Influence of Different Media and Conditions on Probiotics Isolation from Breast Milk

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Abstract. Breast milk is an important food source for infant development and a viable potential probiotics resource for formula and fermentation industry. However, how to enrich the bacteria in human milk at the bacterial level in different media under different conditions is still poorly understood. Our research tested the isolating capability of LBS media, MRS media and MRS (pH=5.2) media under both anaerobic and aerobic conditions via Gram staining and 16s rRNA sequencing for Lactobacillus and Bifidobacterium. As a conclusion, MRS (pH=5.2) media showed a good capability on Lactobacillus isolation while LBS media presented well on Bifidobacterium isolation under anaerobic conditions.

1. Introduction
As the developing of human microbiome researches, a large number of bacteria has been demonstrated to have positive efforts on health improvement and disease treatment[1]. Breast milk microbiota is reported as a critical source for the development of infant intestinal flora, which is also a valued resource of probiotic selection[2,3]. For instance, Lactobacillus and Bifidobacterium isolated from breast milk, which have great application on baby food industry[4], are proved to have various functions such as anti-carcinogenic, anti-inflammatory and growth promotion among infant growth and human health regulation[5,6]. Moreover, the probiotics from breast milk were reported to have higher biosecurity compared with probiotics isolated from other non-human sources and satisfying resistance to gastric digestion and bile shock[7].

At the same time, breast milk is composed with complicated microbiota as Staphylococcus, Pseudomonas and Edwardsiella are major strains with Lactobacillus and Bifidobacterium are minor parts of the whole breast milk flora[8]. Except that, breast yeast infection is not uncommon during breastfeeding[9]. Candida is another important contaminant need to be considered for probiotics isolation from breast milk.

But little attention has been devoted to media and condition optimization for breast milk probiotic isolation. Hence, aiming to explore an optimized condition for isolating Lactobacillus and Bifidobacterium from breast milk, we investigated the microbial community composition on three different frequent-used media anaerobically, or aerobically by 16s rRNA sequencing method.
2. Materials and methods

2.1. Sample collection
The breast milk sample was collected in a sterile bag from a healthy lady and stored under -80 degree before used.

2.2. Sample isolation
The three media used in this experiment were MRS Agar (BR250G, HuanKai Microbial), LBS Agar (HB0385, Hopebio) and MRS (pH=5.2) Agar. LBS Agar was also known as Rogosa Agar. The pH of MRS (pH=5.2) Agar was adjusted by 5M hydrochloric acid solution to 5.2 based on MRS Agar. Anaerobic condition was created with anaerobic culture bag and one-off anaerobic gas bag from Hopebio.

200uL breast milk was smeared evenly on 90mm plates under different conditions including on MRS agar aerobically and anaerobically, on LBS agar aerobically and anaerobically, on MRS (pH=5.2) agar aerobically and anaerobically. Then all the plates were cultured under 37℃ for 48 hours in incubator. After incubation, the strain amounts were counted and 10 strains from each plate were picked for Gram staining. Three replicates were made for each condition.

2.3. 16s rRNA sequencing
The sample were cultured in 6 different conditions as mentioned in 2.2. After that, using 5mL PBS each plate to wash down all the strains on surface. For each condition, three replicates were mixed as one sequencing sample.

DNA of sequencing samples were extracted. Then the concentrations were measured and checked by Qubit. 30ng DNA was used each PCR for 16s rRNA V4 region enrichment with primers (515F: 5’-GTGCCAGCMGC GCGGTAA-3’ and 806R: 5’-GGACTACHVGGGTWTCTAAT-3’). The PCR products were purified by Agencourt AMPure XP Beads and analyzed by Agilent 2100 Bioanalyzer before sequencing on Illumina Hiseq2000 platform.

2.4. Data analysis
Douglas method were used for the original data analyzing from Hiseq2000 as clean data[10]. The clean data were analyzed and jointed by FLASH[11]. OTU analysis was made by USEARCH(v7.0.1090)[12]. Alpha diversity was analysed by mothur(v1.31.2) in R[13]. Beta diversity was analyzed by QIIME(v1.80) and gplots in R with Euclidean and Complete method[14].

3. Results
Process of this experiment is presented by the flow chart in the Figure 1(a). Breast milk was spread and cultured on 3 different media aerobically and anaerobically. For the apparent feature, the number of colonies on each plate and how many conditions were influenced by yeast were tested by counting and microscopy images. Then we sequenced microbiological compositions on each condition to find out the exact strain types isolated by these conditions from breast milk.

3.1. Colony amounts
Colony amounts of each condition were shown in Figure 1(b). Two segments of the amount axis were used because of the giant difference for different conditions. Comparing with 3 used media, LBS media showed the best separative capacity followed by MRS (pH=5.2) media. MRS media had the weakest separative capacity among 3 tested media.

Interestingly, nothing grew up on LBS media under aerobic conditions. Except that, anaerobic conditions seemed like gave all the media better separative capacities. Without any dilution of breast milk sample, there were only around 2 colonies grew in LBS media and around 3 colonies grew in the MRS (pH=5.2) media under anaerobic conditions, which was too little for a productive probiotic isolation. While appropriate around 100 colonies were showed on MRS media under anaerobic
conditions, which made us conclude that on a colony amount level, isolating potential probiotics from breast milk via MRS agar under anaerobic conditions is better than other isolation conditions in this experiment.

Figure 1. (a) Flow chart of the process. (b) Colony amounts on different plates with tested conditions. (c) Percentages of 3 kinds of shapes in tested conditions. d. Morphology of yeast found in breast milk.

3.2. Yeast contamination resistant ability
Yeast grew up a lot under aerobic conditions and was found by the image of Gram staining. As it was shown in Figure 1(d), yeast had the same color with Gram positive strains. The size of yeast was 10 times bigger than Lactobacillus and Bifidobacterium with round or rob shape. Percentage of yeast was counted in Figure 1(c). among colonies selected for Gram staining imaging. Yeast contamination was only appeared under aerobic conditions. 57% of selected strains in MRS media and 97% in MRS (pH=5.2) media were yeast strains.

Figure 1(c) also showed different shapes grew up in 5 conditions. For the anaerobic conditions, only rod shape bacteria grew on LBS agar. The shape of 87% strains on MRS media under anaerobic conditions was round. While for MRS (pH=5.2) media, the shape of 89% strains were rod. Because the yeast contamination in the breast milk influenced the aerobic conditions heavily, there is no point to select potential probiotics under aerobic conditions.

3.3. Microbiological compositions
In order to deeply investigate which kind of bacteria grow up well on 6 different conditions, we sequenced the V4 region of 16s rRNA part based on Illumina Hiseq2000 platform. Around 1.2*10^5 reads were made of each sample. The ReadUtilizationRatios of all the samples were higher.
than 95%. Both the read and ReadUtilizationRatio told us the sequencing data was valid (data was not showed).

From the weighted percentage stacked bar, we can be told that the taxonomic composition of community under each condition except on aerobic LBS agar while no colony showed under that condition.

As can be seen from Figure 2(a), on phylum level, the major phylum in the original breast milk were Proteobacteria and Firmicutes. All the conditions had inhibiting capabilities on Proteobacteria and no Proteobacteria showed on anaerobic LBS agar and anaerobic MRS (pH=5.2) agar. Firmicutes were well enriched in all media anaerobically and aerobically. Compared with the sample taxonomic composition, Actinobacteria could grow up well on LBS agar under anaerobic conditions as a red part of anaerobic LBS represented as Actinobacteria appeared.

Weighted percentage stacked bar in Figure 2(b) showed taxonomic composition on genus level. Breast milk sample were composed mainly by Actinobacter, Pseudomonas, Staphylococcus and Enhydrobacter, as Lactobacillus and Bifidobacterium were not domain strains in the breast milk, which was presented under different conditions, both Lactobacillus and Bifidobacterium only appeared under anaerobic conditions. For aerobic conditions, Staphylococcus was the domain strain of MRS and MRS (pH=5.2) agar. For anaerobic conditions, Lactobacillus appeared on all of 3 kinds of media while

Figure 2. (a) Taxonomic composition of community on phylum level. (b) Taxonomic composition of community on genus level. (c) Venn diagram of oxygen environments. (d) Venn diagram of media. (e) Venn diagram of isolating conditions.
Bifidobacterium only grew up on LBS agar. Anaerobic MRS media was not a good choice for potential probiotics selection as the major strain on that was Staphylococcus. The unclassified strain in Figure 2(b) belonged to Enterobacteriaceae on kind level.

3.4. Alpha Diversity Analysis
We calculated the alpha diversity of each samples via MicrobiomeAnalyst online system using phyloseq package. Chao1 and ACE were chosen to estimate the richness of each condition. The Chao1 data and ACE data were consistent of each sample. Thus, we only presented Chao1 figure in Figure 3. From Figure 3(a) and table 1, it was very clear that anaerobic LBS media presented the most richness of OTUs compared with other conditions, which was followed by other two media under anaerobic conditions.
The OTU richness was lower under aerobic conditions on different media. Interestingly, both Chao1 and ACE of the control sample were lower than the 3 media under anaerobic conditions.

| Conditions             | sobs | Chao1  | ACE       | coverage     |
|------------------------|------|--------|-----------|--------------|
| Aerobic_MRS(pH=5.2)    | 20   | 23     | 42.6498   | 0.999895     |
| Anaerobic_LBS         | 20   | 65     | 396.2054  | 0.999830     |
| Anaerobic_MRS(pH=5.2) | 25   | 47     | 64.14869  | 0.999797     |
| Anaerobic_MRS         | 38   | 53.6   | 57.3441   | 0.999734     |
| Aerobic_MRS           | 23   | 35     | 45.47082  | 0.999843     |
| Mcontrol              | 51   | 55.7143| 61.6643   | 0.999792     |

3.5. Beta Diversity Analysis

Beta diversity analysis is a widely used way for people to estimate the composition distance of tested samples based on OTU composition. We used phyloseq package to calculate and present the results in a 2-D Principle Coordinate Analysis (PCoA) plot chart with bray distance. From Figure 3(b), all the conditions changed the OTU composition of original sample a lot which could be separated in two group via their distributions. One of the groups included aerobic MRS, aerobic MRS (pH=5.2) and anaerobic MRS conditions. In this group, the coordinates of aerobic MRS and aerobic MRS (pH=5.2) were the same. The other group included anaerobic LBS and anaerobic MRS (pH=5.2) conditions. The distribution of each sample indicated the OTU composition similarity among different conditions.

To gain more insight between different media and particular strains, we did hierarchical clustering analysis using hclust function in package stat and presented the result using heatmap and dendrogram. Euclidean was used for distance measure and ward.D was used for clustering algorithm.

The clustering of different conditions was consistent with the observation from PCoA. MRS (pH=5.2) and LBS media under anaerobic conditions were two which presented enrichments of our aiming strains. For Lactobacillaceae on family level, a significant enrichment was found on both anaerobic MRS (pH=5.2) media and anaerobic LBS media. While Bifidobacteriaceae were only widely found on anaerobic LBS media. However, for the rest of the conditions, neither Lactobacillaceae nor Bifidobacteriaceae was found significantly enrichment.

4. Discussion

Considerable research efforts have focus on microbiota isolation and culturing. For example, culturomics play important roles in human health improvement, new probiotics development, new antibiotics discovery and so on[15]. As plenty of new bacteria had been found by optimization of culture conditions and MALDI-TOF mass spectrometry, yet a few reports have been seen to focus on the media and conditions used for bacteria isolation, which really matters the downstream procedure and the further application of the aiming bacteria. In order to trace the connection between different conditions and particular samples, which was breast milk here, we isolated strains from breast milk under different conditions and tried to explain the isolation capability towards particular strains, Lactobacillus and Bifidobacterium, of tested conditions.

The media in this experiment are widely used for lactic acid bacteria isolation. MRS media is a classic non-selective media for Lactobacilli cultivation created by deMan, Rogosa and Sharpe in 1960[16]. LBS media, also known as Rogasa media was created for the isolation and enumeration of oral and fecal Lactobacilli[17]. Because pH had a significant effort on the growth of lactic acid bacteria, we regulated down the pH of MRS media from 6.5 to 5.2 as the third media used in this experiment. Considering breast milk is a critical resource for the infant gut microbiota establishment, we set two oxygen conditions as anaerobic environment and aerobic environment.
Very interesting results were found from colony amount and Gram staining imaging. From the colony amount results, nothing grew up on LBS media aerobically while the colony amounts of both MRS and MRS (pH=5.2) showed a huge difference. Colony amount on both media aerobically was much higher. The observation was explained latter by the Gram staining imaging. A heavy contamination of yeast was found on both MRS media and MRS (pH=5.2) media under aerobic conditions. Plenty of yeast colonies had similar features with lactic acid bacteria, which might be one of the reasons that why amount differences between different oxygen conditions were found on the same media. The salts composed LBS media might have ability to inhabit yeast growth. Although yeast can live in both anaerobic and aerobic conditions, it seemed that it didn’t grow up under the three media under anaerobic conditions. From this result, to select probiotics from breast milk sources, anaerobic conditions are necessary to prevent contaminations from yeast.

According to the sequencing data, Lactobacilli from breast milk only grow up under aerobic conditions although Lactobacilli is a kind of facultative anaerobe and always grew up well under aerobic conditions at 37 degree[18]. Surprisingly, considering the sequencing results, not LBS media but MRS (pH=5.2) media was the best choice for Lactobacilli among the three media under anaerobic conditions. This result might be influenced by oxygen conditions or pH environments. For Lactobacilli selection, although the number of colonies on MRS (pH=5.2) media was lower than expected, anaerobically MRS (pH=5.2) media is still the best choice among 6 tested conditions.

For Bifidobacterium selection, using LBS media under anaerobic conditions was the only condition detected with by 16s rRNA sequencing. But according to the Gram staining imaging, no typical bifid shape was found from the Gram staining imaging, instead of all the strains on LBS were rod shape on LBS media. Which indicated that it is not enough to distinguish Bifidobacterium from breast milk source only via its obvious bifid shape.

After calculating the alpha diversity of each samples, we noticed that all the anaerobic conditions had higher alpha diversities than aerobic conditions and original sample. One of the possible reasons is that some activated strains were not detected by 16s rRNA sequencing because that their abundance were lower than the detective threshold and after been spread and cultured on different conditions for 48h, these strains with low abundance were enriched and became detectable. Therefore, under anaerobic conditions, more strains from breast milk were enriched and gave these conditions higher alpha diversities. Given that breast milk is a critical source for the development of infants’ gut microbiota under the anaerobic environment of intestinal tract, it is not difficult to understand why there were higher alpha diversities under anaerobic conditions rather than aerobic conditions[19]. What’s more, findings like more kinds of strains were detected after enrichment under different conditions also be seen from other samples with low bacteria abundant, like samples from vaginal[20]. These results indicated multiple conditions culturing before low bacteria abundant sample composition detection might be helpful to conclude accurate results.

Although our experiment focused on the composition and diversity of multiplex isolating conditions which haven’t gain much focus so far. There were still some shortages of this research, including only one breast milk sample tested, considering the individual differences in the composition of breast milk bacteria, the sample can not represent the distribution of common bacteria in each case[21]. And no replicates for sequencing samples might introduce bias into results. However, our research provided a new angle of view into how does bacteria distribute under different oxygen conditions and media for specific sources. This result could potentially be applied to lead the selection of different probiotic candidates.

In conclusion, from our bacteria distribution results, according to the six tested conditions, culturing breast milk on LBS media under anaerobic conditions was the most suitable way to isolate Bifidobacterium from breast milk and using MRS (pH=5.2) media under anaerobic conditions was the most suitable way to isolate Lactobacillus in breast milk.

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