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RESEARCH ARTICLE

Effect of ultrasonication on biofilm forming ability of food pathogens in milk model system

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Abstract: Biofilm formation in the dairy industry threatens product safety and can lead to food-borne illness. The study investigated the effect of ultrasonication as a nonthermal technique on biofilm formation, hydrophobicity, and injured-cell recovery of common dairy pathogens. Five reference strains (*Escherichia coli* ATCC 10536, *Staphylococcus aureus* ATCC 700698, *Bacillus cereus* ATCC 10876, *Bacillus subtilis* NCDC 71, *Bacillus megaterium* NCDC 67) were screened by Congo red plate assay and microtiter plate assay. Specific Biofilm Formation (SBF) values identified *B. megaterium* (SBF 2.51) and *S. aureus* (SBF 1.22) as the strongest biofilm-forming strains and selected for further studies. Sterilized skim milk spiked with selected strains was used as sample matrix for ultrasonication experiments. Ultrasonication parameters were standardized at 20 KHz frequency, 750W power 80% amplitude for 20 min. Biofilm formation on stainless steel chips was evaluated using ultrasonicated milk, showing adhered-cell reductions of 2.94 log in *S. aureus* and 3.47 log in *B. megaterium*. Cell surface hydrophobicity declined markedly after treatment, indicating reduced adhesion potential. Post-treatment recovery at 37 °C showed an increase in CFU/mL count for both *S. aureus* and *B. megaterium* after 12 h, indicating potential recovery under favourable conditions. This study underscores that optimized ultrasonication reduces both planktonic and surface-attached populations, providing a product-friendly, non-thermal approach to enhance quality assurance in dairy processing.

Keywords: Biofilm, Hydrophobicity, Injured cell recovery Pathogens, Skim Milk, Stainless steel chip, Ultrasonication

Introduction

Biofilm is a collection of diverse communities of microorganisms embedded in an extracellular matrix that forms on a variety of environmental surfaces. Biofilms are extremely complicated in nature, especially in multi-cultural settings. Microbes build biofilms to follow a protected development path, allowing cells to survive in hard environments, reproduce, and disseminate to other environments (Zhao et al. 2023). Biofilms are related to a range of issues in various situations, including the spread of disease through medical devices, the creation of dental plaque, which leads to tooth damage and cross contamination in food processing areas. Biofilm contamination of dairy products is common and eradicating such germs can be difficult (Jamunkar et al. 2024). Biofilm formation on dairy industry equipment can cause major sanitary issues as well as financial losses due to food spoiling. Because of their resistant nature, biofilms are difficult to eliminate. Conventional cleaning and disinfection methods, on the other hand, may contribute to ineffective biofilm control and resistance spread. Several non-thermal methods such as Pulsed Electric Fields (PEF), Cold Plasma, and High Pressure Processing (HPP) have been explored for biofilm control in food systems (Safwa et al. 2024). PEF is effective in liquid matrices through electroporation; however, its efficacy may be limited by restricted penetration into structured biofilms, dependence on electrical conductivity of the product, and sensitivity of certain food components to high electric fields (Juncker et al. 2022). Cold plasma generates reactive oxygen and nitrogen species capable of disrupting microbial cells and extracellular polymeric substances (EPS), yet limited penetration depth, longer treatment durations, and variability in microbial susceptibility may affect its uniform performance (Rahman et al. 2022). HPP inactivates microorganisms via membrane disruption and protein denaturation, though its effectiveness can vary against pressure-resistant microorganisms, and certain enzymes and nutrients may exhibit sensitivity to high pressure. Applications of HPP is mostly

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limited to packaged products (Abera, 2019). These limitations justify further investigation of alternative non-thermal approaches for biofilm control in milk systems. Ultrasound, a type of energy produced by sound waves, typically exceeding 16 kHz (Bhargava et al. 2021), finds extensive application in the dairy industry for both modifying and analyzing milk products. The utilized sound frequencies fall into two groups: high-frequency ultrasound, and low-frequency ultrasound. The primary purpose of employing ultrasound on microorganisms lies in inducing cavitation.

The development of new biofilm regulator techniques that adhere to the requirements for use in the food industry appears to be a right step towards the fight against biofilm antimicrobial resistance. Biofilm control studies using non thermal ultra-sonication is lacking and this requires further attention. In this research, skim milk will serve as the suspension medium for recovering injured pathogenic cells post-ultra-sonication treatment. The significance of these injured cells lies in scenarios where dairy plants store milk for varying durations before further processing; under such conditions, these cells may potentially recover, proliferate, and contribute to spoilage. Elucidation of these gaps in our study will improve significantly the possibilities of applying these biofilm control measures in a real industrial environment. Hence, the present study is structured to employ ultra-sonication as a non-thermal method to assess its impact on the biofilm forming capacity and recovery of injured cells among foodborne pathogens.

Materials and Methods

Identification of pathogenic strains

Five pathogenic cultures for screening of biofilm formation were obtained from the Department of Dairy Microbiology, College of Dairy Science and Technology, Ludhiana, Punjab (Table 1). Stock cultures were preserved at -80ºC. Before any assay, strains were revived by transferring stock cultures into Brain Heart Infusion (BHI) medium and further placed at 37ºC for 24 h. Purity of every culture was ascertained by doing Gram's staining and catalase test. The storage of cultures was done below 5ºC between transfers.

Biofilm quantification by microtiter plate assay

Biofilm formation by the test pathogens was quantified using the microtiter plate assay described by Sánchez et al. (2016). Specific biofilm formation (SBF) was calculated using the formula,

SBF= (AB-CW)/G

Where, AB is the OD_{570} nm of the attached bacteria, CW is the OD_{570} nm of the control wells containing only broth, G is the OD_{630}nm of cell growth in broth. Strains were compared based on SBF values to identify the strongest biofilm formers for further experimentation in triplicates.

Ultrasonication treatment of spiked milk samples

Skim milk samples were obtained from the Experimental Dairy Plant of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana. Skim milk was autoclaved and cooled to room temperature. 50 mL of milk was inoculated with the highest biofilm-producing strains at a concentration of approximately 10^5 CFU/mL. The inoculated milk samples were subjected to ultrasonication in 100 mL sterile glass beakers placed in an ice bath to maintain temperature at ~0°C. A probe-type ultrasonicator having 12 mm diameter operating at 20 kHz frequency and 750 W power was used. The treatment was applied in pulsed mode with 10 seconds on and 2 seconds off cycles, allowing intermittent energy delivery to prevent overheating and maintain consistent cavitation effects. Sonication was performed at amplitude 80% for 20 minutes.

Evaluation of biofilm formation on stainless steel chip in ultrasonicated milk

Stainless steel chips were fabricated to dimensions of 2.54 × 2.54 cm, providing a total surface area of 6.45 cm^2. The chips were thoroughly cleaned by vigorous brushing in 70% ethanol, followed by washing with sterile distilled water. Chips were placed in sterile glass petri dishes and sterilized by autoclaving. Biofilm formation on stainless steel (SS) chip with selected pathogens under lab condition with sonicated milk was evaluated by using method of Jamunkar et al. (2024). To evaluate biofilm formation, sterile stainless steel (SS) chips were aseptically immersed in both sonicated and control milk samples inoculated with the test organisms. The samples were incubated at 37°C for 48 h in a shaking incubator to facilitate biofilm development on the chip surface. After incubation, the chips were aseptically removed using sterile forceps and gently rinsed three times with sterile phosphate-buffered saline (PBS, pH 7.2) to remove loosely attached cells. The cells remaining attached to the SS surface after PBS washing were considered as biofilm-forming cells for further analysis.Biofilm cells were recovered from stainless steel (SS) chips using a standardized swabbing method. Each SS chip was aseptically transferred to a sterile Petri plate containing 10 mL of sterile 0.85% saline solution. The entire surface area of the chip was swabbed using a sterile cotton-tipped swab in vertical, horizontal, and diagonal directions to ensure maximum recovery of attached cells. The swab was then immersed in the same saline solution to release the dislodged biofilm cells. The resulting suspension was subjected to ten-fold serial dilution in sterile 0.85% saline to obtain countable bacterial concentrations. The log reduction in attached cell counts was calculated by comparing the mean CFU/mL values of ultrasonicated samples with those of the control group. This calculation provided a quantitative

measure of the efficiency of ultrasonication in biofilm removal. All experiments were performed in triplicate to ensure reproducibility and statistical reliability of the results.

Evaluation of adherence capability of pathogenic strains by determining cell surface hydrophobicity

The adherence capability of the selected pathogen was evaluated by determining its cell surface hydrophobicity using assay of Danchik & Casadevall (2021). Absorbance was measured at 600 nm. The percentage hydrophobicity was calculated as:

$(A_{f-} A_i)/A_i$,x100

where A_i represents the initial OD of the bacterial broth before mixing with xylene and A_f represents the OD of the aqueous phase after separation. This assay was performed in triplicates for each sample to ensure reproducibility and to compare the adherence characteristics of bacterial cells before and after ultrasonication treatment.

Evaluation of post ultra-sonication injured cell recovery of selected pathogenic bacteria from treated skim milk samples

Two selected bacterial strain exhibiting the highest biofilm-forming ability, as identified from the microtiter plate assay in experiment, was further evaluated for post-ultrasonication cell recovery in skim milk as described by Ashokkumar et al. (2009). Ultrasonication of the spiked milk sample was performed at 80% amplitude for 20 minutes using a probe-type ultrasonicator (20 kHz frequency, 750 W power). The treated and control samples were then incubated at 37°C, and additional plating was carried out at time intervals of 1, 2, 4, 8, and 12 hours post-treatment using the same serial dilution and pour plate technique. All plates were placed at 37°C for 24–48 hours, after which the colonies were counted. The percentage recovery of injured cells was calculated.

Statistical analysis

Under the supervision of a statistician, data gathered from numerous experiments during the screening and comparative analysis process were analyzed for two-way analysis of variance (ANOVA) using SAS 9.3 version. Microsoft excel was used to calculate the mean, standard error of data, when needed.

Results and Discussion

Assessing purity of commercial pathogenic strains

All the strains were found to be pure under microscope, shown characteristic appearance on their selective growth medium and were catalase positive (Table 1).

Screening and selection of strong biofilm formers

The biofilm-forming ability of the bacterial strains was evaluated using the microtiter plate assay, and their biofilm production was quantified based on Specific Biofilm Formation (SBF) values. The results, as presented in Table 1, show variation in SBF values among the tested bacterial strains.

Among the tested strains, *B. megaterium* and *S. aureus* exhibited the highest SBF values 2.51 and 1.22, indicating their strong ability to form biofilms under the tested conditions. The SBF index allowed for differentiation between high and low biofilm producers by minimizing the influence of growth differences. These results suggest that *S. aureus* and *B. megaterium* possess enhanced adhesion and biofilm maturation capacity on compared to the other tested organisms. This indicates a higher biofilm biomass for these two organisms under the assay conditions, consistent across the triplicate wells.

The high biofilm-forming ability of *Bacillus megaterium* observed in the present study is supported by the findings of Stancu (2020), who demonstrated that *B. megaterium* IBBPo17 exhibited increased production of extracellular polymeric matrix (EPM)—a key indicator of biofilm development. The ability of *B. megaterium* to form substantial biofilm under selective environmental conditions, as demonstrated in their study, reinforces the idea that this species possesses inherent biofilm-forming potential.

The high biofilm formation observed in *Staphylococcus aureus* in the present study is supported by previous research demonstrating the key role of polysaccharide intercellular adhesin (PIA) in the development and stability of *S. aureus* biofilms. According to Idrees et al. (2021), PIA is the major component of the extracellular polymeric substance (EPS) in *S. aureus* biofilms. This cationic polysaccharide mediates strong intercellular adhesion, enhances colonization ability, and contributes significantly to immune evasion, antimicrobial resistance, and

Table1: Purity testing of Pathogenic strains and their Specific biofilm formation (SBF) values

Sr. No.	Name of Bacterial strain	Gram's Staining	Catalase test	SBF Value*
1	*Escherichia coli* ATCC 10536	Gram -ve	Catalase +ve	0.20±0.1
2	*Staphylococcus aureus* ATCC 700698	Gram +ve	Catalase +ve	1.22±0.1
3	*Bacillus cereus* ATCC 10876	Gram +ve	Catalase +ve	0.65±0.17
4	*Bacillus subtilis* NCDC 71	Gram +ve	Catalase +ve	1.12±0.05
5	*Bacillus megaterium* NCDC 67	Gram +ve	Catalase +ve	2.51±0.1

*Average of triplicate trials with standard error

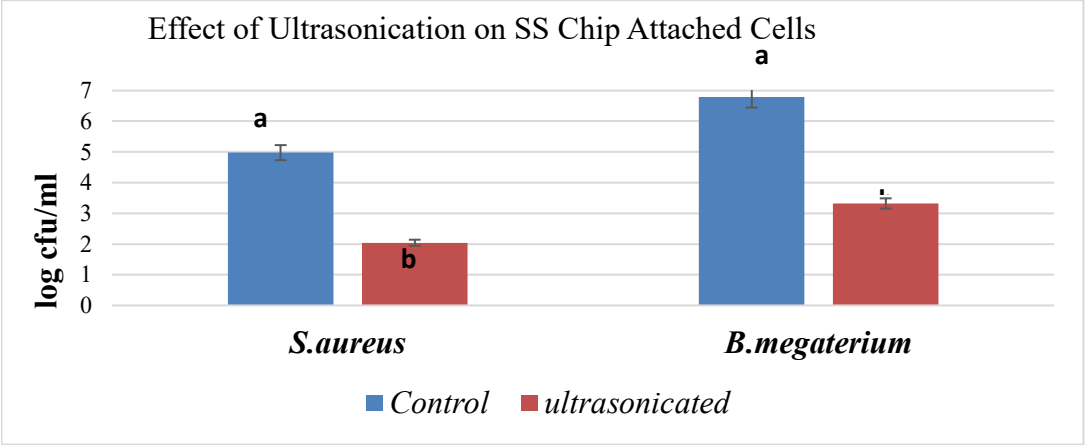


Fig. 1 Log CFU/mL values of *S. aureus* and *B. megaterium* attached to stainless steel chips before (control) and after ultrasonication (80% amplitude for 20 min). Different superscript letters (a & b) within the same row showed significant differences among the groups (P<0.05) and (n=3)

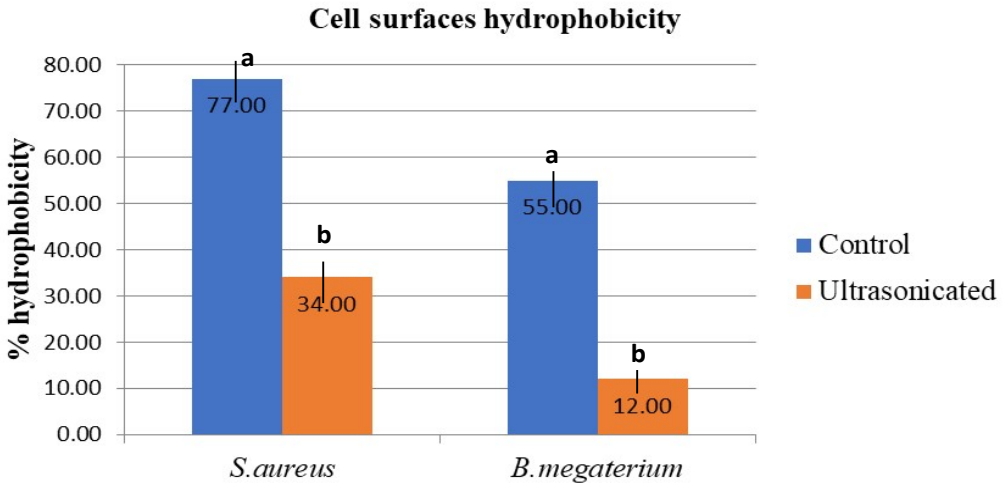


Fig. 2 Cell surfaces hydrophobicity of pathogens. Different superscript letters (a & b) within the same row showed significant differences among the groups (P<0.05) and (n=3).

persistence in biofilm-related infections. The presence and function of PIA thus provide a mechanistic explanation for the high biofilm biomass observed in our study.

Evaluation of Biofilm formation on stainless steel chip in ultrasonicated milk

The results showed that in the control group (non-ultrasonicated), *S. aureus* exhibited a biofilm count of 4.98 log CFU, while *B. megaterium* showed a significantly higher count of 6.79 log CFU (Figure 1). Our findings align with Vargová et al. (2023), who reported that *S. aureus* exhibited a heightened capacity to adhere and form biofilm on stainless steel surface (SS).

The number of *S. aureus* cells attached to SS surfaces after various hours of incubation is analyzed. The analyzed isolation exhibited a number of adherent cells ranging from 4.26 to 6.59 log CFU/cm^2. The largest number of adherent cells (6.59 log CFU/cm^2) on SS surfaces had been identified after 48 hours. In the ultrasonicated samples, a substantial reduction in adhered biofilm cells was observed. *S. aureus* showed a post-treatment biofilm count of 2.04 log CFU, corresponding to a 2.94 log reduction from the control. *B. megaterium* exhibited a count of 3.32 log CFU, with a 3.47 log reduction compared to its control. These findings suggest that ultrasonication significantly disrupted ($p < 0.05$) biofilm development in both organisms. Du et al. (2021), also showed that ultrasonic vibrations led to a decline of *S. aureus* adhesion on stainless-steel surfaces.

Effect of ultrasonication on pathogens attachment tendency (cell hydrophobicity)

Both pathogens were exposed to ultrasonication for 20 minutes at 80% amplitude and subsequently allowed to attach to stainless steel (SS) surfaces at 37 °C for one hour. The attachment behavior is shown in Figure 2. In our study, *B. megaterium* exhibited the lowest hydrophobicity following treatment, while *S. aureus* showed the highest. A positive correlation was observed between cell hydrophobicity and attachment, indicating that greater spore hydrophobicity promotes stronger adhesion to surfaces.

S. aureus typically exhibits high cell surface hydrophobicity, with wild-type strains showing ~70–80% adhesion to hexadecane in the MATH assay (Lather et al. 2016). Fadila (2016) showed the

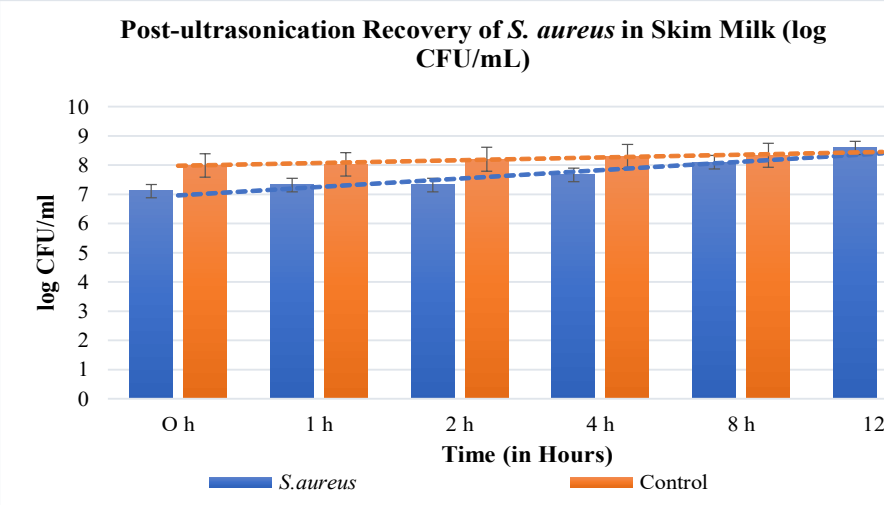


Fig. 3 Post-ultrasonication recovery of *S. aureus* in skim milk (log CFU/mL) at different time intervals. Values are average of triplicate trials with standard error

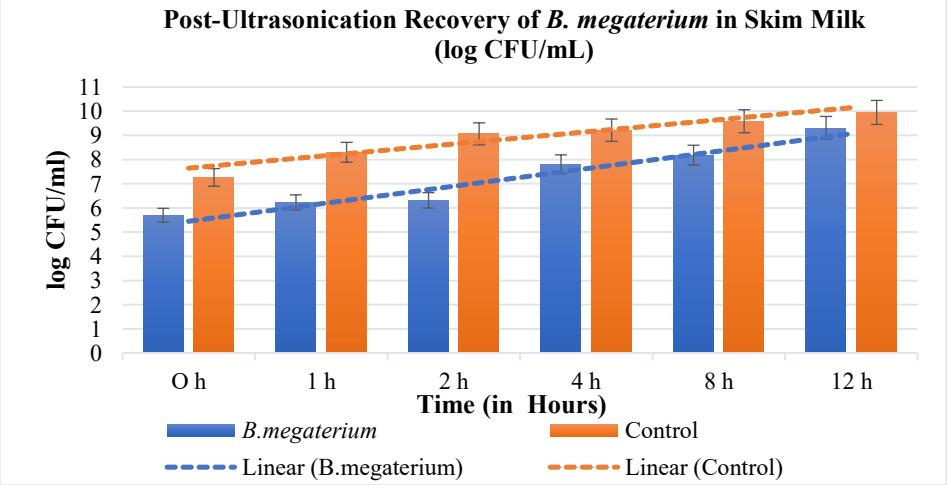


Fig. 4 Post-ultrasonication recovery of *B. megaterium* in skim milk (log CFU/mL) at different time intervals Values are average of triplicate trials with standard error

similar results in which *Bacillus cereus* hydrophobicity values from 42.1 to 51.6%.

Evaluation of post ultra-sonication injured cell recovery of selected pathogenic bacteria from treated skim milk samples

The recovery pattern of ultrasonication-injured *Staphylococcus aureus* in skim milk was evaluated over a 12-hour period and compared with an untreated control. Immediately after treatment (0 h), the viable count of *S. aureus* in the sonicated sample was 7.11 log CFU/mL. This value was considered the baseline for calculating the recovery of injured cells, and the Δ log CFU/mL at 0 hour was taken as zero. A gradual increase in viable count was observed over time, reaching 8.59 log CFU/mL at 12 hours. The untreated control sample started at 7.99 log CFU/mL and showed only a slight increase to 8.45 log CFU/mL over the same period (Figure 3).

In the case of *Bacillus megaterium*, a more substantial recovery trend was observed following ultrasonication. The viable count immediately after treatment (0 h) was 5.70 log CFU/mL and served as the baseline for recovery calculations (Δ log = 0 at 0 hour). Over the 12-hour observation period, the count increased significantly to 9.31 log CFU/mL. The control group started at 7.26 log CFU/mL and reached 9.95 log CFU/mL at 12 hours (Figure 4). This highlights the inherent resilience of *B. megaterium* and underscores the importance of monitoring injured but viable cells in post-processing environments, as they can regain full metabolic activity and potentially compromise food safety. A study done by Fan et al. (2021) reported that on thermosonication treatments of *B.* subtilis spores, partial inactivation was reported along with morphological damage; however, spores sometimes remained able to initiate germination, indicating that some surviving cells retain recovery potential depending on the severity of injury and recovery environment.

Conclusions

From the outcome of this investigation, we can draw the conclusion that *S. aureus* and *B. megaterium* were the strongest biofilm formers among the tested strains, reflecting their

persistence and adaptability in dairy-associated environments. Ultrasonication, when applied under optimized conditions, was effective in reducing planktonic cell populations and significantly disrupting biofilm development on stainless steel surfaces, indicating its potential as a non-thermal intervention for biofilm control in food processing systems. Nevertheless, post-treatment recovery studies demonstrated that both organisms, despite sustaining sublethal injury, retained the ability to recover and proliferate in a favourable growth medium such as skim milk. This resilience highlights the possibility of recontamination and emphasizes the need for integrated control strategies that combine microbial inactivation with measures to prevent the regrowth of injured cells. Overall, the study supports the use of ultrasonication as a promising biofilm control approach in dairy systems offering a non-thermal alternative to target specific pathogenic microorganisms while preserving the quality and nutritional attributes of the product. The challenges faced by ultrasonication in industrial implementation includes high equipment and installation costs, process integration into existing production lines, and operational and maintenance complexity. These factors may limit large-scale adoption despite its promising potential in dairy processing.

These findings provide a strong foundation for future investigations into scaling up ultrasonication technology and integrating it into industrial cleaning and sanitation protocols for enhanced food safety.

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Ethical Considerations: This article does not involve any studies involving human or animal subjects.

Declaration of Conflicts of Interest: The authors confirm that there are no conflicts of interest to disclose.

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