, a situation that is exacerbated if the organisms are gram-negative bacteria (p = < 0.001). Integrity results are crucial for the purpose of this method since losing integrity of the genetic material during extraction may contribute to the loss of susceptible species that are naturally underrepresented in the sample (Figure 3d,e).

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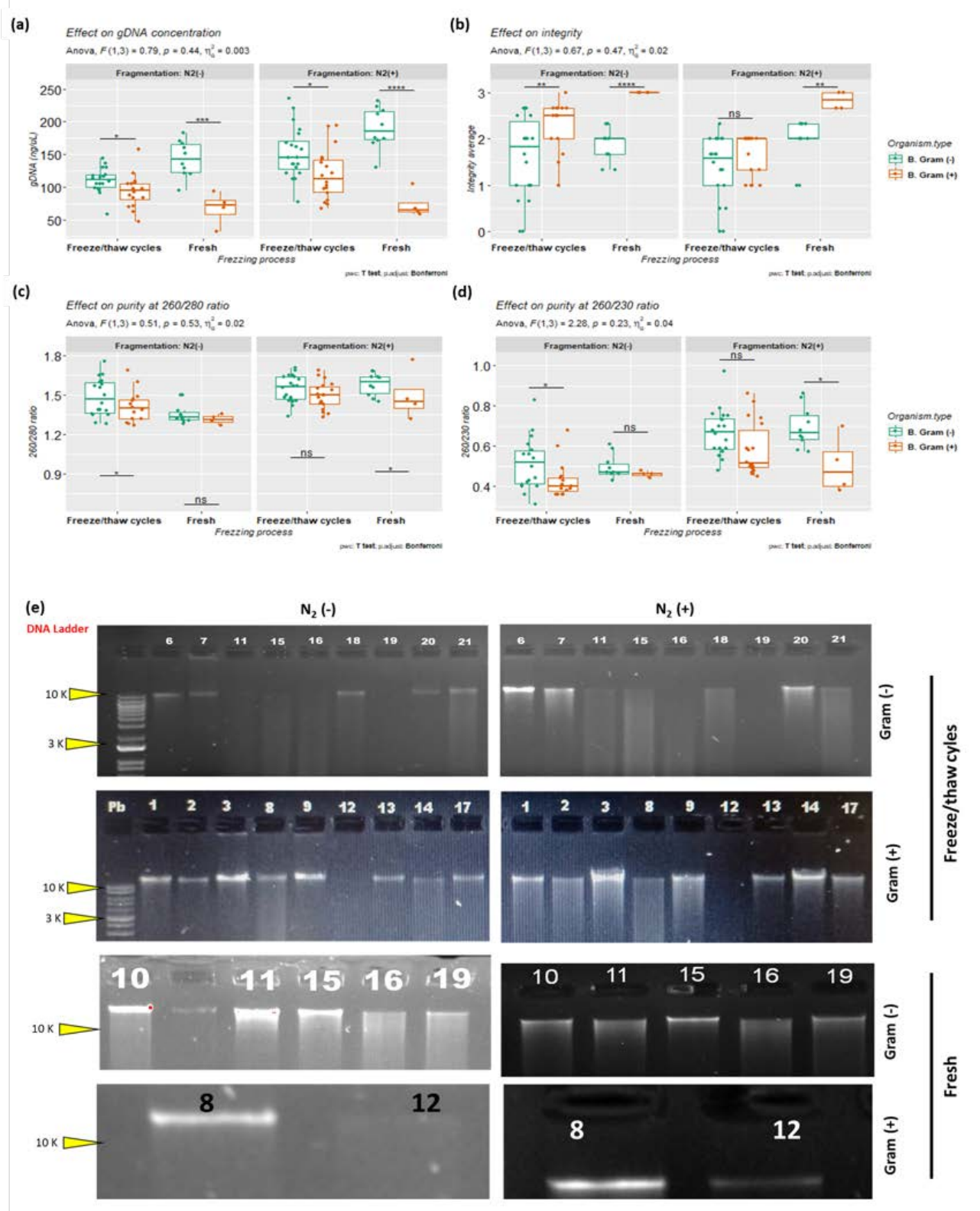

Figure 3. Effect of processing in DNA quantity and quality from isolated microorganism samples. (a) box plots showing the DNA concentration (ng/µL), (b) DNA purity at (260/280 nm), (c) DNA purity at (260/230 nm), and (d) DNA integrity. All boxplots show the gram (+) and gram (-) bacteria, grouped by processing and fragmentation. (e) Representative results from gel electrophoresis analysis of gDNA from microorganisms isolated using the CTAB-STD method. N2 (+) represents maceration with liquid nitrogen, N2 (-) represents extraction without maceration The box signifies the 75% (upper) and 25% (lower) quartile showing the distribution of 50% of the samples. The line inside the box

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plot represents the median. The whiskers (top and bottom) represent the maximum and minimum values. Outliers, which are beyond 1.5 times the interquartile range above the maximum value and below the minimum value, are shown with the corresponding color dot. P-values for differences between the treatments were calculated using a three-way ANOVA statistic. When the difference between regions was significant ($p < 0.05$), all pair-wise comparisons were tested for significance using a t-test procedure with Bonferroni significance correction.*** represents significancy were $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

3.4 Repeated freeze/thaw cycles affect quality of metagenomic DNA in human milk samples

We previously showed the effect of FT cycles on the quality of gDNA in isolated cultures of microorganisms (Figure 3). We hypothesized then that repeated FT cycles could affect the quantity and quality of metagenomic DNA from human milk samples, thereby influencing the reconstruction of the microbiological profile by loss of species and/or abundance as shown previously in different samples [26,46,47]. To test this hypothesis, a total of 84 independent samples from human milk donors were used, which were divided into two groups, 21 were stored frozen for 6 months and subjected to five FT cycles over time (M), the remaining 63 were frozen and extracted with the same storage time but without freeze-thaw cycles, as a control (S). We noticed a very interesting trend in concentrations of M samples, there was a significant increase ($U = 3570$, $p = < 0.001$) in the amount of DNA extracted (261.74 ±127.32 ng/µL) comparing to the S samples (35.23 ± 18.27 ng/µL), perhaps due to the breakdown of cells by crystals formed during multiple FT cycles. Although this could be influenced by the characteristics of the samples themselves, it is also remarkable the wide dispersion observed in the M samples that is not observed in the S samples. In contrast to the DNA concentration, there was a significant decrease in purity at the 260/280 rate ($M = 0.79 \pm 0.13$, $S = 1.67 \pm 0.11$, $U = 3570$, $p = < 0.001$), in rate 260/230 ($M = 0.31 \pm 0.12$, $S = 0.58 \pm 0.24$, $U = 3570$, $p = < 0.001$) and in integrity ($M = 0.38 \pm 0.5$, $S = 2.57 \pm 0.64$, $U = 2485$, $p = < 0.001$) (Figure 4a).

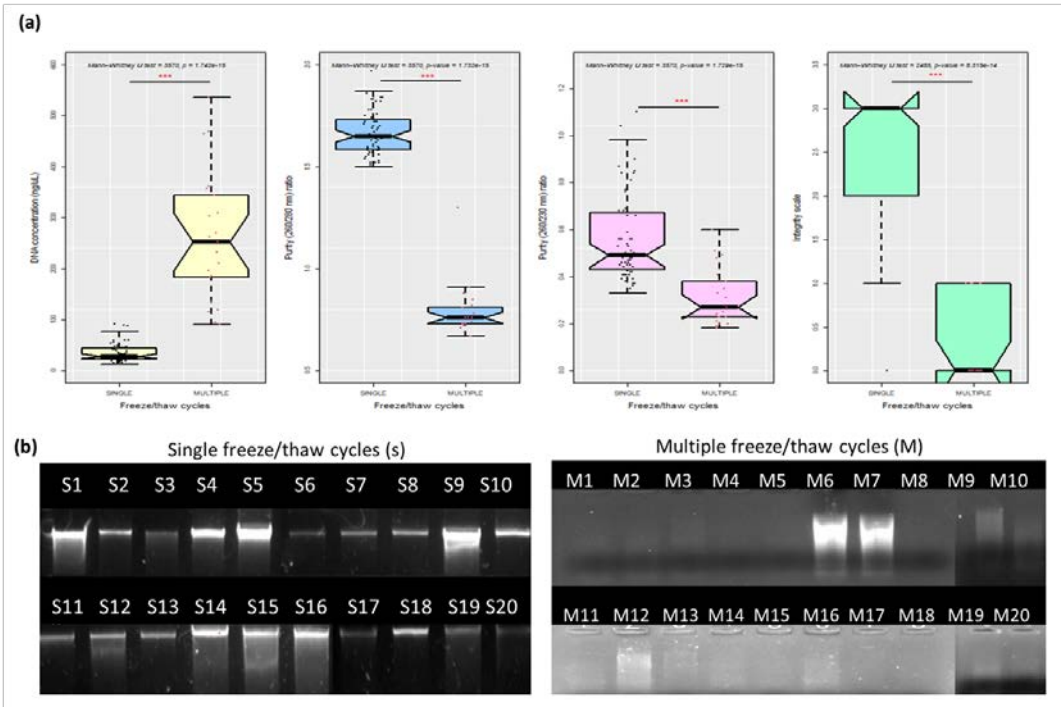

Figure 4 Comparison of genomic DNA quantity and quality according to single or multiple freeze/thaw cycles in human milk samples. (a) box plots showing the DNA concentration (ng/µL), DNA purity (260/280 nm), DNA purity (260/230 nm) and integrity of the human milk samples extracted with CTAB-STD method. The box signifies the 75%

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(upper) and 25% (lower) quartile showing the distribution of 50% of the samples. The line inside the box plot represents the median. The whiskers (top and bottom) represent the maximum and minimum values. Outliers, which are beyond 1.5 times the interquartile range above the maximum value and below the minimum value, are shown with the corresponding color dot. P-values for differences between the comparisons were calculated using the Mann–Whitney U test .* represents significancy were *** $p< 0.001$, ** $p < 0.01$ and * $p < 0.05$ (b) Metagenomic DNA was electrophoresed on a 1 % agarose gel, stained with ethidium bromide, and photographed in a gel documentation system. Lane S1 to S20 correspond to metagenomic DNA isolation by single freeze/thaw cycle. Lane M1 to M20 correspond to metagenomic DNA isolation by multiple freeze/thaw cycles.

In Figure 4b, a characteristic gel of the integrity observed in the samples subjected to the defrost/thaw cycles (samples M1-M20) can be seen. The absence of defined bands at the top, the presence of a smear over the entire electrophoretic run and/or the presence of a diffuse band at the end of the run are characteristic of the degradation suffered compared to the S samples (samples S1-S20). Although some of the M samples were still usable for standard PCR amplification, biased amplification is uncertain due to the degradation observed in the agarose gels, so accurate recovery of the metagenome of the samples would be severely compromised. On the other hand, our experiment suggests that DNA concentration increases in the interaction between the factors, maceration, and freezing processing, however integrity seems to decrease with increasing freeze/thaw cycles. Our experiment reveals that a combined strategy could be used to reach an optimal point between the interaction, i.e., to know at what point maceration and FT cycles maximize DNA concentration and minimize loss of integrity and consequently functionality.

4. Discussion

In the present study, four different methods of DNA extraction from human milk samples were evaluated. Sample quantity and quality were based on spectrophotometer measurements and agarose gel electrophoresis scores. According to the concentration (37.14 ± 22.57 ng/µL) there is no significant difference between the CTAB-based methods, but with the commercial ZYMO method ($p = < 0.001$), which favors that the CTAB-STD method is able to recover more genetic material from the sample and favor the reconstruction of the metagenomic profile (Figure 1a). Yield rates in the extractions are difficult to compare since it depends not only on the efficiency of the method, but also on the quantity and characteristics of the sample itself. Other authors report results below 50 ng/µL (when adjusting results to 1mL of sample) [25,48,49]. A study by Cheema et al. reported a maximum DNA concentration of 0.68 ng/µL per mL of sample [29], in comparison our results are well above the reported value. For genomic DNA to be considered pure, the ThermoFisher Scientific NanoDrop 2000™ protocol indicates that an absorbance ratio of 260/280 nm equal to or greater than 1.80 is required. Our 260/280 ratios had an average ratio of 1.68 ± 0.12 (ranging from 1.5 to 1.9), showing a significant difference compared to the other methods used ($p < 0.001$) demonstrating that the standardization process improved DNA extraction with acceptable purity ratios for further use in sequencing projects. Previous efforts have been made to optimize the purity of DNA extractions in their 260/280 ratio, showing similar or lower results to those reported in this study[25,28,49]. A 260/230 nm ratio less than 2 is indicative of contamination with organic compounds [50]. In our study all methods obtained values below the standard ranging between 0.29 ± 1.54, however, within the observed values the CTAB-STD method obtained the best performance with an average ratio of 0.58 ± 0.26 and a significant difference compared to the GTC and ZYMO methods ($p = < 0.05$). According to previously published literature [25,48] where the performance of different commercial DNA extraction methods has been evaluated, similar 260/230 rate results have been obtained to those reported in this study. These low levels may be due to the high levels of fat present in human milk, which can negatively influence the efficacy of DNA isolation buffers [27,28], as well as the presence of

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various components of sample matrix, such as polysaccharides and polyphenols, or by chemicals from extraction [51]. Gel electrophoresis is a commonly used criterion for assessing DNA integrity. The results of this study suggest a significant increase in integrity compared to the methods evaluated (Figure 1c). There are few studies where the integrity of the evaluated samples is reported, in this regard Gaur et al., show gels where a characteristic sweep is observed along the entire electrophoretic run [48], a particularity that we observed in our CTAB-2PH method, but that was minimized during the standardization process, showing a superior performance in the CTAB-STD method (Figure 1b). Taken together, these results suggest that the purity, integrity, and quantity of DNA is higher in human milk samples when using our standardized method for human milk (CTAB-SDT) compared to the other previously reported and commercially available DNA extraction protocols tested in this study. It is important to highlight that we are aware that it is still necessary to work on improving the 260/230 rate to avoid errors in subsequent analyses, with the intention of improving the level of resolution of human milk research.

The functionality test revealed not only the ability of the CTAB-STD method to amplify the V3-V4 fragment in all human milk samples, but also a 95 % prevalence in the amplification of the fungal ITS-1 fragment, contrary to that reported by Moossaavi et al, where they report only 21.4 % of amplified samples [10] when reviewing the extraction method we observed that they used a commercial package [20] from ZYMO (Quick-DNA™ Fungal/Bacterial Kit from ZYMO research (Zymo Research Corp., Irvine, CA, USA) whose base is the use of GTC in its lysis buffer and fragmentation with beads, in our results a similar commercial package was used (Quick-DNA™ Fecal/Soil Microbe Kit from ZYMO research (Zymo Research Corp., Irvine, CA, USA) obtaining an amplification prevalence of 40 %, very close, even higher, than that reported by Moossavi et al. Another study conducted by Boix-amorox et al. obtained a higher prevalence of fungal positive samples, reporting a range between 35 and 80 % [35], being even lower than the one reported here. The extraction method used was InviMag stool DNA kit (Stratec Molecular, Berlin, Germany), specifying a mechanical and chemical lysis treatment. Similarly, our method uses a mechanical and chemical lysis with proteinases to increase the cell wall fragmentation, however, the standardization process for human milk improves the performance given the complexity of the sample, low biomass, and high diversity of organisms with different cell structure. This suggests that the result reported previously could be influenced by the extraction method used, situation that could avoided using the CTAB-STD proposed in this study. Nevertheless, further studies should be carried out.

To evaluate our standardized CTAB method for human milk (CTAB-STD) in its ability to extract gDNA from microorganisms with different cell wall composition, such as gram-positive, gram-negative bacteria and yeasts, we selected different organisms mimicking the human milk diversity and performed isolated cultures, then evaluated the quality and quantity of the extracted DNA. The results show that the purity at a 260/280 rate reaches the optimal range with an average of 1.83 ± 0.09 and there is no significant difference between the different groups of organisms ($p = 0.20$), as for the 260/230 rate, an average of 1.47 ± 0.35 is reached, without significant differences among organisms ($p = 0.22$), which although it is not an optimal value is considerably higher than that obtained in human milk samples (0.58 ± 0.26) suggesting that the complexity of the sample contributes greatly to the low value in the 260/230 rate in human milk samples. As far as we have been able to consult the literature there is no other publication using isolated cultures to evaluate the functionality of a DNA extraction method in its concentration and quality. Previous studies have used synthetic communities to evaluate the efficiency of different methods but do not report the effect on the purity and integrity of the extractions [28,31]. DNA concentration reached a significant reduction ($p = < 0.05$) between gram-positive (33.74 ± 25.83 ng/µL) and gram-negative (80.68 ± 32.89 ng/µL) bacteria, as well as with yeasts (85.86 ± 34.84 ng/µL), suggesting that the difference in cell wall composition may be biasing the outcome of DNA extractions. In the previous study evaluating the efficacy of different

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DNA extraction methods in their ability to reconstruct a microbiological profile, an underrepresentation of gram-positive bacteria was observed [28,52], particularly bacteria of the genus Lactobacillus and Streptococcus which have cell walls that are difficult to break down [31]. Previous results suggested a significant decrease in the extraction of gram-positive bacteria (Figure 2a). In an attempt to get a broader picture of what might influence this observed difference, a three-factor experiment was designed, including gram-positive and gram-negative bacteria, the use of liquid nitrogen as a cell fragmentation mechanism, and multiple FT cycles. All cultures were standardized to an optical density of 0.47 ± 0.13 at 600 nm and extractions were performed in duplicate and by two different analysts. The results of the three-way ANOVA suggest that none of the three-way interactions for each of the response variables (DNA concentration, purity, and integrity) is statically significant, however in all response variables an interaction between fragmentation and freeze-thaw cycles is observed ($P = < 0.001$), particularly influencing the result obtained in gram-positive bacteria which is found to be decreased in all combinations compared to gram-negative bacteria. Previously reported results precisely suggested an under representation of gram-positive bacteria when reconstructing microbiological profiles of human milk samples [28–30,53]. It is interesting that when comparing the results of fresh samples without maceration to samples macerated with liquid nitrogen and exposed to multiple FT cycles, the significant difference in DNA concentration between gram-positive and gram-negative bacteria reaches its minimum value, suggesting that both maceration and FT cycles contribute to fragmenting the cells and maximizing DNA concentration. A study by Lyons et al., evaluating the effect of storage, extraction method and temperature concluded that the most effective method for accurately reconstructing the biological community they evaluated was short storage at -80 °C, furthermore the extraction methods included physical fragmentation [26]. Similarly, the results reported by Xin et al., showed that a storing at −20 °C and three FT cycles were the best freezing condition and could markedly enhance DNA extraction efficiency, but preserve the species diversity of meconium microbiota [47]. Another study by Männistö et al., on artic bacterial communities showed that FT cycles do not change drastically the microbiological profiles of the samples [46].

The purity at 260/280 does not reach significant differences when comparing gram positive and negative bacteria in any of the conditions, however when comparing fresh samples against those treated with multiple FT cycles there is a significant increase in purity, which is even greater in the combined effect, suggesting that fragmentation is contributing to the removal of protein residues from the sample. Purity at a rate of 260/230 shows a significant improvement ($p = 0.05$) in comparisons between bacteria when using any fragmentation method, suggesting that the modifications made to the protocol contribute substantially to improving the quality of DNA extraction. In contrast to previous observations, integrity appears to decrease in all cases when either fragmentation method is used, showing the worst performance in the combination of these two factors, with gram-negative bacteria being most affected (1.36 ± 0.687). Together these results explain some previously reported results [26,46,54] where an overrepresentation of gram-negative bacteria was observed, since they are more susceptible to fragmentation than their gram-positive counterparts, but also suggests that the integrity of their DNA could be compromised and bias the result on those gram-negative species less represented in the samples. The representative gel in Figure 3e shows how integrity is more affected in gram-negative bacteria than in gram-positive bacteria. These results highlight the need for precautions during processing and storage complex samples where a mixture of organisms may be present.

Keeping in mind the observed effect of storage, TF cycles and physical fragmentation on gram-positive and gram-negative bacterial populations, we decided to compare the results of our fresh, liquid nitrogen-fragmented samples with a set of samples that had been stored for 6 months and had undergone five TF cycles. The results consistently showed us a significant increase in DNA concentration, but a significant loss of purity and

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integrity, which affected even the functionality of the DNA (Figure 4). In the results published by Shao et al., it is observed that TF cycles induce a progressive degradation of genomic DNA, which is greater as the number of TF cycles increases. On the other hand, it is described that the larger the DNA size, the more susceptible it is to degradation by TF cycles. This peculiarity of extracting high molecular weight DNA occurs consistently in phenol-chloroform extractions. Our method uses this strategy, which would explain why storage time, successive FT cycles and the combined effect of fragmentation with liquid nitrogen substantially degraded the human milk samples, which could result in, at best, asymmetric amplification and compromise the veracity of the microbiological profile.

5. Conclusions

Microbial DNA in milk samples was difficult to extract using commercial DNA extraction kits due to the extremely low microbial biomass and richness in organic components. The CTAB-based extraction method standardized for human milk demonstrated better performance in the concentration, purity, and integrity of the extracted DNA. DNA extraction methods for complex samples and metagenomic purposes must be able to extract total DNA from a wide range of species [53], in this study we show that the CTAB-STD method is able to extract DNA from gram-positive, gram-negative bacteria and yeasts. Furthermore, the proposed method was able to amplify a 100 % of V3-V4 fragment and 95 % of the milk samples for the fungal ITS1 fragment, thus its metagenomic application could contribute significantly to the identification of fungi as part of the human milk microbiota. We also discovered the effect of sample processing on DNA concentration and hypothesized that a process of optimizing freeze-thaw cycles will favor the extraction of total DNA from the sample, minimizing fragmentation and the effect caused by the difference in cell wall composition. Finally, we conclude that proper storage, processing, and extraction of human milk samples can contribute to the quantity, purity, and integrity of gDNA samples for metagenomic purposes. In summary, this work offers a protocol to improve the extraction of bacterial and fungal DNA from human milk samples, which may be useful to explain their ecological dynamics and susceptibility to changes, given by internal and external conditions of the mothers.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1; Table S1: Strains and cultivation condition used in this study; Table S2: Summary of DNA extraction methods used in this study; Table S3: DNA Quality based on electrophoresis of genomic DNA

Author Contributions: Conceptualization, Edgar Balcázar-López; Data curation, Guadalupe García-Robles and Felipe Ramírez-Salazar; Formal analysis, Mario Alemán-Duarte; Funding acquisition, Blanca Aguilar-Uscanga, Israel Benítez-García, Edgar Balcázar-López and Josué Solís-Pacheco; Investigation, Mario Alemán-Duarte, Guadalupe García-Robles and Felipe Ramírez-Salazar; Methodology, Edgar Balcázar-López; Project administration, Blanca Aguilar-Uscanga, Israel Benítez-García and Josué Solís-Pacheco; Resources, Israel Benítez-García and Josué Solís-Pacheco; Supervision, Edgar Balcázar-López and Josué Solís-Pacheco; Validation, Edgar Balcázar-López and Josué Solís-Pacheco; Visualization, Mario Alemán-Duarte; Writing – original draft, Mario Alemán-Duarte; Writing – review & editing, Blanca Aguilar-Uscanga, Israel Benítez-García, Edgar Balcázar-López and Josué Solís-Pacheco

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of the civil hospital "Fray Antonio Alcalde" (HCFAA) of the University of Guadalajara (HCG/CEI-0907/22 with Record 141/22 dated 8th June 2020).

Informed Consent Statement: Written informed consent has been obtained from all subjects involved in the study to publish this paper.

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Conflicts of Interest: The authors declare no conflict of interest.

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