# Biodiversity Analysis of Lactic Acid Bacteria (LAB) in Naturally Fermented *Garcinia Mangostana* (GM) Pericarp

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***Abstract*— This study aims to isolate bacterial species and to enumerate the Lactic acid bacteria (LAB) species of Garcinia Mangostana (GM) pericarp cider at different fermentation stages as well as to establish bacterial growth profile during fermentation of GM pericarp. Throughout the fermentation process, cell mass concentration is determined by using cellular dry weight method. The highest cell dry mass obtained was 0.008 g at day 29 meanwhile the lowest was 0.001 g at day 88. Due to secretion of Lactic acid by LAB, the pH of the fermentation broth decreasing and overall pH range recorded at 3.46 to 4.00. Since the main concern of this paper is biopreservation and food safety of the GM cider, at this range of pH common foodborne pathogen unable to growth and survive. It is detected that LAB is a predominant species of microorganism present in the cider (2.85 – 6.04 log CFU/ml). The Gram stain done on the samples show the bacteria present starting at the beginning until the end the fermentation has a purple, violet stain when observed under microscope. Most of the sample appeared Staphylococcus, Staphylobacilli, Streptococcus and Streptobacilli. Based on this gram stain, LAB is the only type of bacteria that present during the fermentation period. During fermentation carried out, there were three phase where different strain of LAB dominated the cider.**

***Keywords*— Biodiversity, Lactic Acid Bacteria, *Natural fermentation, Garcinia mangostana pericarp, Purple Mangosteen.***

## I. INTRODUCTION

Garcinia Mangostana (GM) or known as mangosteen by local is common tropical fruit in subtropical region. Also known as "queen of fruit", it is believed that Mangosteen pericarp has a lot of medicinal values that can acts as immunomodulatory, antioxidant, antifungal, anti-inflammatory, antiviral and antibacterial. These therapeutic values are contributed by the richness of polyphenols especially xanthone, where α-mangostin, β-mangostin and γ-mangostin form its major constituents (Yu, et al., 2009). GM pericarps can be utilized as nutraceutical product and currently with sale worth of USD 800 million in 2008. Epidemiological reports suggested the reduction of cancer and degenerative diseases through consumption of active ingredients contained in the pericarp

GM fruit has its own defense system against microbial where it secretes a yellowish substance when the pericarp is damaged. This antimicrobial substance contained various bioactive components such as α-mangostin, β-mangostin, γ-mangostin, garcinone B, and garcinone E. These compounds can be classified as xanthone alongside other compounds such as mangostinone, tanins and falavonoid (Shibata, et al., 2013). Study showed α-mangostin and γ-mangostin acted as metastasis inhibitor of cancerous p53 mutant cell that caused breast cancer to human in animal model (Chae, et al., 2012). α-mangostin and γ-mangostin were shown inhibit the release of biological component from bone marrow-derived mass cell (BNMC) which related to the immune response. Response such as skin inflammation and allergic has been studied using BNMC obtained from male mice at different concentration of α-mangostin and γ-mangostin. The results show that these components suppressed the secretion of Interleukin (IL)-6 in inversely dose-dependent behavior as the concentration and mRNA decreasing compared to the latter. Similar results were obtained using prostaglandin D2 (PGD2) and leukotriene C4 (LTC4) where the expression of the RNA and β-Hexosaminidase (β-HEX) were diminished.

Cancer disease such as breast, prostate and ovary commonly caused by irregular production of fatty acid synthase (FAS) which promote the growth of the malicious tumor. Most cancer drug in the market is consumed to inhibit the FAS in order to reduce the spread of the tumor. Once the drug is taken, it helps lower the FAS without affecting healthy cell since FAS serves as anabolic energy storage for liver and adipose tissue. This has been proven by (Li, et al., 2014) where the cytotoxic properties of α-mangostin effective against breast cancer and reduce anti-apoptosis molecule. The effectiveness against the cancer lies on the FAS intracellular activity and intracellular synthesis of fatty acid required by the tumor cell to proliferate. These activity inhibit P12K/AKT and MAPK/ERK1/ERK2 which responsible for FAS expression when used in manner dose-dependent. The reduction of FAS providing anti-metastatic effects against FAK since the FAK is responsible for mediating growth factor of the cancer cell to extracellular environment.

## II. METHODOLOGY

### *A. Fermentation process*

GM pericarp was purchased from a local market in Kelantan. The pericarp part was washed with clean water and ground. The fermentation was carried out in 5L bioreactor (Sartorius) according to the following recipe: ground GM pericarp (10% w/v), glucose (10% w/v) and distilled water (90 %v/v). The fermentation was carried out for 88 days at ambient conditions with no agitation or pH control. Fermenter was air-tight and air vent was created to remove excess $CO_2$. Mild agitation was made and the pH of the cider was recorded before each sample withdrawal to obtain more representative bacteria sampling as anaerobic bacteria tend to settle down on the bottom of the fermenter.

### *B. Microbiological analysis: Growth Agar Media and broth preparation*

Analyses on microorganism were carried out on day 10, 17, 23, 29, 37, 45, 51, 59, 73, 80 and 88. Three kinds of media were used in this section for analysis and were obtained from the Biotechnology Laboratory of UiTM Shah Alam. Enumeration of total microorganisms and LAB bacteria present was carried out by adding 1 ml of fermentation broth into 9 ml of nutrient broth. The solution was diluted appropriately and plated on Nutrient agar and MRS agar. The plates were incubated at 37°C for an overnight. Colonies range of 1 to 200 with different morphology were calculated from the plate and expressed as log colony forming units (CFU) per ml of sample. The colony forming unit can be calculated using formula below

$$CFU/ml = \frac{no\ of\ colonies\ \times dilution\ factor}{volume\ of\ culture\ plate}$$

2 ml of sample also withdrawn and centrifuge two times and the supernatant were discarded from a falcon tube to obtain only the bacterial cell. The bacterial cell was dried at 80°C for 24 hours. All experiments were performed in duplicate.

### *C. Phenotypic characterization of present microorganisms*

Gram staining and cell morphology were observed under a Biological microscope MT 4200H (Meiji Techno, USA). The gram staining was determined by using Gram Staining Kit (Sigma-Aldrich, USA) according to the manufacturer's instruction. Both sample from Nutrient agar and MRS agar were observed under 400x magnification factor and tabulated.

## III. RESULTS

### *A. Cell density and pH change during fermentation.*

Figure 1 show a graph of cell dry mass against days of fermentation meanwhile for figure 2 show a plot of pH of the ferment cider against days of fermentation.

Generally, both graphs show fluctuation pattern throughout the fermentation durations. The plot of the cell dry mass increased from day 10 to day 29. At day 10, the cell dry mass was 0.006 g and increased 0.001 g for day 24 and keep increasing by 0.001 g at day 29. After day 29, change of pattern can be observed where the cell dry mass suddenly drop to 0.002 g at day 44 and static for day 51 and 59.

Later at day 66, the dry mass increased with value of 0.006 g and eventually drops drastically to 0.004 g for day 73, 0.002 g for day 80 and 0.001 g for day 88. From the statistic, day 29 shows the highest cell dry mass recorded meanwhile the lowest dry mass was at day 88. The results obtained are not very accurate even though the reading was carried out more than twice and two samples collected specifically for cell dry mass. Once the moisture and water has been evaporates from the sample, debris from GM pericarp was visible together with the dry mass and affecting the reading. This happens due to some of the pericarp's debris still floating in the sample although has been centrifuge twice at elevated speed and time.

The extraction of supernatant has been carried out very carefully but some bacterial cell managed to get sucked together with the supernatant. The growth of the cell can be said in a slow rate since no inoculation was carried out prior to fermentation. Due to that, the bacteria cells are still adapting to the changes of environment and nutrient resulting a small increases of cell density (Shuler, et al., 2014).

The readings of the pH were taken, observed and compared to cell dry mass. The pH was recorded and plotted as figure 2 in order to observe any changes to the pH of the cider which indicates the lactic acid released by LAB. Generally, similar to cell dry mass the pattern of the plot shows fluctuation from the start to the end of the process. The pH range of the fermentation about 3.46 to 4.01. The highest pH recorded throughout the fermentation was at three days after the fermentation.

By observing considering the graph trendline of the change of pH throughout the fermentation process, the pH of the cider decreases as the fermentation period increases. The decline in pH value resulting from the accumulation of organic acid in the fermentation broth, which mainly lactic acid as it is the main product of the fermentation. Since the fermentation was carried out in batch mode where there was no outflow and inflow of material, the bacteria keep consuming glucose available and convert it into lactic acid. The accumulations of protonated lactic acid are eventually changing the pH of the cider.

### *B. Colonial morphology and colony forming unit (CFU)*

Different colony morphology indicates more than one species inhabit on the agar surface. Most of the colonies appeared on the surface of the agar shown creamy like color with except for day 88 where three colors can be seen which creamy like, brownish and pinkish color. From the color itself it can be conclude that more than one species present in the Nutrient agar. The shape also varies from pin point, circular and unsymmetrical shape. Despite these differences, they also share similarities which all of them had smooth surface with wet texture.

The number of viable cell in the sample was calculated and expressed in terms of CFU/ml. The calculation was made based on the number of bacteria's colony appeared on both Nutrient and MRS agar. Since serial dilution was not carried out consistently, the number of viable cell of the day was taken based on the physical morphology of the colony whether the colony were apart from each other similar to study carried out by (Chuah, et al.,2016). The viable cell for total plate count was ranging from 2.48 CFU/ml to 6.04 meanwhile for LAB count ranging from 2.85 CFU/ml to 6.04. Complete list of plate count and the cell morphology are shown on table 2.

### *C. Gram stain and cell phenotype of microorganism present*

From the observation under microscope, all sample taken from both Nutrient agar and MRS agar shown purple violet stain. Theoretically, bacterial cell with this kind of stain is a gram positive bacteria cell. LAB is a gram-positive bacteria cell consists of coccus or bacillus phenotype that only able to grow in presence of oxygen. Mostly the bacteria were visible in clump or in chain arrangement and classified using Bergey's Manual of Determinative Bacteriology. The type of strain appeared in the sample were varies from Staphylococci, Staphylobacilli, Streptococci, Streptobacilli and Coccobacillus. At day 17, coccus shaped bacteria was detected with purple stain for Nutrient agar meanwhile for MRS agar, gram positive, staphylobacilli strain is observed. Different from day 17, day 23 and 29 shown staphylococci arrangement for nutrient agar meanwhile for MRS the results obtained for day 23 and day 24 was the same as previous day.

Recalling result obtained for pH changes of the cider, starting from the beginning of the fermentation period until day 29, the pH value decrease significantly thus shown the bacteria dominate the process at this stage were LAB strain with clump configuration. From day 37 until at the end of the fermentation period, Streptococci were observed for Nutrient agar sample meanwhile Streptobacilli arrangement for MRS agar. By analyzing overall result obtained, there were three phase in this fermentation. The first phase occurred at day 0 to day 29, where dominated by coccus and bacillus bacteria with clump configuration. Streptococci and Streptobacilli arrangement were dominate the second phase which happened at day 37 until day 59. The third phase occurred after day

59 where the LAB strain with same morphology as the phase two strain with ability to withstand pH below typical optimum range pH (pH 4 to 4.5).

## IV. Discussion

Up until today, there is no literature published on the naturally fermented Garcinia Mangostana fermentation as well as the isolation and identification of lactic acid bacteria related to this natural fermentation. The microbiological changes during 88 days of fermentation period was examined and studied to identify any LAB species present based on the morphology of the bacterial cell itself. The fermentation of the GM peel was carried out naturally without adding any starting culture to initiate the fermentation process. Although the GM peel was obtained in the market without knowing whether the fruits in the same orchard and from the same cultivars, it is believed that the microflora inhabit almost similar to one another. This is because the identification of the LAB bacteria is characterized based on the shape of the strain, gram stain and its dominance throughout the fermentation process. LAB is usually detected in naturally occurred traditional food fermentation such as Tempoyak, Silage, Kimchi and Mahewu (Chuah, et al., 2016; Caplice, et al., 1999; Hu, et al., 2015). To support the presence of LAB in the fermentation broth, the growth of the cell was measured by plate count method which expressed in colony-forming unit along with pH change along the 88 days of fermentation.

For the first three times sample taken which at day 10, day 24 and day 29 shown increases in cell mass meanwhile for the pH changes in fermentation broth decreases. According to (Chuah, et al.,2016) rapid acidification in medium can attributed to high initial LAB counts. As the LAB consumed carbon source available in the medium, the cell tend to carried out reproduction as survival method to sustain life. As the cell multiplied, more carbon source was being consumed resulting from more secretion of lactic acid resulting from respiration process. By referring to the gram-staining of day 17, day 23 and day 29, all staining for both nutrient agar and MRS agar show the cell were a gram-positive and has coccus and bacillus shape. The LAB bacteria are normally has coccus or bacillus shaped and gives purple violet stain when observed under microscope (Teuber, et al., 2008).

After day 29, the cell dry mass of the decreases drastically at day 44 and the cell density remain unchanged for day 51 and 59. This pattern is similar and reported by (Muyanja, et al., 2003). Meanwhile for pH, the pH increases from day 29 to day 51 and eventually drops at day 59. The sudden drop of cell mass of LAB because the growth of lactic acid bacteria was slow at low pH. Furthermore, at low pH, the cell loss its viability and the acidification causing damage to the cell as well (Hutkins, et al., 1993). At day 29, the pH value was lower than optimum pH range for the growth of LAB (range of 4.0 to 4.5). According to (Hutkins, et al., 1993), due to this over acidification, the bacterial cell tend to lose activity and the growth of LAB species such as streptococci and lactococci decreased significantly at pH 5. Below than pH 5, cell lysis and internal component leakage occur. By observing the gram stain at day 44, day 51 and day 59 shows purple violet stain when observed under microscope at 400x magnifications. Sample taken from Nutrient agar shows the bacteria grow on the agar surface has shape of coccus meanwhile bacillus shape for MRS agar.

The depletion of pH medium throughout the fermentation process because of the accumulation of organic acid which mostly lactic acid. Although the accumulation of lactic acid decreased the pH of the medium, the internal pH of the cell is alkaline since the cell rapidly transports the protonated lactic acid out of the cell. The membrane of the cell is relatively impermeable to the proton outside of the cell. Difference in pH between intracellular and extracellular of the cell creates pH gradient. The maintenance and formation of this pH gradient is vital not only to achieved relative stable equilibrium as well as a component of proton motive force. When the pH of the fermentation medium was slightly decreases, the LAB bacteria able to maintain and regulate the pH homeostasis of the medium. However, when the pH drop drastically, the cell unable to maintained pH difference and eventually the pH difference collapse and the cell viability drops (Hutkins, et al., 1993)

Since there was no starter culture added into the fermentation broth, more than one LAB species might present. This is because although the cell density remained unchanged at day 44, 51 and 59, there is increased in cell dry mass at day 66. By recalling information from (Caplice, et al., 1999), typical pH range from pH 4.0 to 4.5 but some are active at pH 9.6 and 3.2. This shows that other strain of LAB bacteria dominate after day 59. By referring to the gram stain at day 59 the sample appeared as gram positive for both Nutrient agar and MRS agar. After day 59, the average cell density decreasing until it reached 0.001g at day 88. The cell depletion might resulting from ran out of carbohydrate, amino acid and other growth promoting nutrient, accumulation of toxic and inhibiting compounds, as well as sudden drop or increased in hydrogen ions that out of range the bacteria can tolerate (Hutkins, et al., 1993). At these point, the trendline of the graph shows the pH decreasing until it reached pH 3.59. According to (Muyanja, et al., 2003), the decreased in pH shows decreased in lactic acid concentration due to utilization of yeast present in the fermentation broth. This is because according to their research, at the initial state of the fermentation bushera, an Uruguan fermented beverages, is dominated by LAB and coliforms meanwhile for late stage, LAB and yeast dominate by LAB and yeast. Coliform bacteria are gram negative bacteria that ferment lactose with production of acid and gas as product of respiration. This rod shaped bacteria is usually found in soil, aquatic environment and on vegetation (Feng, et al., 2002). This type of bacteria might also inhabit the GM pericarp but there is no gram negative stain reported for day 44, 51 and 59.

In some cases, yeast also present in the fermentation medium and create mutualism with lactic acid bacteria. It is postulate that the LAB carried out acidification which optimum for yeast proliferation meanwhile yeast provides vitamin and other growth nutrient for the LAB (Steinkraus, 1996). The staining of the sample might as well contain yeast cell since in this research, the fermentation process is carried out without starter culture thus mixed culture of microflora is present (Chuah, et al., 2016). Different from (Steinkraus, 1996), (Chuah, et al., 2016) reported that the yeast only present on the first day of the fermentation and not detected the day after that. According to the literature, as the LAB increases, the proliferation of yeast decreases since the yeast cells inhibit by the acetic and propionic acid secreted by the LAB.

Decreases in pH of the fermentation broth due to accumulation of organic acid especially lactic acid inhibits the growth of coliform microorganisms which causing food poisoning to human. Since fermented food high in acidity, its consumptions without any heat treatment such as cooking and pasteurization is off concern (Chuah, et al., 2016). This condition indirectly provides bio-preservative to the fermented food since most coliform bacteria are acid intolerance (Muyanja, et al., 2003). Furthermore, LAB has ability to secrete bacteriocins that can lyse the pathogenic microorganism and kill them. Thus, the shelf life of the fermented food can be extended since the LAB strain overcompetes with other non-desirable bacteria (Mokoena, et al., 2010). However, (Cho., et al., 2001) reported survival of E.coli and L.monocytogenes in the kimchi sample although the number of cells is low and not fully inhibited. They conclude that the coliform bacteria might adapt to acid environment thus prolong the survival in the fermentation medium. The coliform bacteria present at any point of fermentation are able to survive 8-12 day after contamination (Chuah, et al., 2016).

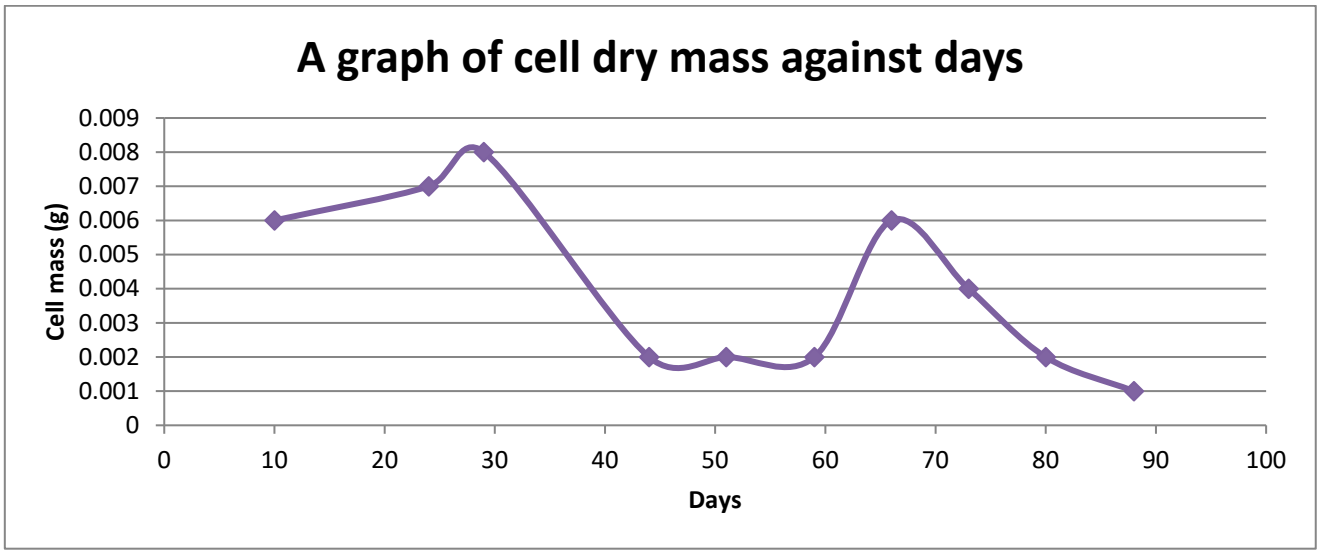


Figure 1: A graph of cell dry mass against days of GM pericarp fermentation.

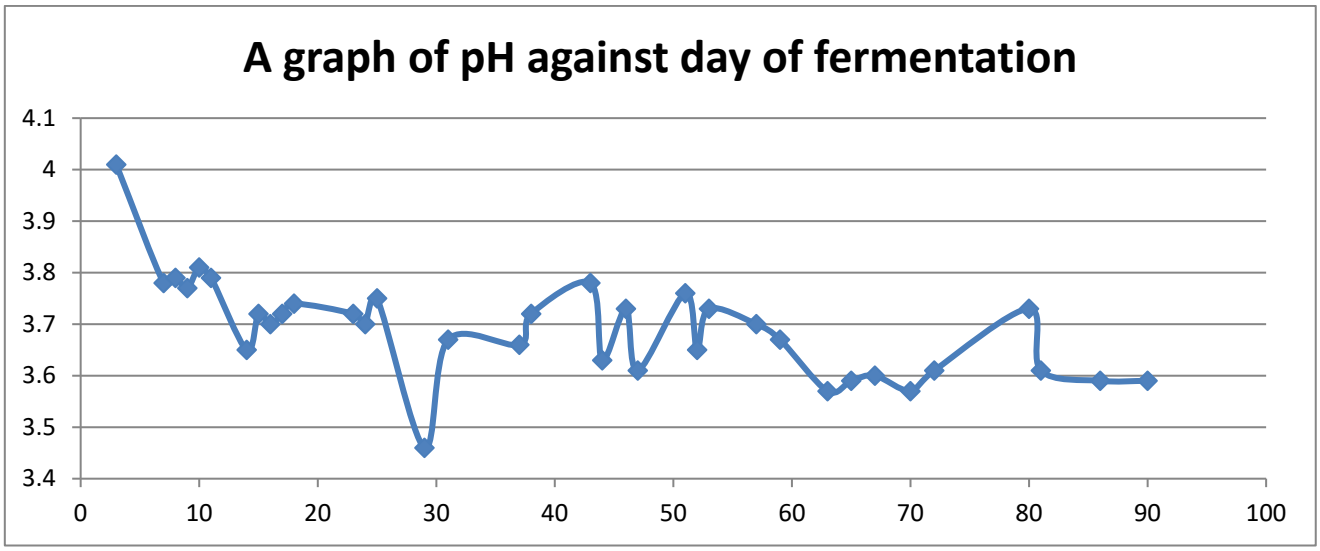


Figure 2: A plot of pH of fermentation medium against days of fermentation

Table 1: Plate count for both Nutrient agar and MRS agar with respective cell morphology of sample. (Note: N.D ., no data)

| Days | Plate count (log CFU/ml) | | Cell morphology | | | |
|---|---|---|---|---|---|---|
| | Total plate count | LAB count | Nutrient agar | Gram stain | MRS agar | Gram stain |
| 10 | 4.60 | 3.78 | N.D | N.D | N.D | N.D |
| 17 | 4.00 | 3.00 | Coccus | + | Staphylobacilli | + |
| 23 | 2.48 | 3.13 | Staphylococci | + | Staphylobacilli | + |
| 29 | 2.90 | 4.04 | Staphylococci | + | Staphylobacilli | + |
| 37 | 3.48 | 3.00 | Streptococci | + | Streptobacilli | + |
| 45 | 3.32 | 2.85 | Streptococci | + | Streptobacilli | + |
| 51 | 6.49 | 5.08 | Streptococci | + | Streptobacilli | + |
| 59 | 5.60 | 6.04 | Streptococci | + | Streptobacilli | + |
| 66 | 4.00 | 4.04 | Streptococci | + | Streptobacilli | + |
| 73 | 5.83 | 5.08 | Streptococci | + | Streptobacilli | + |
| 80 | 5.86 | 4.23 | Streptococci | + | Streptobacilli | + |
| 88 | 5.68 | 5.18 | Streptococci | + | Streptobacilli | + |

## V. Conclusion

Based on the results obtained, it can be concluded that LAB were present from the start till the end of the fermentation period. This postulation can be support by analyzing the pH changes of the broth, the cell dry weight and the stain of the sample. Based on the plotted cell dry weight and pH against time, more than one strain of LAB dominated the fermentation process. Additionally, the gram stain shown both coccus and bacillus shaped bacteria present in chain and clump arrangement at the same time. The stain also shown purple-violet color when observe under microscope. Since there are no food pathogen able to invade and dominate the fermentation process based on the gram stain, it can be said that LAB able to provide biopreservation and food safety against the foodborne pathogen to the cider through secretion of the bacteriocins. In future works, differentiation and genotyping of LAB strain should be carried out thus dominant LAB can be used as inoculant. Furthermore, the strains' resistivity towards antibiotic and survival of foodborne pathogen has to be done to ensure the viability of the strain.

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