# Poultry processing and the application of microbiome mapping

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**ABSTRACT** Chicken is globally one of the most popular food animals. However, it is also one of the major reservoirs for foodborne pathogens, annually resulting in continued morbidity and mortality incidences worldwide. In an effort to reduce the threat of foodborne disease, the poultry industry has implemented a multifaceted antimicrobial program that incorporates not only chemical compounds, but also extensive amounts of water application and pathogen monitoring. Unfortunately, the pathogen detection methods currently used by the poultry industry lack speed, relying on microbiological plate methods and molecular detection systems that take time and lack precision. In many cases, the time to data acquisition can take 12 to 24 h. This is problematic if shorter-term answers are required which is becoming more likely as the public demand for chicken meat is only increasing, leading to new pressures to increase line speed. Therefore, new innovations in detection methods must occur to mitigate the risk of foodborne pathogens that could result from faster slaughter and processing speeds. Future technology will have 2 tracks: rapid methods that are meant to detect pathogens and indicator organisms within a few hours, and long-term methods that use microbiome mapping to evaluate sanitation and antimicrobial efficacy. Together, these methods will provide rapid, comprehensive data capable of being applied in both risk-assessment algorithms and used by management to safeguard the public.

**Key words:** poultry processing, foodborne pathogens, rapid methods, microbiome mapping, bioinformatics

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## INTRODUCTION

The United States Department of Agriculture's (**USDA**) Poultry-Inspection System monitors the processing environment at multiple stages of the slaughter process (Hogue et al., 1998). This has ultimately resulted in the poultry processing industry being relatively successful at limiting foodborne pathogens; however, several emerging challenges could make the current success less sustainable in the near future. As the processing lines increase speed to boost the production of poultry meat, the pathogen monitoring methods currently used by regulatory agencies and the poultry industry will become increasingly limited and necessitate innovation. The pressure to step-up line speeds has resulted in demand to modernize the USDA Poultry-Inspection System, which is primarily dependent on the "visual inspection of blemishes and bruises" that are indicative of poor processing to a more direct, automated, and rapid foodborne pathogen risk-based method (Allain et al., 2018). Eventually, this will require the poultry processing industry to become even more integrative, incorporating large data sets, next generation sequencing (**NGS**) technologies, and real-time sensors on processing lines for at-the-minute management decisions to mitigate the likely increased risk in foodborne disease (Ricke et al., 2017). In turn, the industry need will necessitate academic and government research organizations to aid in that transformation with novel, real-time, highly-accurate, and relatively user friendly methodologies that can be used by employees with broad academic and varied backgrounds (Ricke et al., 2017; Thompson et al., 2017).

Historically, the poultry industry has recruited from a minimally trained labor workforce pool, which will become incongruent with automation and detection innovations without extensive training (Thompson et al., 2017). That being said, the modernization of the industry will result in the available qualified labor force shrinking into a more educated, more skilled group of individuals (Ricke et al., 2017; Thompson et al., 2017). This will likely require the pedagogy associated with poultry processing and academic training to change. This will become true of most of the food industry as trade globalization continues to accelerate and

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consumer markets expand for a wider range of food products (Ricke et al., 2015). Concomitant with these anticipated increases will be the demand for more accountability and improvements in traceability technology (Ricke et al., 2015).

Therefore, while the total financial burden on the poultry industry decreases with automation, the cost per employee will rise as highly skilled employees capable of handling automation challenges are more expensive along with the expectation that a processing employee, laboratory technician, or manager will need to become trained to assimilate "Big Data" and interpret the resulting complex nature of these types of data sets (Strawn et al., 2015; Ricke et al., 2017; Thompson et al., 2017). Training will have to move towards a more integrative, multi-disciplinary understanding that involves multiple fields. And this goes beyond the poultry industry. Agriculture in general will require individuals at all levels of food production who possess extensive backgrounds in "omics," computer science, machine learning, microbiology, genomics, next-generation proteomics, and biology, among other fields of discipline (Strawn et al., 2015; Cao et al., 2017; Taboada et al., 2017; Liakos et al., 2018; Yiannas, 2018). Therein lies the challenge with agriculture education and training as these programs must not only adapt much more rapidly away from traditional training programs, but they must become more integrative, even at the advanced degree level. This reality is already being observed with the advent of "Big Data" entering into the Blockchain pipeline, which has revolutionized tracking and traceability throughout the food production cycle and will soon be the gold standard (Strawn et al., 2015; Thompson et al., 2017; Yiannas, 2018). Changes in educational philosophy at all levels of training will be a critical component of technological advancement in the food industry (Thompson et al., 2017).

While technology and economics may drive the poultry industry to a more real-time, automated approach, the detection of foodborne pathogens will always be a bottleneck as bacterial species all have their unique idiosyncrasies. Fortunately, molecular detection-based approaches have markedly improved over the past decade and become much more accessible to the poultry and food industry (Baker et al., 2016a,b; Ricke et al., 2018, 2019; Collineau et al., 2019). The molecular revolution has resulted in the enhanced detection of pathogens more rapidly at a lower limit of detection and quantitation than traditional microbiological methods. Yet, the different matrices present in food processing still mask pathogen detection, continuing to create the "needle in the haystack" dilemma for pathogen sampling and monitoring (Handley et al., 2015; Ricke et al., 2015; Blevins et al., 2017). In addition, actual *Salmonella* load as a performance standard may be on the horizon as the detection technology improves (Ricke et al., 2018; FSIS, 2019).

The application of 16S gene-based microbiome sequencing has been considered as a potential alternative for pathogen risk assessment via the assessment of the complete microbial profile of a poultry sample. However, microbiome sequencing is not necessarily the answer to this dilemma either, as even microbiome sequencing is relatively slow and requires considerable resolution as some pathogens such as *Salmonella* are below the limits of detection of stringent bioinformatic pipelines (Grim et al., 2017; Ricke et al., 2018). Significant standardization and baseline data for new technology entering the industry will have to occur before it becomes practical. Therefore, the current methods (microbiology and molecular detection) will be the initial step for integration and innovation. In many ways, while traditional microbiological methods may change over time, they will always be necessary to complement emerging innovations.

The preference for both regulatory agencies and the poultry industry would be to quantify baseline levels of *Salmonella* and other foodborne pathogens, identify important strains or serovars, and assign quantitative risk-based models to assess where food safety risk is the greatest on the processing line (Handley et al., 2015; Rajan et al., 2017; Thompson et al., 2017; Sampedo et al., 2018; Collineau et al., 2019; Godefroy et al., 2019). Traditionally, the poultry industry adopted a "biomapping" approach based on microbial plate counts of microorganisms referred to as "indicator organisms," such as the use of aerobic plate counts (**APC**), coliforms, or other microorganisms indicative of potential pathogen load (Blevins et al., 2017; Handley et al., 2018, Bourassa et al., 2019). Using a period of time to establish the acceptable baseline standard, importing data into large databases, and using computer-based risk assessment algorithms that factor in flock history, plant hygiene, and historical standards will be a logical next step. Additionally, commercial molecular detection techniques have become standard, where the prevalence and quantitation of pathogens is actively being developed for food industry use by numerous instrument companies (Ricke et al., 2018, 2019).

The rote commercial molecular technologies have significant promise in alleviating some of the burdens associated with speed and detection limits, are complementary to plate counts, and absolutely have a role in the overall pathogen detection and control strategy of the industry. In continuing to adopt and transform current commercially available molecular technologies, the industry will push regulatory agencies to more sensitive, specific, and rapid techniques that increase the detection capabilities and burden of responsibility of poultry processors to reduce pathogens. Challenges will continue to emerge to make this difficult and will continue to necessitate academic and federal research innovation. For instance, the matrix associated with processing environments such as ceca, crop, environmental swabs, or rinsates, all pose unique, matrix specific challenges for current molecular-based approaches as various inhibitors are present that can decrease detection methods (Stevens and Jaykus, 2004; Wang, and Salazar,

2016). The need for approval is necessary; therefore, the implementation of new technologies requires stepwise, Food Safety and Inspection Services and Association of Official Analytical Chemists (FSIS/AOAC)-guided methodologies and approvals. All of this takes time and the perpetual generation of new insight into traditional techniques tends to confound definitive interpretation. For instance, total APC are used to indicate the microbial load on a surface and can be associated sanitation efficacy based on general bacterial enumeration. However, each APC plate may not necessarily represent similar microbial populations. Recently, microbiome sequencing of the CFUs on Petrifilm APC culture plates indicated that the organisms recovered from APC microbial populations are not consistently representative of the actual microbial populations on the carcass (Kim et al., 2017).

Microbiome sequencing to generate a corresponding microbiome map from the same carcass rinsate samples used for APC enumeration could be an immediate innovation based on the ability to retrieve a more comprehensive microbial profile (Blevins et al., 2017). However, currently, the time to data acquisition requires at least a week along with significant informatics and biostatistics, and finally it is still a very relative interpretation. Management decisions stemming from microbiome studies require considerable time, data, and training to achieve accurate and relevant interpretation. Therefore, while important for future applications, the problems associated with microbiome data may actually serve as a reminder of the complexity of microbial ecology of the poultry processing environment and the difficulty with deriving meaningful action plans for process control measures. An opportunity for exploring solutions to this challenge directly exists with microbiome mapping in the poultry industry for that reason.

The science of these emerging technologies will not only evolve but also be accompanied by decreases in their respective costs to encourage commercial implementation in a wide range of food production industries. Certainly, the advances in microbiome analyses and whole genome sequencing offer new opportunities for the poultry processing industry to gather more complete and potentially meaningful data. However, to truly develop practical guidelines for routine use of these technologies will require recruiting a workforce with independent and nontraditional backgrounds. To achieve such a workforce will require a revolution in educational philosophy through cross-training and diversified academic curricula that incorporates not only traditional food science and food safety information but also introduces sophisticated data logistics training and a background in managing computer programs (Thompson et al., 2017). By combining an understanding of the biology of poultry processing with cross training will force academic, industry, and government research organizations to recognize and demand more integrative backgrounds of candidates in their subsequent hiring strategies. Introduction of individuals with diversified backgrounds at all levels from industry, government agencies, and academia, will lead to the potential of fresh innovative approaches for novel discoveries in the development of antimicrobials and other mitigation strategies as well as more comprehensive approaches to accelerate implementation of new detection technologies. Recruiting scientists from independent and varied backgrounds, through improved cross-training and education, and by continuing to implement science-based standards, many of the current technological hurdles of microbial analyses with the more advanced molecular approaches will be overcome. By evaluating and understanding the microbial ecology and advancing microbial mapping, this may be the first step to a number of successful changes to the poultry industry as its innovation may lead to online sensors, novel snap tests for indicator organisms, risk-reduction management, and antimicrobial selection. In the remainder of this review, the opportunities for where such changes such as the introduction of microbiome technologies might lead poultry processing and the ensuing expected outcomes will be discussed.

## GOVERNMENT MANDATES AND POTENTIAL AVENUES FOR SUCCESS INVOLVING MICROBIOME MAPPING

Due to years of data analysis that integrates foodborne related disease and microbial loads, current microbiological and molecular techniques are in place to ensure food safety. Importantly, these data benchmarks are representative of the microbial load that is considered safe across all seasons, geographical locations, and processing lines. The U.S. FSIS mandates that poultry plants sample carcasses at various processing stages in order to ensure that plants do not exceed national standards for microbial contamination (FSIS, 2015). Yet, when innovations such as the implementation of microbiome mapping to evaluate long-term changes in the plant environment, as with other microbiological data are introduced it becomes critically important that a method of sample collection and interpretation is standardized in a similar fashion as traditional methods (FSIS, 2015). This reduces sample acquisition time and enables a better comparison of traditional methods with NGS technologies.

While plate methodologies should absolutely still be used as an adjunct monitoring strategy as it provides the fastest data to date and a relative assessment of the true risk to the consumer, microbiome mapping using NGS technologies as the avenue of the future for the long-term assessment of risk-assessment represents an entirely new approach to a much more in-depth profile of the farm to fork microbial ecology (Blevins et al., 2017; Ricke et al., 2017). By improving and understanding microbial ecology and subsequently combining technologies, real-time innovations in online sensors

that enable immediate decisions are possible in the future. For instance, if a cornucopia of microorganisms becomes a true predictor of pathogen risk or food spoilage or even meat quality, sampling carcasses for these core populations could actively monitor the environmental effects on poultry processing microbial ecology. Compared to plate counts, microbiome sequencing is an unconstrained method allowing for complete novel discovery independent of media selection or growth conditions.

An example for the utility of microbiome mapping is the long-term evaluation of multi-hurdle antimicrobials in a plant processing environment. Currently, APC and post hoc analyses of specific populations identified using selective and differential media are the standard. While APC serve as indicators for the total microbial load and therefore evidence of level of microbial contamination in the system, each APC plate is different and results in unique microbial profiles based on sample location (Kim et al., 2017). Additionally, different media may apply different selective pressures that allow certain populations to show up on plates but remain below the limit of detection (Kim et al., 2017). Therefore, while APC are established, microbiome mapping may provide better resolution as to the populations present and how they fluctuate with time and treatment. Populations that rise or fall may increase or decrease quality or pathogen risk assessment depending on the microbiome mapping vs. no difference overall with APC (Kim et al., 2017).

Changes in microbial populations that can actively be monitored by microbiome sequencing may correlate with important industry standards outside of the rise or fall of a specific microorganism. Over time, resistance to antimicrobials by various microbial populations mount and the population composition can shift. If baselines are established and monitoring is consistent, the resurgence of a specific group of organisms may predict future outbreaks and the requirement to change antimicrobial approaches and enable a proactive management approach to food safety. Ultimately, if innovations continue, the ability to create real-time microbial load monitoring or pathogen monitoring on the line may be possible and could be coupled with microbiome sequencing for enhanced resolution. For instance, a sensor mounted on the chiller tanks that can sample and subsequently quantify bacterial population shifts, total dissolved solids, and pH would substantially revolutionize the industry. If the sensor can collect the rinsates for analysis using microbiome sequencing or targeted quantification techniques, active monitoring of the line becomes very rapid. As a result, commercial poultry processing line speeds could increase to meet consumer demand.

Currently, the common sampling practices have specific requirements to ensure the accuracy of the data and ultimately that must be continued and expanded for the use of any NGS for development of sensor technology. A critical component of this is the uniformity of data collection among individual processing lines, processing plants, and geographical location which would be universally true regardless of type of data being collected. In addition, standards remain extremely important as they not only provide a historical baseline, but can actively be built upon for assessment of new technologies. This is especially true with microbiome sequencing, which requires standardization for any and all direct comparisons among sample sources. If the DNA is properly harvested and contextualized as the long-range monitoring system for all of the poultry processing plants in the U.S., this could actually become data that is actively monitored by companies as a whole and ultimately provide very useful data for applications such as traceability.

For example, at the moment, selecting whole bird carcasses may only be sampled randomly one time and at one location. Further sampling must occur across all of the plant shifts as well as across time and all personnel sampling the independent carcasses should be organized and adhere to at least some definitive basic standards. They must also ensure that any culture media used to collect the sample such as buffered peptone water should be sterile (FSIS, 2015). This requires time, man-hours, training, and consequences for noncompliance that may even be enforced after a lengthy investigation resulting from a foodborne disease if management is not actively engaged.

The real power in online monitoring systems combined with microbial mapping is the utilization of a central facility, such as the corporate laboratory offices of a poultry processing company, to monitor the lines directly and remotely. The integration of important information like the time of day the sample was collected, the data, and the geographical location are important covariates for more advanced analyses as they can impact meat quality and pathogen monitoring. In the future, detailed record keeping has the potential to incorporate advanced mathematical approaches to analyze microbiome data for development of predictive models via advanced programming tools such as machine learning (Ricke et al., 2017; Thompson et al., 2017). Likely, sampling regimens such as the ones used by FSIS and the poultry industry will still be utilized, and they should in order to complement and validate the real-time data.

## ESTABLISHING STANDARDIZATION RIGOR FOR MICROBIOME MAPPING

Likely, real-time sensors and other innovations will complement microbiome mapping as integrating pathogen and spoilage risk assessment and finding new indicator methods with enhanced precision is appealing. However, ultimately, the projected power of long-term facility management and monitoring via microbiome mapping will yield potentially novel discoveries that will not only facilitate the innovation of

other technologies but also the appreciation of more traditional techniques. Just like microbial and molecular detection techniques, microbiome sequencing, as previously mentioned, requires standardization as each stage of microbiome processing has the capacity to induce bias that reduces the reliability and repeatability of the data. Every difference, be it sample collection or DNA extraction, introduces bias to the data and will result in data that are not comparable across time and space. There are numerous options available for 16S rDNA extraction from bacterial pellets and they all introduce bias to the data that will alter the endpoint data analyses (Kim et al., 2012; Rothrock et al., 2014; Becker et al., 2016; Golob et al., 2017; Brandt and Albertsen, 2018; Stinson et al., 2018). It will be important to reach a consensus as a scientific community before implementing this technology. As compositional differences are important, and as risk assessment may be based off of the ecological changes in bacterial communities, this single method approach is invaluable (Becker et al., 2016). For example, while TRIzol is economical, it also selectively reduces GC-rich regions and has been associated with data bias and in certain cases may result in misleading conclusions (Kim et al., 2012; Becker et al., 2016). The use of celite, which exploits the binding chemistry of fossilized diatoms, is extremely economical but unproven. However, it has not been validated for microbiome sequencing, requires considerable time and technical skills to acquire data, and as a result may alter the final data in unknown ways (Kim et al., 2012).

Just like with the production of microbiological plate media-based data, the details going into how microbiome sequencing must occur will need to happen. For example, there is a consensus from the human microbiome side, and confirmed by the animal microbiome groups, that suggests that the hybridization of protocols between the "gold standard" kit, QIAamp DNA Stool Mini Kit, and mechanical homogenization of bacterial pellets would provide the highest data consistency for samples such as poultry processing rinsates (Kim et al., 2012; Rothrock et al., 2014; Becker et al., 2016; Golob et al., 2017). Studies evaluating the microbiome have used this protocol with repeatable success (Kim et al., 2012; Rothrock et al., 2014; Becker et al., 2016; Golob et al., 2017). Additionally, all DNA that is purified from rinsate samples must meet quality standards prior to downstream processing, otherwise there is risk for high chimera frequency occurring resulting in unuseable data. At a minimum, the 260/280 ratio should be greater than 1.7 and less than 2.2 for successful sequencing (Illumina MiSeq V2 Reagent Kit). An optimal yield for extraction should be between 75 and 300 ng/µL according to the QIAamp DNA extraction kit (Qiagen QIAmp DNA extraction kit).

Beyond extractions, library preparations will also become an important point of standardization. The polymerase selected for the PCR part of the kit needs to be an ultra-high-fidelity polymerase, ideally the *Pfx* kit, to ensure as few chimeras as possible with the highest fidelity. But, the most important component may be the primer selection for the library preparations. At the same time, the target for sequencing must be identified. Fortunately, the work of Carl Woese and his colleagues determined that the phylogenetic relationship between prokaryotic species could be determined based on conserved gene sequences that are not subject to horizontal gene transfer (Woese and Fox, 1977; Clarridge, 2004). The focus on the 16S rDNA region has revolutionized microbial ecology and evolutionary biology and can certainly be implemented by the poultry industry if microbiome mapping is to be further developed and standardized as a routine microbiological procedure.

However, just selecting which 16S rDNA region of interest industry wide will be controversial as each region confers both benefits and caveats. This discovery by Woese and his colleagues ultimately led to the current microbiome revolution. As a result, the evolution of microorganisms could be delineated based on genomic analyses to assess taxonomic relatedness and subsequently quantified, ultimately led to early revolutionary phylogenetic concepts such as the recognition of the Archaea as a separate kingdom all the way to the more recent flurry of studies describing food animal microbiomes (Woese and Fox, 1977, Clarridge, 2004; Diaz-Sanchez et al., 2013; Hanning and Diaz-Sanchez, 2015; Yoon et al., 2015; Cao et al., 2017; Ricke et al., 2017; Huws et al., 2018; Shi et al., 2019). Currently, Eubacteria populations are heavily focused on for their contribution to the pre-and post-harvest food animal production systems. There may be some unexpected outcomes. For example, with the power of microbiome sequencing, the phylogenetics of the Archaea that contribute to the food animal gastrointestinal microbiome and are not culturable under standard conditions may actually have functional importance for the processing environment (Woese and Fox, 1977; Clarridge, 2004). By relying solely on traditional microbiological culture techniques, this contribution could easily be overlooked. However, what genomic region should be focused on? The 16S rDNA gene, a component of the 30S small ribosomal subunit in Eubacteria and Archaea, emerged as the premier target as it enabled the differentiation between Eubacteria versus Archaea and contains 9 hypervariable regions. This small molecule has conserved regions that have the exact same sequences that are actively targeted by universal primers with 9 intragenic regions varying between 30 and 100 base pairs that are termed hypervariable regions (Robinson et al., 2016; Amato, 2017; Ricke et al., 2017). Phylogenetic relationships are a function of dissimilarity and similarity within those hypervariable regions. Over time, these hypervariable regions accumulate point mutations while the conserved regions universally do not change and can be targeted with conserved and universal primers. The more closely related a species is to another species, the more similar those mutation patterns are in the hypervariable

regions (Robinson et al., 2016; Amato, 2017; Ricke et al., 2017). Altogether, the section of DNA flanked by conservative primers and containing the hypervariable region is identified as the operational taxonomical unit (**OTU**). Currently, short reads are the standard and only amplify one specific hypervariable region (Ricke et al., 2017). Until long-read technology becomes accessible, identifying the single region to focus on that will have the greatest resolution for the poultry processing industry will require development of baseline data sets.

The selection of the specific hypervariable region of interest will absolutely influence the microbiome data and its subsequent interpretation (Comeau et al., 2017; Ricke et al., 2017; Brandt and Albertsen, 2018). For example, targeting the (hypervariable) HV3 to HV4 region also favors enteric pathogens and gut microorganisms, which ultimately may be what the poultry industry needs to focus on as that is one of the more likely sources of microbial populations associated with potential food safety and spoilage organisms. However, that remains purely an assumption at this time. While universal in nature, the primers that target specific hypervariable regions could also favor certain strains, allowing for non-proportional amplifications of specific populations resulting in a strong bias that could, in turn, be problematic for processing management decisions (Yu and Morrison, 2004; Hugerth and Andersson, 2017).

In order for microbiome mapping to become prevalent and to be implemented as a tool for the industry, a consensus on the target regions and pressure on the sequencing companies to generate accessible kits and sequencers for long reads may become an important and necessary next step. Additionally, without plate counts to verify populations and to enable a faster detection of microbial shifts, microbiome sequencing alone will lead to potentially misplaced interpretations. However, combining microbiome mapping with improved microbiological and molecular screening as part of a long-range monitoring system for plant sanitation efficacy, antimicrobial effects, and microbial ecology may reveal spoilage and pathogen prevalence in a more predictable fashion.

## QUALITY CONTROL AND ASSURANCE OF MICROBIOME MAPPING

In order to prepare a sequencing run, quality needs to be ensured through the process. Sequencing is still relatively expensive for routine application and failures can push back data acquisition for at least a week, if not more depending on the identified stage of the failure. The detailed protocols for poultry processing samples have been provided in a review by Feye and Ricke (2019) and will only be briefly discussed here. Having library preparations meet specific standards sets the sequencing run up for the greatest probability of success. It also ensures that the sequencing data received is of optimal quality as well, which is conducted with both cleaning up reactions post-library construction as well as ensuring homogeneity in the library preparation with Qbit, Bioanalyzer, and qPCR. In order to remove the PCR primers, polymerases, and reagents, and to ensure a homogenous sample preparation, reactions must be cleaned up between library preparation and sequencing. There are a few ways to accomplish this, including exo-zap, gel purifications, and normalization kits. As simplicity is the goal, the Sequal Prep Normalization kit enables a one-step amplicon purification and copy number standardization, which is important for downstream processes to succeed (Invitrogen, 2018). The kit is easy to use, has a relatively long shelf-life (6 mo), and is relatively rapid without introducing new bias (Invitrogen, 2018). This step is important as the plates are then pooled post-normalization resulting in the final library. In order to verify that the post-PCR product is within the range of any kit or method used, it is highly encouraged that the Qbit, which is a fluorescent kit that binds to dsDNA, is used as an approximate measurement of the concentration. Importantly, the Qbit system does not reveal quality, but it does ensure the sample is within the range of the normalization and qPCR kits. It is also a good final library check prior to sequencing.

The quantification of the library, as well as purification verification, is conducted in 2 steps: bioanalyzer and qPCR with qPCR being the most informative step. That being said, each step will be essential as it fully determines the quality of the library preparations. The use of the bioanalyzer is important as it provides necessary information on library integrity, the formation of primer dimers, and an estimation of the DNA concentration based on the area under the curve. In short, the bioanalyzer should be the first step after library normalization as it quickly and accurately indicates the quality and integrity of the DNA library preparation. However, the absolute determination of library copy numbers requires qPCR. It is important for 2 reasons: (1) the qPCR kit will ensure that the final library is within the optimal copy-number range that corresponds with the best depth of sequencing of the Illumina sequencing platform, and (2) contamination will be detected or confirmed. The KAPA Biosystems Library Quantification kit (Roche International, LLC, Wilmington, MA) is specifically tailored to the Illumina platform (Kappa Biosciences). The rigorous testing by KAPA Biosystems (Roche International, LLC) ensures that kit consistency combined with the uncomplicated protocol aids in the reliable standardization of sequencing, principally leading to the production of precise, contamination-free microbiome data (Kappa Biosciences). To aid in interpretation, the company also provides a “plug and play” spreadsheet with expected ranges and outcomes, calculations of the efficiency curves, and the dilutions. To save money, reducing the reaction volume from 20 to

10 µL is acceptable and recommended by the manufacturer (Kappa Biosciences). In order to ensure quantitation is absolute, it is recommended that the sample be diluted as it confirms the accuracy of the copy number (Kappa Biosciences). It should be noted that any library prep should also be assayed in its final, undiluted state as back-calculations off of the standard curve are not necessarily accurate (Kappa Biosciences). Although not recommended, if batches of libraries are produced at different times or by different personnel, then any qPCR analysis should include samples representative of those library preps. This is because assuming homogeneity among batches should never be a common practice. First tier troubleshooting and data interpretation assistance is found in the kit manual (Kappa Biosciences).

## SEQUENCING OPTIONS, YET ANOTHER POINT OF STANDARDIZATION

The Illumina platform is one of several NGS options that have emerged as a result of rapid advancements in genomic technologies (Heather and Chain, 2016). PacBio, SOLiD, and Ion Torrent platforms continue to impact the genomics revolution and have taken aim at becoming the instrument of choice in microbiome sequencing (Kozich et al., 2013, Luo et al., 2012; Golob et al., 2017). However, due to the multi-parallel, sequence-by-synthesis technology employed by Illumina (San Diego, CA), Illumina exhibits specific advantageous characteristics for the analysis of complex microbial communities (Luo et al., 2012; Hiergeist et al, 2015). The tailored 16S rDNA sequencing library kit, the lower cost per instillation and analysis, a low error rate, and the optimal contig length for assembly make the Illumina platform one of the more popular platforms (Luo et al., 2012; Hiergeist et al, 2015). Illumina currently markets 3 sequencer platforms: HiSeq, MiSeq, and the desktop sequencer NextSeq500 with several of these technologies now being introduced and being implemented by government-oriented systematic whole genomic sequencing (Allard, 2016, Manley et al., 2016; Taboada et al., 2017; Pightling et al., 2018). Illumina produces a 1% error rate over A/T-rich homopolymer regions, and in total a 3% error rate due to single point errors in GGC motifs and 5' bias, which is minor compared to other methods (Luo et al., 2012). Additionally, Illumina reads are between 250 and 400 basepairs per read and are optimal for coverage across the V3 to V4 region of 16S rDNA (Luo et al., 2012; Kozich et al, 2013; Hiergeist et al, 2015). Other methods, namely the PacBio and long read sequencers, can have significant problems with error rates and de novo sequencing. These technologies are rapidly improving and will likely be very accessible to the poultry industry in the near future. Importantly, as MiSeq continues to remain popular, many of the simple to use bioinformatic pipelines, such as QIIME2, are tailored to MiSeq. Until that changes, and it will need to, the MiSeq platform will probably remain the gold standard.

Data indicate that the library prep chosen, the concentration of DNA entering library construction, the method of barcode annealing, and the concentration of DNA loaded onto the flow cell are all critical points for standardization (Luo et al., 2012; Kozich et al, 2013; Hiergeist et al, 2015). To resolve this issue, all microbiome kits need to be the standard Illumina 16S rDNA sequencing kit, as well as their ancillary preparatory kits, such as the normalization and library prep kit. Furthermore, mock communities, or cocktails of equal-copy number bacteria 16S rDNA, are recommended as optimal internal standards for sequencing (Kozich et al., 2013; Park et al., 2016). Like other recommendations, in order to ensure universal monitoring in poultry processing plants, baseline mock communities will need to be developed after a systematic broad survey of microbiome communities from poultry production and processing sources (Kozich et al., 2013; Oakley et al., 2013; Park et al., 2016; Rothrock et al., 2016; Kim et al., 2017; Handley et al., 2018; Wages et al., 2019). A defined community that is the standard across all microbiome sequencing analyses throughout poultry processing monitoring will enable uniform comparisons between plants both across time and location.

## DATA ANALYSIS

The revolution of bioinformatics platforms parallels the creation of high-throughput data techniques, such as the MiSeq. However, there is a concern associated with selecting or relying on a single platform. Just as with laboratory wet-bench work, there will always be stipulations associated with a single bioinformatics approach. Comparable data are difficult to create as each stage of this protocol, and corresponding platform, can introduce variability that impacts the final data analysis (Luo et al., 2012**;** Kozich et al., 2013; Hiergeist et al, 2015; Plummer et al., 2015; Golub et al., 2017). Second, bioinformatics continues to evolve and produce novel avenues for analysis. The platform chosen and the options available to researchers and industry personnel will perpetually change.

Currently, there are 2 main categories available for data analysis: multi-platform analysis and single platform analysis. While multi-platform analyses are becoming easier to use, complex coding languages make it difficult to engage all of the available options. Therefore, the single platform analysis is suggested as the pipeline easier to use and understand (Knight et al., 2007; Schloss et al., 2009; Plummer et al., 2015). Single platform analysis is fully integrated and can produce data outputs that are easy to interpret (Luo et al., 2012, Kozich et al., 2013; Hiergeist et al, 2015; Golub et al., 2017**;** Ricke et al., 2017; Bolyen et al., 2019). Likely, single platform analysis will predominate every aspect of research in the near future. While some power is lost, the standardization of pipelines, the statistical

analyses, and the repeatable pipelines are essential components of microbiome research that should be required by a research and scientific community, even beyond poultry applications.

There are 2 main platforms that are commonly cited in the published literature for single platform analysis: QIIME and MOTHUR (Schloss et al., 2009; Bolyen et al., 2019). Failure to create a standardized bioinformatics pipeline will impact the ability to share and compare data among processing plants and can lead to data adjustments that may not necessarily be appropriate and/or accurate. It will also make regulations based off of microbiome data difficult to generate and implement. However, the data between QIIME and MOTHUR seem to be comparable, with recent improvements allowing the pipelines to merge (Plummer et al., 2015; Golub et al., 2017). Therefore, it is essential to select one pipeline. As the specific protocols executing the bioinformatics programs become easier, they are not discussed in length herein.

The QIIME acronym stands for "Quantitative Insights into Microbial Ecology," and was developed by Caporaso et al. (2010) off of the PyCogent toolkit (Knight et al., 2007). Since then, QIIME2 has emerged with a different mission—to become the bioinformatics clearinghouse that is accessible by a broad audience. Ideally, by taking R coded backbone programs, like DADA2, sk-learn, and ANCOM, simplifying, and creating an easy to use Python interface, less trained users can evaluate the data using a reproduceable pipeline. This overcomes many issues associated with R packages and individualized pipelines where the manipulation of the data can be user specific. QIIME2 also intends to standardize the statistical output, incorporating a false discovery rate that accounts for the compositional challenges of NGS data. The data analysis pipeline is highly interactive and fluid, enabling the semi-customization of repeatable data analyses pipelines and the dissemination of results (Schloss et al., 2009; Ricke et al., 2017; Bolyen et al., 2019).

MOTHUR is another platform that is completely integrated, and although not considered as user friendly as QIIME remains a preferred choice by some (Ricke et al., 2017). Updates to this platform are meant to be more plentiful, open sourced, and malleable. Instead of using Python like QIIME, MOTHUR is written in C++ and is universally applicable to all known sequencing platforms and is fast (Ricke et al., 2017). The general pipeline for MOTHUR is the same as QIIME in terms of trimming the reads, assigning OTU and taxonomical identification, and the production of diversity plots (Ricke et al., 2017).

## THE EFFORTS TO OVERCOME THE CURRENT LIMITATIONS OF MICROBIOME DATA

Microbiome data produces 3 main sets of information: alpha diversity, beta diversity, and compositional information. Currently, most of this is relative, where the assumption is that 10 ng input of sample DNA into each library has rDNA that is equally contributed by all prokaryotes present and all of those species are able to contribute to the microbial ecology of the system. Additionally, it is assumed that the specific time the microbiome data is generated is representative of the environment. Efforts to overcome these limitations exist, but the microbial ecology must still be considered. While amplicon-based classification of OTUs is informative in microbiome analysis, biases are introduced during DNA extraction and during OTU clustering and identification. As mentioned earlier, extraction of genomic material can be greatly influenced by the methodology used for extraction, as well as the starting sample matrix (Kim et al., 2012; Rothrock et al., 2014; Becker et al., 2016; Golob et al., 2017; Brandt and Albertsen, 2018; Stinson et al., 2018).

Currently, there are 4 major limitations, with several caveats existing in the methodology associated with sequencing and bioinformatics. The biggest limitation to the microbiome sequencing to date is that it is absolutely not quantitative. Ultimately, everything is based off of the relative abundance of 16S rDNA within a sample, with a normalized sample input. This assumes that the copy numbers of 16S rDNA are consistent between bacterial species, which is not true. Developing a quantitative approach using flow cytometry will overcome that limitation; however, the assumption of the same copy numbers per bacterial cell will still exist. Therefore, ultimately, the microbiome must be deconvoluted which entails a number of goals. First, the copy number per species must be determined, or at the very least at the phylum level. Second, high-throughput, absolutely quantitative primers must be developed to immediately quantify the viable populations in aerobic samples and total populations in mixed atmospheric samples. In accomplishing this task, biological activity and function can then be assigned to these organisms. This will enhance predictive bioinformatic capabilities and interpretations, which are both key for food and poultry industry applications.

Another limitation to microbiome sequencing is that it includes all of the microbial DNA present on the surface of a carcass or the environment. This does not mean the populations present are actively contributing to the microbial ecology or risk, such as the case with pathogens, or are associated with microbial load. While microbiome mapping has been proposed as the next generation of microbial mapping within a plant, this reality underscores the importance of coupling microbiome sequencing with actual phenotypes, or microbiological plate counts. Not only will this provide faster, more immediate answers than microbiome sequencing alone, it is more likely to immediately reveal the specific risk identified from culturable and non-culturable enriched samples in the plant. Microbiome sequencing provides significant information, deconvolution and quantitation provides even more precision, but without microbiological analyses,

precise decisions may not be possible. This is especially true if other phenotypes, such as antibiotic resistance, become important to monitor. Just as in whole genome sequencing and RNA-Seq, the absence of phenotype information weakens the strength and applicability of the data.

The validation of the microbiome sequencing and the speed of data acquisition are the final major limitations of microbiome sequencing. Validation of NGS data is common in whole genome sequencing and RNA-Seq, which ensures that the copy number present is truly real. This can be accomplished through Fluidigm and sometimes microarray technology. Currently, validation of the copy number of 16S rDNA sequences is not a common practice. As each technology (sequencing and qPCR-based technologies) exists, validation may be problematic. Therefore, to overcome this limitation, species of interest should also be plated on their respective media. This will not only overcome some of the time-consuming issues of microbiome sequencing, but also the phenotype and a final validation of the data. Ultimately, the time element of microbiome sequencing will be addressed with newer technologies and more commercial kits that reduce the intellectual contribution required for successful library preps. Additionally, line sensors may also need to be developed that hybridize absolute cell counts, such as with flow cytometry and with pathogen detection if this technology will ever become useful. In the short term, plate data and traditional microbiological methods will be required to meet immediate plant management quality control needs.

While not a limitation of the technology, another problem with microbiome data is the lack of statistical understanding and background that even highly skilled scientists and statisticians have when it comes to NGS data. Importantly, not every statistician is a bioinformaticist. For instance, a statistician with expertise in non-biological disciplines and prediction may not fully understand a biological process such as poultry processing and microbiome sequencing. Therefore, not only is the poultry industry going to need to change the training paradigms, the limitations and strengths of other fields must be evaluated in this context as well.

There are a few important key fundamentals associated with microbiome sequencing that must be understood prior to any data planning or implementation. Microbiome data is absolutely not qPCR, it is not plate count data, it is not a single response element or uniform hypothesis per tube. There can be millions of responses per tube, which leads to significant false discoveries if populations are arbitrarily selected based on interest and not based on statistically significant rationales. Instead of *P*-values for pairwise and individual compositional effects, a q-value must be used. Statistical analyses such as t-tests, ANOVAs, MANOVAs, and other tests are irrelevant as the assumptions associated with microbiome sequencing and the statistical methods are incongruent. In canned protocols such as QIIME2, analysis of communities of the microbiome (ANCOM) and Gneiss are available. These methods incorporate strict false discovery rates and ensure statistical power is satisfactory. Unfortunately, statistical power is a bit of a controversial topic to date with microbiome sequencing, but considerable progress has been made to incorporate that knowledge into the appropriate research designs. Multiple R-based and web-based methods exist for this and absolutely must drive the power of the analysis and the applicability of the results. Without proper statistical power and compositional analyses, the data are not informative and could be easily misinterpreted.

## CONCLUSIONS

Ultimately, technology will always evolve and it will always open the door for the poultry processing industry to improve its ability to safeguard the public against foodborne pathogens. However, without recognizing the opportunity and becoming more highly integrative, it will become difficult to evolve. There are several aspects of more advanced integration to consider. Certainly, the adoption and incorporation of new technologies such as NGS and microbiome analyses represents the introduction of new concepts, but the truly valuable component is the ability to integrate these new technologies with existing methodologies such as plate counts for biomapping. Both are still relevant in their own right, and efforts to overlay the data of otherwise quite different methodologies will result in much more meaningful interpretations for assessing the real-world aspects of microbial ecological changes occurring in a poultry processing plant. Likewise, educational and training approaches for incoming workforce members will require paradigms that are much more integrative across a multitude of diversified curricula and philosophies. This essentially means that the standard baselines for fundamental knowledge and experience will be need to be complemented by developing skills to adapt and embrace new delivery methods for training and learning that may to some extent actually occur on the job. Finally, while certain aspects of NGS and microbiome applications may appear at the moment to be hypothetical as they relate to poultry processing, these technologies much like previous innovations will in time become a reality as commercialization advances are made and expanded markets are sought. Ultimately, applications will be co-developed as technology manufacturers and end users interact and align technology with practical questions that need to be addressed.

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