Invited review: Current perspectives for analyzing the dairy biofilms by integrated multiomics

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ABSTRACT

Biofilms formed by pathogenic or spoilage microorganisms have become serious issues in the dairy industry, as this mode of life renders such microorganisms highly resistant to cleaning-in-place (CIP) procedures, disinfectants, desiccation, and other control strategies. The advent of omics techniques, especially the integration of different omics tools, has greatly improved our understanding of the features of microbial biofilms, and provided in-depth knowledge on developing effective methods that are directly against deleterious biofilms. This review provides novel insights into the single use of each omics tool and the application of multiomics tools to unravel the mechanisms of biofilm formation, specific molecular phenotypes exhibited by biofilms, and biofilm control strategies. Challenges and future perspective on the integration of omics tools for biofilm studies are also addressed.

Key words: biofilm formation, dairy safety, dairy quality, integration of omics

INTRODUCTION

Biofilms are defined as complicated communities of microorganisms adhering on solid, liquid, or air interfaces, and forming a spatially 3-dimension structure consisting of self-secreted extracellular polymeric substances (EPS; Sauer et al., 2022). The biofilm lifestyle has been confirmed as the most predominant mode of microorganisms in different food sectors, including the dairy industry (Yuan et al., 2020a). Studies have proved that biofilms formed by lactic acid bacteria (LAB) may promote the quality of foods and inhibit the growth of foodborne pathogens (Yao et al., 2022; Yuan et al., 2022). However, microbial biofilms in dairy environments are generally composed of a variety of pathogenic or spoilage microorganisms (Flint et al., 2020). The most distinguishing behavior of microbial biofilms is their great capacity to withstand control methods, including the cleaning-in-place procedures (CIP) normally used in the dairy industry (Toushik et al., 2020; Yuan et al., 2021). This in turn, elevates the persistence of microbiological contamination in the dairy industry, and eventually affects the safety and quality of dairy products.

Considering the complex nature of biofilms in the dairy industry, the use of omics techniques, including genomics, transcriptomics, proteomics, metabolomics, and even their integrations, significantly benefits the biofilm studies (Wang et al., 2020, 2021a; Yuan et al., 2023). Especially, the integration of omics tools provides the multilayer insight into microbial compositions, content and function of genes, and metabolites of biofilm cells (Du et al., 2020; Liu et al., 2021b). However, to the best of our knowledge, studies providing a comprehensive picture of dairy biofilms by integrating different omics tools are scare.

This review summarizes the most updated studies where single-omics tool and their integrations have been applied for dairy biofilm studies. Challenges and perspectives on multiomics tools for biofilm studies are also included. From a practical view, an improved knowledge of events may help the industry to reduce the biofilms formed by pathogenic or spoilage microorganisms and therefore enhance the safety and quality of final dairy products.

BIOFILM IN THE DAIRY INDUSTRY

Distribution of Biofilms in the Dairy Industry

Milk is generally believed to be the perfect medium for the growth and biofilm formation of microorganisms in the dairy environment. In addition, the dairy processing line provides an ideal environment for biofilm formation because of the extreme complexity
of facilities. The complex biofilm matrix and altered physiology of biofilm cells protect microorganisms against disinfectants, antibiotics, pH, UV radiation, and desiccation strategies in the dairy industry (Kumari and Sarkar, 2018; Yuan et al., 2020b; Wang et al., 2022a). Therefore, biofilms may occur at any point through various dairy processing sites, including raw milk tank, spiral-wound membranes, wooden vats for cheese production, dairy reverse osmosis, ultrafiltration membranes, sprinklers from dairy farm cooling systems, and even the dairy waste water system (Shpigel et al., 2015; Dixon et al., 2018; Verma et al., 2021; Sun and D’Amico, 2023). Previous studies have indicated the capacity of both gram-positive bacteria (Lactobacillus, Bacillus, Staphylococcus, Streptococcus, Listeria, Lactococcus, Exiguobacterium, Kocuria, and Micrococcus) and gram-negative bacteria (Acinetobacter, Pseudomonas, Cronobacter, and Chryseobacterium) to adhere on surfaces of metals, glass, rubbers, and plastics, and then form biofilms within several days or even hours (Zou and Liu, 2018; Woo et al., 2023). Such results strongly indicate the need for optimizing the dairy plant design and establishing effective CIP procedures to avoid unwanted dairy biofilms.

**Role of Microbial Biofilms in the Dairy Industry**

*Cronobacter sakazakii*, Bacillus cereus, Listeria monocytogenes, *Staphylococcus aureus*, and *Escherichia coli* are the important pathogens with great ability to form biofilms on surfaces in dairy plants (Boor et al., 2017). Pathogenic biofilms are regarded as a major public health concern, and a heavy economic burden caused by biofilm-associated diseases has also risen in last years. It has been estimated that around 80% of microbial infections are caused by microbial biofilms (National Institutes of Health, 2002).

Biofilms have also notoriously been identified as potential source to reduce the quality of dairy products. In 2 previous studies, either the proteolysis of Pseudomonas fluorescens biofilm cells or the lipolysis of *S. aureus* biofilms cells was significantly higher than those from planktonic cells (Teh et al., 2012, 2013). The increased heat stability of enzymes from biofilms cells can be explained by the protection from the biofilm matrix. The enzymes produced by biofilm cells may even hydrolyze the amino acids in dairy products, followed by the production of biogenic amine (Diaz et al., 2016). Biogenic amine at high concentrations may have toxicological effects on human health (Moniente et al., 2022).

In addition, biofilms formed on inner surfaces of tanks and pipes could cause corrosion, and ultimately decrease the heat transfer efficiency of machine (Kimbell et al., 2020). For example, prolonged contact with biofilms formed by of *Bacillus sporothermodurans* and Geobacillus stearothermophilus caused large cracks on stainless steel coupons. Energy-dispersive spectroscopy also showed an increased content of oxygen and sulfur, but a decreased content of iron in stainless steel (Gupta and Anand, 2018).

**Key Factors Affecting Biofilm Formation**

In general, biofilm formation can be affected by many factors, including the strains, environmental conditions, characteristics of different contact surfaces, and bacterial interactions (Govaert et al., 2018; Yuan et al., 2018a; Wang et al., 2022b). When referring to the real situations in the dairy environment, specific conditions may also induce the formation of biofilms. For example, lactose in milk promoted the biofilm formation of dairy isolates by increasing polysaccharide adhesion and activating the quorum sensing system (Xue et al., 2014; Duanis-Assaf et al., 2016; Yuan et al., 2018b). The calcium in milk altered the biofilm formation of G. stearothermophilus by changing EPS production; however, a clear mechanism for this phenomenon needs investigation by omics experiments (Wang et al., 2021b). Chamberland et al. (2017b) observed that operating parameters, including feed temperature, filtered fluid, and cleaning cycle, affected the bacterial biofilm on ultrafiltration membrane. Marka and Anand (2018) confirmed that different feed substrates caused variations in biofilms on reverse osmosis membranes, and resulted in differences in biofilm resistance to CIP procedures.

**Traditional Techniques for Biofilm Studies**

The disturbing properties of microbial biofilms have promoted the necessity of using reliable tools to unravel the community and behavior of biofilms in the dairy industry. Swabbing, vortexing, and sonication, combined with culture-dependent methods, are generally applied to analyze the microbial structure of biofilms on food-contact surfaces. Using this strategy, isolates of Micrococcus spp., Bacillus spp., Clostridium spp., Serratia marcescens, Staphylococcus epidermidis, Pseudomonas spp., Microbacterium oxydans, and other strains have been isolated and identified from different dairy processing sites (Zou and Liu, 2018). However, this procedure usually depends on selective media, some of which may cause false-positive results. Another problem of this culture-dependent method is the missing information from unculturables cells in biofilms. This can be solved by genomics tools, as we will discuss.
Crystal violet staining is an indirect tool for estimating dead and viable cells and matrix components in biofilms under various culture conditions (Merritt et al., 1998). However, the lack of a standardized protocol make it difficult to compare the biofilm-forming capacity of strains from different studies. Advanced microscopy techniques, including confocal laser scanning microscopy, scanning electron microscopy, and atomic force microscopy, can picture the bacterial morphology, biofilm architectures, and the interaction between surface and biofilms (Sauer et al., 2022). However, these traditional methods do not provide in-depth molecular mechanisms for the formation and elimination of dairy biofilms.

**INTERPRETATION OF DAIRY BIOFILMS BY OMICS TOOLS**

In recent years, advanced omics tools have been widely applied for elucidating the key regulators for biofilm formation, biofilm stress tolerance, and biofilm elimination efficiency at levels of genes, RNA, proteins, and metabolites.

**Genomics**

Multispecies biofilms have been considered as the most frequent state of bacterial contamination in the dairy industry, and analyzing the microbial composition of mixed-species biofilms is vital to ensure the dairy safety and quality thereof (Sauer et al., 2022). In recent years, genomics tools including metagenomics and whole-genome sequencing have been widely used to analyze the microbial communities and related genes implicated for biofilms, due to its increasing throughput and decreasing sequencing cost.

Metagenomics has shown high-throughput and in-depth identification of microbial composition, including unculturtable cells in mixed-species biofilms in dairy environments. For example, Chamberland et al. (2017a) performed a metagenomics experiment to unravel the biofilm population of spiral-wound membranes in dairy plants, and *Lactococcus*, *Streptococcus*, *Arthrobacter*, *Methylobacterium*, *Enterococcus*, were confirmed as the predominant microorganisms in the mixed-species biofilm. This study confirmed that the long-term fouling of membranes is a main issue that may decrease the performance of membrane systems and lead to expensive membrane replacements. As reported by Wang et al. (2022a), this technique also helped confirming the critical control points during milk powder processing by tracing the remarkable change of bacterial community, which is of great significance for the quality guarantee of dairy products. However, main functional components relating to the critical functions of biofilms are uncovered (Sun and D’Amico, 2023). Moreover, metagenomics cannot predict how bacterial interactions affect cell metabolites, due to the inability to distinguish between dead and living cells.

Whole-genome sequencing acts as a reliable tool for understanding genes implicated for biofilms formed by a variety of dairy isolates. For example, functional genes (*guaA*, *pgaC*, and *yqiG*) involved in *Pediococcus pentosaceus* biofilm formation were revealed by WGS (Mgomi et al., 2022). In another study, the genetic variants of *L. monocytogenes* with regard to biofilm phenotypes was observed by Pan-Genome-Wide Association Study, which reflects the persistence and prevalence of *L. monocytogenes* at low temperature under nutrition-deprived conditions in the dairy industry (Lee et al., 2019).

**Transcriptomics**

Unlike the high stability of DNA, the RNA of microorganisms is dynamically changing. Therefore, the aim of transcriptomics is to identify genes differentially expressed under specific conditions at any given time. Transcriptomics tools, including RNA-sequencing (RNA-seq), microarray, and even metatranscriptomics, could decipher potential molecular mechanisms for biofilm formation, response of biofilms to stress conditions, and efficiency of control strategies in the dairy industry (Yao et al., 2022; Zheng et al., 2022).

DNA microarrays are the collection of short oligonucleotide probes attached to a solid surface that are specific to thousands of known transcripts. This technique has been widely used to identify the expression levels of biofilm-related genes. Efthimiou et al. (2019) performed a DNA microarray study to analysis the response of *S. aureus* biofilm cells to acidic and alkaline environments in the dairy industry. The significantly differentially expressed genes were involved in stress response, virulence and antibiotic resistance pathways. Despite the relatively low cost, major issues for microarrays are the unavailable for unknown transcripts, high background noise from the cross-hybridization, and low accuracy of expression for transcripts in low or high abundance. The popularity of DNA microarray is decreasing due to the increasing availability of next-generation sequencing platforms.

A powerful tool for the detection of both known and unknown transcripts, RNA-seq offers advantages of high capacity, sensitivity, and reproducibility. Despite its relatively high cost and the requirement of intensive labor, the comprehensive results obtained from RNA-seq analysis make it ideal for biofilm studies. For example, RNA-seq has been applied to elucidate the
global gene expressions during the biofilm formation by *C. sakazakii*. A large number of genes coding for flagellar assembly, LysR and AraC family transcriptional regulators, bacterial outer membrane, and virulence factors, have been found to be differentially expressed, which provides a better understanding of the control of foodborne disease induced by *C. sakazakii* (Xu et al., 2021b). This technique has also been used to study the bacterial interactions between *Lactobacillus casei* and *Streptococcus mutans* in a mixed-species biofilm. A total of 134 genes of *Streptococcus mutans* were significantly regulated by the presence of *L. casei* in the mixed-species biofilm, and the regulated genes were involved in superoxide dismutase, NADH oxidase, and members of the mutanobactin biosynthesis (Wen et al., 2017). However, availability of genome sequences mapping the reads is limited. Moreover, single transcriptomics results only profile the first stage of gene expressions, and the transcriptome from each biofilm sample is still subject to variations at translational or post-translational level. This may ultimately lead to phenotypes that may not reflect the observed transcriptomic profiles (Ma et al., 2022). Hence, results derived from transcriptomic studies should be treated with caution, and need further validation by other omics tools.

Metatrascriptomics is a tool to provide knowledge on the whole gene expression profiles of mixed-species biofilm communities, due to the changes of environmental conditions. This technique unraveled the complex assembly and bacterial functions in biofilms on tooth, fixed-bed biofilm reactors, and sediments (Edlund et al., 2018; Zhang et al., 2020; Centurion et al., 2022), but has not been applied for dairy biofilm studies.

**Proteomics**

Proteomics has been successfully applied to identify the precise changes in proteins, and generate key information about behavior and pathways of microorganisms during biofilm formation. At this stage, the 2 proteomics techniques that used to elucidate putative factors involved in biofilms are 2-dimensional (2D)-PAGE coupled to MS, and label-based proteomics.

Use of 2D-PAGE linked to MS, one of the earliest tools for proteomics studies, has been widely applied to analyze differently expressed proteins during biofilm formation. In 2 previous studies, functional proteins of *C. sakazakii* and *Lactobacillus sakei* involved in biofilm formation on stainless steel surface at low temperature were identified by 2D-PAGE linked to MALDI-TOF MS (Ye et al., 2016; Pérez-Ibarreche et al., 2017). However, this technique is always time-consuming, and is insensitive to analysis of hydrophobic and low-abundance proteins.

Label-based proteomics methods, such as isobaric tags for relative and absolute quantitation (iTRAQ), and tandem mass tags (TMT), have provided precise measurement of differently expressed proteins and protein–protein interactions. In a previous study, a TMT-based proteomics analysis was applied to reveal the regulators in biofilm formation by Bacillus *licheniformis*. Flagellar assembly, matrix production and sporulation, 2-component system, and bacterial chemotaxis are the important pathways involved in *B. licheniformis* biofilm formation (Wang et al., 2020).

Unlike using a small peptide as a tag by TMT, the tag introduced by iTRAQ is based on N-methylpiperazine derivatization. A total of 177 proteins of *Cronobacter* spp. were significantly differentially expressed during its biofilm formation, and these proteins were involved in cell adhesion, signal transduction, biological binding, and cellular interaction (Yang et al., 2016).

However, the limitations of single proteomics are the contaminations from undigested dairy proteins, and detected microbial proteins not being in the database or having no known functions (Ma et al., 2022).

**Metabolomics**

Metabolites are low molecular weight end products of enzyme-catalyzed reactions in microorganisms, which better reflect bacterial activities at the phenotypic level. Metabolomics, including the targeted and untargeted metabolomics, are the end-point monitoring tool of describing global metabolic profiles, together with understanding the mechanistic roles metabolites play in relation to biofilm cells (Ma et al., 2021). Different metabolomics tools have been developed based on high-resolution chromatography (high-performance liquid chromatography, ultra-performance liquid chromatography, and gas chromatography) linked to detection methods (nuclear magnetic resonance, MS).

Targeted metabolomics methods are usually driven by a specific hypothesis or biological question. In targeted metabolomics, metabolites involved in specific pathways linked with biofilm formation are quantified. It is highly sensitive and allows for very low limits of metabolites detection (Guo et al., 2021). However, targeted metabolomics approaches cannot discover novel compounds, and usually require prior knowledge of metabolites derived from a more global tool similar to untargeted metabolomics or proteomics (Rocchetti et al., 2019).

Untargeted approaches are used to detect metabolites from biofilms as many as possible. However, it is laborious to extract metabolites with diverse polarity, and the implementation of different extraction methods is necessary to avoid bias during the extraction stage.
Purpose 1: Unraveling the Population and Functions of Dairy Biofilms

Knowledge of the microbial population and their functions on different sites during dairy processing can benefit the identification of primary colonizers on surfaces, the assessment of biofilms risk, and the optimization of cleaning procedures. The combination of metagenomics and proteomics analysis provided both microbial communities and metabolic profiles in a full-scale pre-denitrification biofilter and clarified the mechanism of denitrification. In detail, the most abundant genera in the mixed-species biofilm on a biofilter were Chryseobacterium, Acidovorax, Polaromonas, Bossea, and Dechloromonas. The most abundant pathways in proteomic annotations were ATP binding cassette (ABC) transporters, glyoxylate and dicarboxylate metabolism, biosynthesis of amino acids, 2-component system, and carbon metabolism (Tian and Wang, 2021). Unfortunately, the combination of metagenomics with other omics techniques has not been used for dairy biofilm studies yet.

Purpose 2: Unraveling the Mechanisms for Biofilm Formation

In a previous study, the integration of proteomics and transcriptomics was performed to obtain solid evidence of the physiological and functional changes involved in biofilm formation by B. cereus, to avoid large differences between mRNA transcripts and protein function of bacterial cells (Caro-Astorga et al., 2020). The RNA-seq results in the above study were obtained for a reference for gene expression, and data from iTRAQ analysis were used to confirm that variations in gene expressions in the same direction as variations of translation. The results confirmed a high similarity between both methods in the number of differentially expressed genes with down or up expression levels. In details, different metabolic pathways were observed between the planktonic cells and biofilm cells. Rearrangement of nucleotides, sugars, amino acids, and energy metabolism was observed in biofilm cells. The changes in the metabolic activity of biofilm cells also satisfied other pathways, including the synthesis of extracellular matrix, activation of reactive oxygen species detoxification machinery, reinforcement their cell wall, and production of higher amounts of secondary metabolites. However, some differences in pyrimidine metabolism between the transcriptome and proteome were observed, which highlights the importance of omics integration analysis (Caro-Astorga et al., 2020).

Purpose 3: Unraveling the Bacterial Interactions in Mixed-Species Biofilms

Bacterial interactions, usually in ways of cooperative, neutral, or competitive, could affect the structure and functions of mixed-species biofilms (Röder et al., 2015). Many studies confirmed the social interactions in mixed-species biofilms formed by dairy strains. For example, the cooperative interaction of B. licheniformis and G. stearothermophilus, and competitive interaction of L. monocytogenes and Lactobacillus paraplanatarum were reported in previous studies (Winkelströter et al., 2015; Wang et al., 2022b). However, their molecular mechanisms of bacterial interactions in biofilms have not been studied.

Ellepola et al. (2019) conducted a study to reveal the cross-kingdom interactions in a dual-species biofilm by Candida albicans and Streptococcus mutans by the integration of transcriptomics and proteomics tools. This study confirmed a high level of consistency between the proteomics and transcriptomics data, and in particular, genes and proteins involved in pyruvate degradation to ethanol and acetate production, glycolysis, carbo-
hydrate metabolism, the tricarboxylic acid cycle, sugar transport systems, and the electron transport chain were significantly influenced at both the transcription and translation levels. However, different results between these 2 methods related to nucleotide metabolic processes, AA metabolic processes, stress response, and cell cycle-related processes were also observed. More importantly, the integration of transcriptomics and proteomics also provided additional information that could explain the cross-kingdom interactions in the dual-species biofilm. Genes and proteins related to fungal vacuolar development and hyphal growth of \textit{C. albicans} were upregulated in the dual-species biofilm. In addition, some unknown functions were proved to be differentially regulated, which may provide interesting gene targets for future studies (Ellepola et al., 2019).

<table>
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<th>Multimomics tool</th>
<th>Purpose of the study</th>
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<td>RNA-seq + iTRAQ and LC-MS/MS</td>
<td>Characterize the inhibitory effect of plantaricin GZ1-27 on biofilm formation by \textit{Staphylococcus aureus}.</td>
<td>Plantaricin GZ1-27 prevents the biofilm formation by inhibiting production of surface matrix-associated proteins and restraining functions of regulatory factors.</td>
<td>Du et al., 2020</td>
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<tr>
<td>RNA-seq + LC-MS/MS</td>
<td>Analysis the adhesion characteristics of \textit{Lactobacillus reuteri} upon gastrointestinal fluid stress.</td>
<td>Biofilm production was decreased when \textit{L. reuteri} passed through the harsh gastrointestinal tract. Gene expressions involved in cell envelope, metabolic processes, stress response, regulatory systems, transporters were affected. Proteins related to ABC transporters and LpxTG anchor domain proteins were upregulated.</td>
<td>Xu et al., 2021</td>
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<td>RNA-seq + TMT and LC-MS/MS</td>
<td>Analysis the role of \textit{isaA} in \textit{Staphylococcus aureus} biofilm formation.</td>
<td>Biofilm-related genes and hemolysin genes, such as \textit{sasF}, \textit{sarX}, and \textit{hlyC}, were downregulated with \textit{isaA} gene disruption.</td>
<td>Ma et al., 2022</td>
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<td>WGS + RNA-seq</td>
<td>Analysis of biofilm formation by persistent and transient \textit{Listeria monocytogenes}.</td>
<td>Differences in gene expression for biofilm formation or stress-tolerance genes enable \textit{L. monocytogenes} to persist in a food processing environment.</td>
<td>Assisi et al., 2021</td>
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<tr>
<td>HRMS + FTIR</td>
<td>Response of \textit{Candida albicans} biofilm to zosteric acid treatment.</td>
<td>Proteins associated with the membrane and cell wall are the target of zosteric acid. Proteins involved in biogenesis, structure and integrity of cell walls, and adhesion and stable attachment of hyphae were downregulated, whereas proteins involved in stress response were overexpressed.</td>
<td>Cattò et al., 2022</td>
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<td>RNA-seq + UHPLC-MS/MS</td>
<td>Analysis of changes in \textit{Staphylococcus aureus} exposed to terpinen-4-ol.</td>
<td>Terpinen-4-ol strongly inhibited DNA and RNA biosynthesis in \textit{S. aureus} by affecting genes and metabolites related to purine and pyrimidine metabolic pathways.</td>
<td>Cheng et al., 2021</td>
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<td>WGS + 1D-SDS-PAGE and LC-MS/MS</td>
<td>Analysis the antibiofilm activity of pepsin-digested bovine lactoferrin on \textit{Pseudomonas fluorescens} biofilm formation.</td>
<td>Bovine lactoferrin hydrolysat caused a significant reduction in biofilm biomass by repressed \textit{PleD}, \textit{TycC}, and \textit{GbrS}, and induced negative regulators of alginate biosynthesis and cyclic-di-GMP-binding biofilm dispersal mediator.</td>
<td>Quintieri et al., 2019</td>
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<tr>
<td>RNA-seq + iTRAQ and LC ESI-MS/MS</td>
<td>Reveal the intrinsic offensive and defensive features of \textit{Bacillus cereus} biofilms.</td>
<td>Biofilm cells rearrange nucleotides, sugars, amino acids, and energy metabolism. This metabolic rearrangement coexists with the synthesis of extracellular matrix, sporulation, reinforcement of the cell wall, activation of reactive oxygen species detoxification machinery, and production of secondary metabolites.</td>
<td>Caro-Astorga et al., 2020</td>
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<tr>
<td>RNA-seq + TMT and LC-MS/MS</td>
<td>Analysis of RpoS in biofilm formation of \textit{Pseudomonas fluorescens}.</td>
<td>Involvement of RpoS in polysaccharide metabolism, intracellular secretion, extracellular structures, cell wall biogenesis, stress responses, and AA and biogenic amine metabolism, which may contribute to biofilm formation.</td>
<td>Liu et al., 2019</td>
</tr>
<tr>
<td>RNA-seq + iTRAQ and LC-MS/MS</td>
<td>Analysis of biofilm formation of \textit{Escherichia coli} under microaerobic versus aerobic conditions.</td>
<td>Transcripts under microaerobiosis highlighted genes controlling biofilm formation (\textit{pgaABCD} and \textit{csgDEFG} operons). Biofilm formation is an adaptive strategy allowing \textit{E. coli} to survive in low-oxygen environments.</td>
<td>Liu et al., 2022</td>
</tr>
<tr>
<td>Metagenomics + UHPLC-MS/MS</td>
<td>Metabolic profiles of \textit{Lactobacillus paraplantarum} in biofilms, and the investigation of its intestinal modulation.</td>
<td>Significant distinction between planktonic and biofilm cells was observed, with amino acids and carbohydrate metabolism more active in biofilms. Biofilm cells increased the abundance of \textit{Lactobacillus} in gut microbiota. Relative abundance of intestinal microbiota participating in carbohydrate metabolism was higher in the biofilm probiotic-treated group.</td>
<td>Liu et al., 2021</td>
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\textsuperscript{1}RNA-seq = RNA-sequencing; iTRAQ = isobaric tags for relative and absolute quantitation; LC-MS/MS = liquid chromatography-tandem mass spectrometry; TMT = tandem mass tags; WGS = whole-genome sequencing; HRMS = high-resolution mass spectrometry; FTIR = Fourier-transform infrared spectroscopy; UHPLC-MS/MS = ultra-high-performance LC-MS/MS; 1D-SDS-PAGE = 1-dimensional SDS-PAGE; LC ESI-MS/MS = liquid chromatography/electrospray ionization-tandem mass spectrometry.

\textsuperscript{2}Plantaricin GZ1-27 prevents the biofilm formation by inhibiting production of surface matrix-associated proteins and restraining functions of regulatory factors.

\textbf{Table 1.} Summary of reported integration of multimomics tools for dairy biofilm studies\textsuperscript{1}

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Purpose 4: Unraveling the Response to Stress

Increased ability of biofilms to stress can be comprehensively analyzed by multiomics tools. An integration of transcriptomics and proteomics revealed the high tolerance to desiccation by \textit{C. sakazakii} biofilm. A moderate correlation between the proteomics and transcriptomics data were observed, with 331 genes found regulated at both transcriptional and protein levels. Results indicated that the decreased O-antigen chain length of \textit{C. sakazakii} positively affected its biofilm formation and desiccation tolerance. Moreover, arginine and its transport system, biosynthesis of flagella, and Fe/S cluster were also regulated in desiccated \textit{C. sakazakii} cells. However, gene expressions in transcriptome and proteome involved in flagellar and chemotaxis, arginine biosynthesis, iron-sulfur cluster, and lipopolysaccharide synthesis were opposite (Qian et al., 2022).

Purpose 5: Development of Novel and Efficient Methods to Control Biofilms

CIP regimens have shown varied effectiveness in removing biofilms from dairy processing line, which depends on greatly depends on parameters (exposure time, temperature and concentration of cleaning agents) of CIP regimens (İpek and Demirel Zorba, 2018; Kumari and Sarkar, 2018). Even high cleaning intensity of CIP regimen (1.5% NaOH at 65°C for 30 min, 1% HNO₃ at 65°C for 10 min) was used, the highest reduction of biofilm cells on stainless steel surfaces was observed at 4.77 log cfu/cm² (Kumari and Sarkar, 2014). Therefore, alternative control strategies are required at this stage to eliminate the resistant biofilm microflora in the dairy industry in addition to reducing bacterial cross-contamination. Multiomics tools usually provide additional information on the biofilm control mechanism based on the novel control strategies. For example, Lin et al. (2021) integrated proteomics and metabolomics techniques to explore the antibiofilm activity of actinomycin D on \textit{S. aureus} biofilm. By proteomics analysis, the top enriched metabolic pathways were glycerolipid metabolism, \textit{S. aureus} infection, the phosphotransferase system, fluid shear stress and atherosclerosis, and glyoxylate and dicarboxylate metabolism. The metabolomics analysis showed that the top Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched by the differentiated proteins and metabolites were purine metabolism, ABC transporters, protein digestion and absorption, pyrimidine metabolism, and aminoaacyl-tRNA biosynthesis. In addition, the correlation network analysis provided novel insights between differentiated proteins and metabolites. Eighteen annotated pathways were associated with the disruption of energy metabolism and oxidative stress of \textit{S. aureus} (Lin et al., 2021).

Purpose 6: Benefits of LAB Biofilms

Application of LAB biofilms is generally recognized as a promising way of interfering with the formation of pathogenic biofilms (Mgomi et al., 2022). Previous studies have indicated that some antibacterial substances were produced only when LAB cells attached to surfaces and shifted into a biofilm state (Gao et al., 2022). In addition, LAB biofilms showed enhanced survival strategies in response to the stress (low pH, digestive enzymes, and bile salts) during the gastrointestinal transit (Gao et al., 2022). Therefore, the biofilm-based delivery has been proved as a promising approach for LAB delivery. The application of multiomics tools would unravel a comprehensive microbial interaction between LAB biofilms and gut microbiome, and further provide the precise mechanisms by which the gut microbiota is in health or not.

Here, we concluded some advantages of multiomics tools over single-omics technique. First, the results of multiomics analysis could avoid the detection bias from each technique, and provide a better coverage of metabolic pathways. Second, the cross-validation for a high level of consistency between different omics data can make conclusions with higher confidence. Third, multiomics analysis could provide new biological insights into biofilm metabolic and regulatory mechanisms that could not be accessed from each single-omics dataset.

CURRENT CHALLENGES AND PERSPECTIVES OF MULTIOMICS-BASED BIOFILM STUDIES

In this review, in addition to the high cost, we concluded that the other challenges of integrated omics approaches in biofilm studies are experimental challenges, data issues, and biological knowledge in data interpretation.

Experimental Challenges

Careful consideration and appropriate design of a biofilm experiment could improve the reproducibility of final results. A standardized sample preparation protocol is the first step for multiomics studies. Effective sample collections for nucleic acid, proteins, and metabolites from biofilms with few losses are usually
challenged. The unstandardized preparation protocol provides unequal and low quality of samples, especially at the early stages of biofilm formation. The heterogeneity and variability of biofilm cells collected from different regions on surfaces are also the major issues (Meléndez-Carmona et al., 2022). In addition, one omics layer may be the downstream of others, and simply using the same culturing time for sample collection may sometime make the final results incredible and confusing (Caro-Astorga et al., 2020; Fernández-Gómez et al., 2021). Therefore, when designing the multiomics experiment, different samples should be prepared at different time points to avoid the noise from time biases (Stipetic et al., 2016).

Moreover, most multiomics-based biofilm studies have just integrated data from an integration of 2 different omics tools, and significant discrepancies between each omics analysis have always been observed (Caro-Astorga et al., 2020). Integration of omics data across 3 or more layers has become feasible, with the significant advancement in software development.

Lastly, despite the large number of dairy biofilm studies by using omics techniques, limited studies have simulated the dairy-associated environment, including the temperature, flow conditions, pH, contact material, and nutrients variability. It was indicated that biofilms formed by dairy strains may differ from biofilms formed by nondairy strains in the dairy-associated environment. For example, Ostrov et al. (2019) performed a study to analyze the adaptability of Bacillus strains to dairy-associated environments. Dairy-associated Bacillus strains were characterized by formation of robust biofilms, and were more resistant to CIP procedure.

**Data Issues**

Many previous multiomics studies simply performed analysis of the results of genes, transcripts, proteins, and metabolites from each omics data, and to further interpret the biological pathways. The internal interactions across different data from each omics experiment is considered as the driving factor that affect the final results (Subramanian et al., 2020). In general, data handling from each omics analysis should address the issues of data cleaning, transformation, and normalization; however, a gold standard for the analysis of omics data is lacking.

The interspecies interactions in mixed-species biofilms, include the interspecies adherence, metabolic interactions, interspecies signaling, competition for nutrient sources and space, and the production of secondary metabolites for selective killing of other community members, make it difficult to analyze the omics data (Yuan et al., 2020a). In addition, data from different omics experiment does not always fit well, which remains one of the confounding factors limiting data integration. For example, recent studies failed to find a good correlation between protein abundance and mRNA expression levels (Caro-Astorga et al., 2020). The discrepancy arises from factors including differences in the half-lives of mRNA and proteins, post-transcriptional, translational and post-translational regulation, and experimental error (Caro-Astorga et al., 2020). Therefore, development of adequate statistical tools for the integration and analysis of multiomics data, and increasing the size and availability of normalized databases, could greatly accelerate biofilm studies by multiomics tools (González-Plaza et al., 2022).

The ability to annotate unknown biomarkers is also a major limitation of multiomics studies. Confer annotations of unknown biomarkers by coexpression, structural and chemical similarities or abundance cannot effectively map all the unknowns, and usually cause contradictory results in multiomics studies (He et al., 2021; Liu et al., 2021a).

Finally, data archiving and sharing is also the challenge posed by integrated omics methods. There is a growing need for the reproducibility of multiomics studies, and relevant databases are necessary for reproducible studies and generating novel hypotheses by using integrated omics techniques. Although some public databases for archiving high-throughput molecular abundance data do exist, such as the National Center for Biotechnology Information, no such archive exists for integrated omics datasets. For data sharing, results of large multiomics studies may facilitate available resources for further studies.

**Biological Knowledge**

Biological knowledge in data interpretation is another concern for single or multiomics studies. In most cases, a large quantity of data cannot be completely analyzed by the rapid development of bioinformatics. Discovery of key biomarkers and complex multilayered networks related to biofilm cells is challenging, which requires researchers having in-depth knowledge of biofilm cells, software, and databases (Ferrocino and Cocolin, 2017; Gutleben et al., 2018). Here, we hope the availability of multiomics platforms and the rapid advancements in machine learning and modeling tools may make it possible to design models for the prediction of pathways related to biofilm formation.

**CONCLUSIONS**

This review showed that there are a limited but growing number of studies to understand the dairy biofilms
by the application of multiomics. Multiomics tools have largely increased our knowledge of microbial community and the bacterial interactions in mixed-species biofilms, biofilm response to stressful conditions encountered in the dairy industry, and molecular mechanisms for the elimination of biofilms. However, challenges still exist, including experimental design, professional data analysis, and reproducible and high-throughput software and database development. Despite such challenges, the integration of omics tools is encouraged for biofilm studies, and will greatly benefit us by developing efficient methods to control biofilms in the dairy industry.

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