

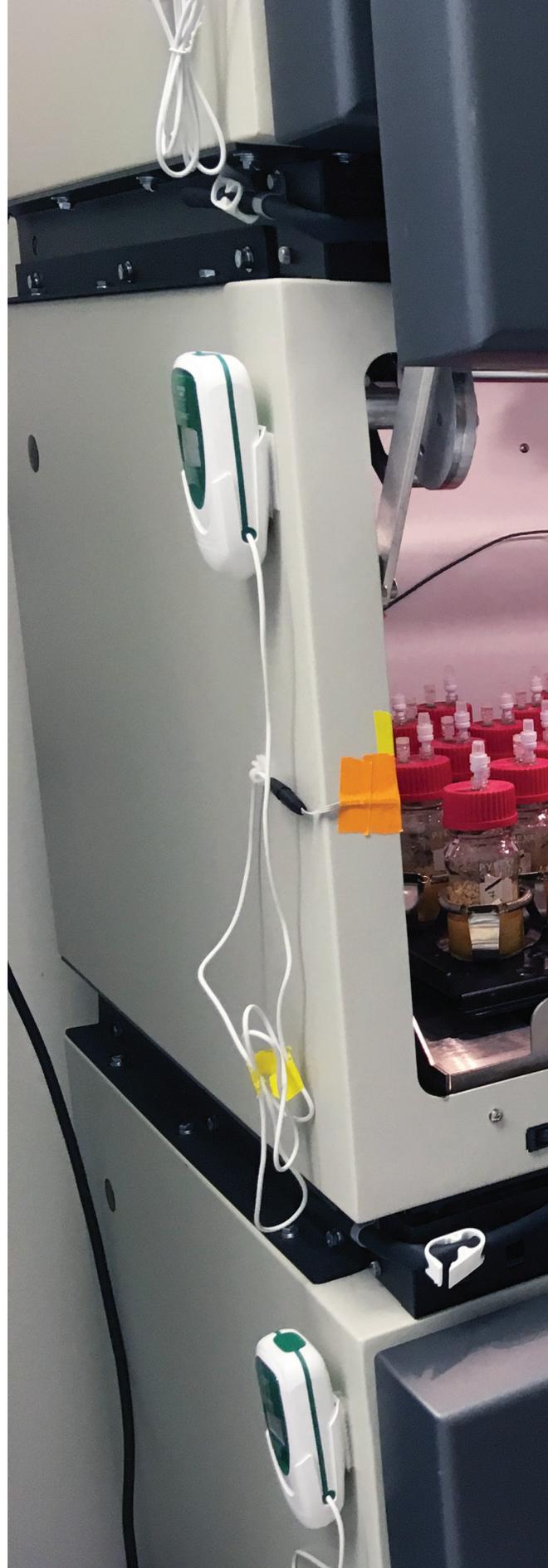
BITS OF THE **BATCH**

Researchers use new molecular biology tools to identify inhabitants of ethanol plant bacterial communities.

By Lisa Gibson

Lactic acid bacteria cells grow and reproduce much more quickly than yeast cells do, consuming the nutrients meant for the yeast in fermentation and producing organic acids instead of ethanol at a molecule ratio of 1:1. That lost production can cost a plant more than \$1 million per year, says Jim Steele, lactic solutions general manager at Lallemand Biofuels & Distilled Spirits.

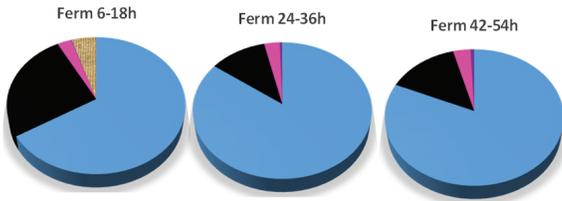
Steele is a co-author of a research paper that used modern methods to identify the bacteria species present in four U.S. ethanol plants over six months. Researchers found that microbiota in ethanol plants are not significantly diversified, with just 13 types of bacterial contaminants making up 90% of the total population, predominantly lactobacilli.





MINI MICROBIOTA: Jim Steele, lactic solutions general manager at Lallemand Biofuels & Distilled Spirits, works with small fermentations in Lallemand's lab. Steele co-authored a study that characterizes bacterial communities in ethanol plants, detailing microbiota from mash to beer well.

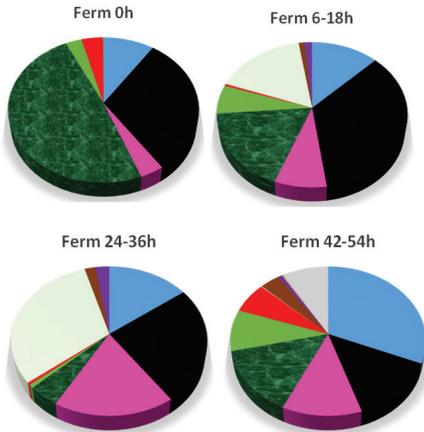
PHOTO: LALLEMAND BIOFUELS & DISTILLED SPIRITS



NOTE: There was not sufficient DNA recover for analysis at Time 0h

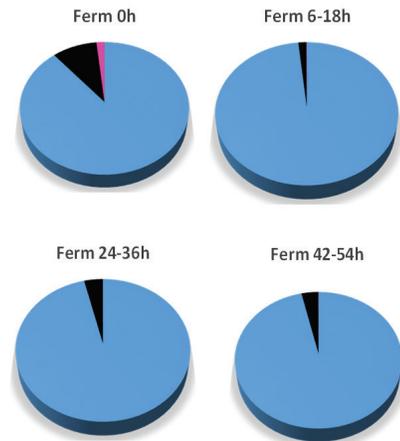
Ethanol Plant 1

- *Bacillus sp.*
- *Clostridium sp.*
- *Lb. agilis cluster*
- *Lb. brevis cluster*
- *Lb. delbrueckii cluster*
- *Lb. plantarum cluster*
- *Lb. reuteri cluster*
- *Lb. zeae cluster*
- *Streptococcus sp.*
- *Weissella sp.*
- *Unclassified*



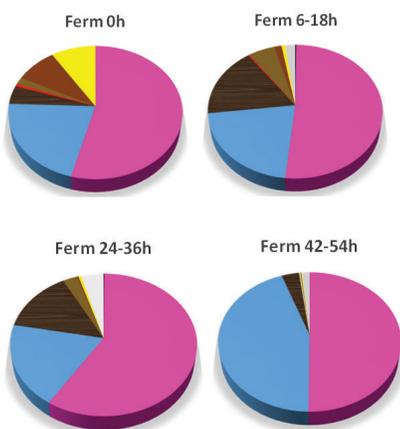
Ethanol Plant 2

- *Bacillus sp.*
- *Clostridium sp.*
- *Lb. agilis cluster*
- *Lb. brevis cluster*
- *Lb. delbrueckii cluster*
- *Lb. plantarum cluster*
- *Lb. reuteri cluster*
- *Lb. zeae cluster*
- *Streptococcus sp.*
- *Weissella sp.*
- *Unclassified*



Ethanol Plant 3

- *Bacillus sp.*
- *Clostridium sp.*
- *Lb. agilis cluster*
- *Lb. brevis cluster*
- *Lb. delbrueckii cluster*
- *Lb. plantarum cluster*
- *Lb. reuteri cluster*
- *Lb. zeae cluster*
- *Streptococcus sp.*
- *Weissella sp.*
- *Unclassified*



Ethanol Plant 4

- *Bacillus sp.*
- *Clostridium sp.*
- *Lb. agilis cluster*
- *Lb. brevis cluster*
- *Lb. delbrueckii cluster*
- *Lb. plantarum cluster*
- *Lb. reuteri cluster*
- *Lb. zeae cluster*
- *Streptococcus sp.*
- *Weissella sp.*
- *Unclassified*

Bacteria

The methods used in the research allowed the identification of bacteria on a species level, even those that don't grow on typical lab media, offering more insight than is gained using traditional plating methods, Steele says. Bacteria species varied, sometimes widely, between plants, as well as in different parts of the processes.

"We found that the microbiota is relatively simple and plant-specific," he says.

Methods and Results

The research was led by Fernanda Firmino, now a research scientist with lactic solutions and sales supervisor in Brazil for Lallemand, as part of her Ph.D. research at the University of Wisconsin-Madison. The paper, "Characterization of Microbial Communities in Ethanol Biorefineries," appeared in the *Journal of Industrial Microbiology & Biotechnology* in December 2019.

Researchers used both partial length 16S rRNA sequencing and shotgun metagenomics sequencing, as 16S rRNA sequences cannot resolve bacterial classifications beyond the genus level. Shotgun metagenomics allows the comprehensive sampling of all genes present.

"We're using modern molecular biology techniques to look at the organisms present," Steele says. "The resolution we have is significantly better than with previous methods."

Samples were drawn from four Midwest biorefineries three times in six months, to obtain 77 primary samples for the study. The samples were taken from three different stages of ethanol production: cooled mash, fermentation and the beer well. Additional samples were taken two years later from one plant to evaluate stability of the microbiota.

The vast majority of operational taxonomic units (OTUs—a common measurement in gene sequencing) collected from the plants belonged to the Firmicutes (89%) and Proteobacteria (11%) phyla. Within Firmicutes, 80% of the OTUs belonged to the genus *Lactobacillus*, 11% to *Lactococcus*, 2.5% *Weissella* and 1.8% to *Clostridium*.

Per plant, the abundance of *Lactobacillus* ranged from 37% to 95%. "They're different because the majority of organisms present during fermentation are actually organisms that live there," Steele says. "That difference is critical because a lot of people think that most of the organisms in fermentation came directly from the corn, which happens, but they are a relatively minor component of those in the fermenter."

READING RESULTS: The results of research to characterize bacterial communities shows variances among plants.

SOURCE: LALLEMAND BIOFUELS & DISTILLED SPIRITS

Within *Lactobacillus*, five clades were identified: *Lb. delbrueckii* (55%), *Lb. reuteri* (15%), *Lb. casei* (1.4%), *Lb. perolens* (0.5%) and *Lb. brevis* (0.3%).

Over the two years of research, the main contaminants in the studied communities didn't show significant changes, Steele says. In general, some plants' bacterial communities will change more than others, as a result of how they're run or how often they change their processes, specifically antimicrobials.

"We don't know for sure, but the idea is, in many of these environments, there's an advantage to the organism who first lands there," Steele says. Most competitive bacteria are going to live, but a plant with a certain species was likely contaminated by that species early on. For example, plant 2 showed samples with far more *Lactococcus* than any of the other plants.

Throughout the production process, the research showed bacterial diversity was richest in the mash, decreased during fermentation and decreased even further in the

beer well. For example, during the return visit in 2018, the *Lb. delbrueckii* clade was the most abundant in the beer well sample. It increased from 10% of the population in the corn mash to 89% in the beer well. That sample showed four *Lactobacillus* clades present in the corn mash, and two in the beer well. *Lb. delbrueckii* and *Lb. reuteri* clades commonly dominated microbiota by the end of sampled fermentations, a clear indication of their fitness for that environment, Firmino's paper says.

Population dynamics change throughout the process in an ethanol plant because the pH drops, the ethanol content increas-



Firmino

es, antimicrobials are added, and only the strongest can hold on and continue to grow, Steele says.

The low complexity of the bacterial community in ethanol plants is likely a reflection of the low microbial load in the cooled mash, sanitation, antimicrobials and the restrictive environment in fermentation, the research paper says. Three of the four plants had relatively stable microbiotas across the six-month and two-year time scales of the research. Firmino posits this is likely because of control measures and the fermentation restraints.

The plants studied are all clean, Steele says. "The one we looked at over the longest period of time is one of the cleanest plants I've ever been in."

Bacterial Reproduction

Typically, a very clean plant will have a lactic growth of about 0.05%, says Jayne Kalbfleisch, technical development manager for Lallemand. "When you start seeing lactic growth climb near or above 0.1%,

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BACTERIA IN BLUE: Phibro Ethanol Performance Group has a three-tiered system for fighting bacterial contaminations that starts with proper CIP and robust, healthy yeast.

PHOTO: PHIBRO ETHANOL PERFORMANCE GROUP

there's concern because those bacteria are all growing much faster (than yeast), and consuming the glucose meant for the yeast."

Under perfect conditions, bacteria can double their numbers in 20 minutes. With no yeast, perfect temperatures and glucose, it takes just six hours for 10 bacterial cells to become 2 million. Of course, fermentation doesn't allow those perfect conditions, but a mistake such as a forgotten antibiotic dose, or a yeast prop sent to a different fermenter can create good conditions for growth. "Bacteria can just take over in a very fast amount of time," Kalbfleisch says.

Unlike yeast, bacteria reproduce through binary fission. They duplicate their DNA and then split in half. Those two daughter cells can begin splitting again immediately. "That process is much faster than the budding of a yeast cell," Kalbfleisch says. "They're very efficient."

As Steele's and Firmino's research showed, bacteria can be present throughout the ethanol-production process. "They're pretty thrifty at adapting to their environment," Kalbfleisch says. "Bacteria populations can take over based on the conditions

they encounter, so we tend to see bacteria that are more tolerant to ethanol growing in the beer well.

"Bacteria are everywhere where we exist," Kalbfleisch says. "They support our life." A "fun fact" she shares is that humans are 10% human cells and 90% bacterial cells. "We're not even all human. We're bacteria.

"But when we think of fermenting and making alcohol, they're the nasty bugs. Those are the ones we're trying to control," she says.

Controlling Bugs

Producers have their pick of providers when looking for bacterial control, as many products and services are on the market, including from Lallemand, Leaf, Dupont, BetaTec Hop Products, Phibro Ethanol Performance Group and many others.

At Phibro, the approach is to find where the bacteria are originating and effectively clean that area, reducing dependence on antibiotics. In the fermenters, for instance, the approach is threefold: effective clean-in-place methods, healthy yeast, and effective antimicrobial additions. "Antibiot-

ics are a good bacteria-control mechanism, but it shouldn't be your only control mechanism," says Jenny Forbes, vice president of products and services for Phibro.

Healthy yeast is a crucial tool for bacterial control, Forbes says. "Having a healthy, strong and robust yeast helps competitively exclude bacteria, so we put a lot of emphasis on yeast health and ensuring that your yeast is in the right growth stage, that it's fast-acting and that it's healthy," she says.

When antibiotics are added, Phibro recommends dosing early in propagation or fermentation, and adding to the proper pH and temperature areas. "You don't ever want to add your antibiotic product to materials that are just going to denature it right away," Forbes says. "So make sure where you're adding it is compatible for the antibiotics. You want to ensure that you get the most value from the product as possible."

Virginamycin and penicillin, two common classes of antibiotics used in ethanol plants, each works differently. Virginamycin penetrates the bacterial cell wall and attaches to the ribosome, the protein factory essential to all the cell's functions. It binds to the ribosome, halting all the cell's processes. Penicillin binds to the cross linking in the cell wall, causing a rupture there.

Forbes says different species of bacteria will respond to one, the other, or both. "Most bacteria we find in an ethanol plant respond to the common actives that everybody's already selling," she says.

The dose differs from plant to plant, but depends on the bacterial load, Forbes says, adding providers have testing procedures to identify optimal actives and dosage. Like Steele, Forbes attributes that bacterial load to a few factors, including operation and CIP methods.

The ethanol industry has refined and polished its bacterial control methods, Forbes says, with effective options in place.

"Bacteria will always be there," Kalbfleisch says. "Plants will always be taking measures to keep those levels low."

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