CHR HANSEN
Improving food & health

FEATURES AND MODES OF ACTION
between probiotics and prebiotics often fed to ruminants

INDEX

KEY HIGHLIGHTS

- **Probiotics and prebiotics have different modes of action that bring benefits to cattle herds.**
- **The differences among the types of probiotics (bacteria vs. yeast) should be considered when choosing a probiotic to be included in the milk replacer, supplement, or total diet of your herd.**
- **The inclusion of both probiotics and prebiotics together may bring additive benefits for beef and dairy cattle herds.**
- **Overall, several different modes of action have been presented from the different technologies discussed herein.**

In different regions around the globe, a wide variety of probiotic and prebiotic types and brands are available for beef and dairy cattle producers, raising questions regarding the (1) potential differences of the modes of action (**MoA**) between probiotics and prebiotics, (2) potential differences among probiotic strains, (3) the effects of these differences on health and performance of ruminants, and (4) whether prebiotics and probiotics are antagonistic or complementary to each other when fed at the same time to cattle. It is not the idea of this document to discuss all the questions, but to provide an initial differentiation on potential MoA regarding how these different technologies might benefit the host.

When probiotics are fed, they must be alive to support the health of the host, while also benefiting other bacteria in the same environment. On the other hand, prebiotics are "food for the resident bacteria to grow and survive" (Uyeno et al., 2015). Nonetheless, it is important to mention that together, they may potentially bring additional benefits to the animal. Table 1 summarizes the benefits of all these different compounds and as often seen when fed together they can maximize the health benefits and, consequently, the performance of the beef and dairy cattle herd.

Table 1. Main proposed modes of action (MoA) of bacteria- and yeast-based probiotics, as well as prebiotics discussed throughout the document (+ = positive effect; - = no effect; NA = not applicable).

Item	Live bacteria	Live yeast	Prebiotic
Production of antimicrobial compounds	+	NA	NA
Biofilm formation	+	NA	NA
Competitive exclusion	+	+	+
Mucin production	+	NA	NA
Supporting gut barrier integrity	+	NA	+
Modulation of immune response	+	+	+
Enzyme production (digestibility)	+	Indirect	NA
Rumen health	NA	+	+
Thermostability[1]	+	-+	+
Heat stress protection	+	+	+

[1] Thermostability in live yeast is not natural and additional protection is required.

BACTERIAL- AND YEAST-BASED PROBIOTICS: *BACILLUS SPP.*

Bacteria-based probiotics administered as direct-fed microbials to feedlot beef animals, dairy cows, calves, and small ruminants include many species and strains of *Bacillus, Lactobacillus, Bifidobacterium,* and *Enterococcus*. In contrast, most live yeast-based products contain different strains of the same microorganism, *Saccharomyces cerevisiae* or *boulardii*, for example. Each probiotic organism may have different MoA resulting in different benefits to beef and dairy cattle. Let's review the main effects of each one.

BACILLUS SPP.

Bacillus spp. are bacteria that - in their spore state – are resistant to a range of environmental conditions, such as acidic pH, absence of oxygen, high temperatures, exposure to UV rays, and any other toxic compound (Bernardeau *et al.*, 2017; Cappellozza *et al.*, 2023a). It is this remarkable resistance against harsh environmental conditions that make *Bacillus spp.* probiotics suitable for inclusion in pelleted feeds, licking blocks, and many other dietary options for cattle.

Among the probiotic solutions for cattle offered by Chr. Hansen, the **BOVACILLUS™** brand contains two strains of Bacilli: *Bacillus licheniformis* and *Bacillus subtilis*. This unique combination of strains supports the health of the host by:

Producing bioactive compounds, including lichenysin and subtilin (Sumi *et al.*, 2015) that directly inhibit the growth of potentially harmful bacteria such as *Clostridium perfringens* type A (Figure 1; Segura *et al.*, 2020).

More recently, our Innovation Department concluded a series of *in vitro* assays to demonstrate the efficacy of **BOVACILLUS™** probiotic against the different serotypes of *C. perfringens* (Trial ID 80946). In summary, our results demonstrate that **BOVACILLUS™** directly inhibit the five serotypes of *C. perfringens* (from A through E) under an *in vitro* setting, proving the direct inhibition efficacy of the *Bacillus* spp. included in **BOVACILLUS™** against this opportunistic and important spore-forming bacteria.

Biofilm formation: Biofilms are complex communities of microorganisms (Tremblay *et al.*, 2013) that strongly attach to each other and to surfaces of contact (Raghupathi *et al.*, 2017). In ruminants, the ability of probiotic *Bacillus spp.* strains to promote the formation of biofilm is a beneficial mechanism that may help reduce the binding of potentially harmful bacteria to the intestinal mucosa and the subsequent damage that these bacteria could cause to the host. In fact, recent research conducted by the Innovation Department from Chr. Hansen demonstrated *in vitro* that in **BOVACILLUS™**, *B. licheniformis* is a strong biofilm producer, whereas *B. subtilis* stimulates biofilm at a lower rate (Segura *et al.*, 2020).

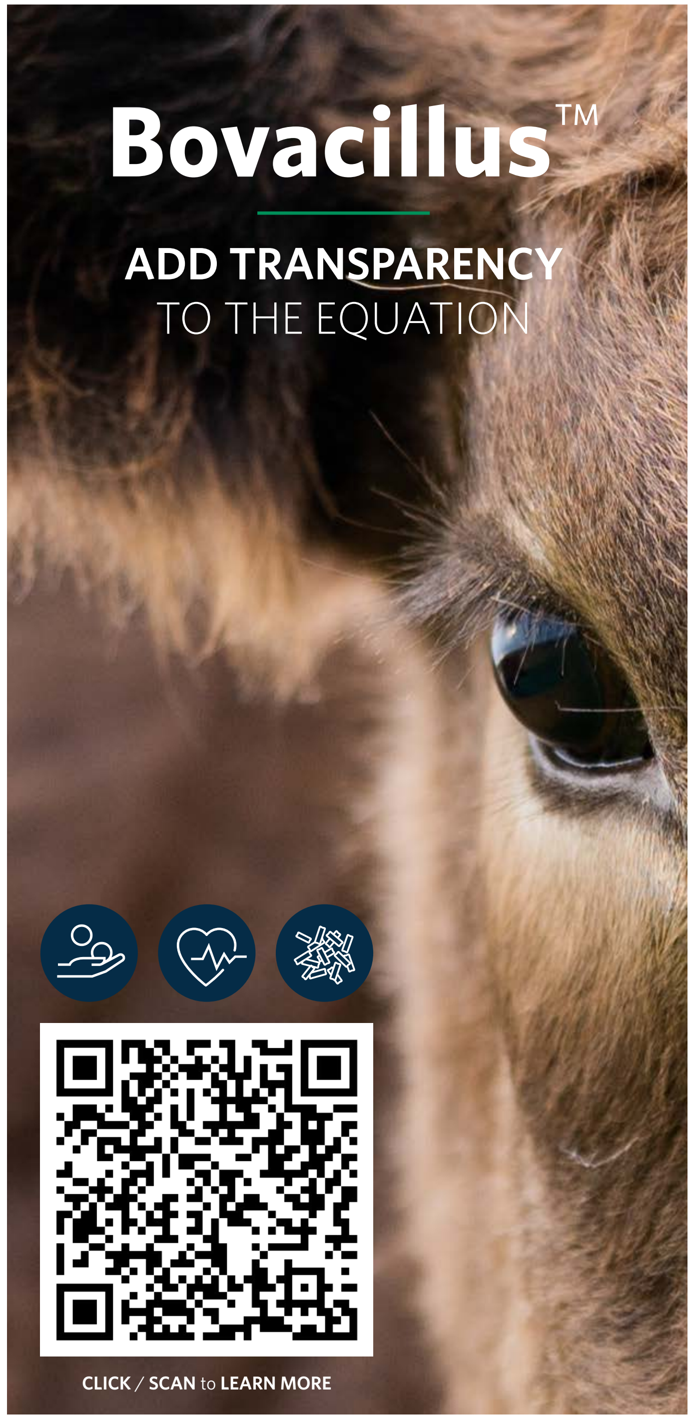
Competitive exclusion: Another mechanism by which *Bacillus spp.* may reduce the negative effects of pathogens in the ruminant digestive tract is through competition for nutrients and binding sites on the intestinal epithelial surface. Copani and colleagues (2020) demonstrated under an *in vitro* setting that co-incubating the *B. licheniformis* or *B. subtilis* (strains found in **BOVACILLUS™**) with a pathogenic *E. coli* O157 (DSM17076) strain significantly reduced the binding of the latter to intestinal cells.

It is also important to highlight that the reduction in pathogen binding was also observed in different types of epithelial cells with different types of *E. coli* (Trial M1086).

Stimulation of mucin production: One of the main protective mechanisms of the gut is the mucous barrier that works as a trap for pathogens and as a reservoir for secretory peptides and immunoglobulin A (**IgA**). Santano *et al.* (2020) demonstrated *in vitro* that the combination of *B. licheniformis* and *B. subtilis* strains found in our newest probiotic, **BOVACILLUS™**, can stimulate the production of the two types of mucins that support the host defenses against any damage.

Maintenance of the intestinal barrier integrity: Different situations may lead to the syndrome of hyperpermeable gut, also known as ***leaky gut***. Among these situations, stress and the result of stress-related responses may compromise the integrity of the intestinal cells, allowing undesirable compounds to reach the circulation and cause health issues in the animals. Recently, our Innovation group demonstrated, through an *in vitro* model, that stress-related metabolites, such as reactive oxygen species (**ROS**), compromise the intestinal barrier function integrity. However, when the *B. licheniformis* and *B. subtilis strains* of **BOVACILLUS™** are co-incubated with ROS, the integrity of the intestinal barrier was supported (Boll *et al.*; submitted). An additional *in vitro* study also demonstrated that gut barrier integrity is supported when *B. subtilis* is co-incubated with the mycotoxin deoxynivalenol (**DON**), which can also lead to leaky gut in ruminants (Boll *et al.*; submitted).

Modulation of the immune response: *Bacillus spp.* probiotics may show immunomodulatory properties, including stimulation of intestinal epithelial cells, activation of dendritic cells and macrophages, modulation of T-cell responses, and stimulation of IgA and IgM secretion from B-cells. By activating and modulating the profiles of cytokines released by dendritic cells, *Bacillus spp.* probiotics can influence *in vitro* T-cell responses, and thus may support normal host immune responses in the absence or in the presence of infection.



Enzyme production (nutrient utilization): *Bacillus spp.* produce enzymes that can facilitate the access of microorganisms to nutrients and, therefore, improve digestibility of forages and concentrates (Rojo *et al.*, 2005; Pech-Cervantes *et al.*, 2019). A recent study conducted by researchers of the University of Queensland (Gatton, Australia) demonstrated that incubation of **BOVACILLUS™** resulted in a greater *in vitro* dry matter and neutral detergent fiber digestibility in 8 out of 10 forage sources with varying composition (crude protein content of the forages ranged from 2.9 to 25.7%; Pan *et al.*, 2022). More specifically, the improvement on dry matter and neutral detergent fiber digestibility averaged 5.4 and 7.7 percentage points, respectively. Regardless of the quality of the forage, **BOVACILLUS™** was able to benefit fiber digestibility in a similar manner. Similar benefits were also reported when high starch grains (barley, corn, sorghum, and wheat) were incubated with the two strains of Bacilli present in **BOVACILLUS™** (+ 6.5 percentage points of improvement on mean *in vitro* starch digestibility; Pan *et al.*, 2022).

More recently, another *in vitro* study was conducted to evaluate the effects of **BOVACILLUS™** probiotic on gas production and nutrient degradability when incubated with different feed ingredients originated from China and South Africa (Cappellozza *et al.*, 2023b). Incubating **BOVACILLUS™** probiotic with the feedstuffs improved overall gas production and fiber degradability when compared with the feedstuffs not incubated with a probiotic.

Lastly, a step forward was given by the same authors in the sense that commercial dairy TMR diets were also tested under the same in vitro setting. Adding **BOVACILLUS™** probiotic improved fiber degradability by 11.8 and 12.0% at 24 and 48 hours of incubation, respectively, highlighting the enzyme-producing ability of **BOVACILLUS™** probiotic in feedstuffs and diets from different nutritional profiles.

Thermostability capacity: Probiotics must be alive to promote the benefits to the host. Spore-forming bacteria, such as *Bacillus spp.* can withstand the heat, pressure, and osmolarity variation applied in feed preparation and within the animal gastrointestinal tract (**GIT**; pH and bile salts). **BOVACILLUS™** can be included into any type of supplement fed to beef and dairy cattle herds, including milk replacer, pelleted feed, mineral premix (Cappellozza *et al.*, 2023a), licking blocks (chemical and/or molasses-based), pasteurized milk, liquid supplements, mashed feed, and total mixed rations. We have confirmed that the two strains of probiotic bacteria remain active following the preparation of all these supplement types.

The stability of **BOVACILLUS™** probiotic was also put to the test in the sense of long-term stability following the pellet preparation. In other words, we counted the spores of *Bacillus* spp. on feed up to 6 months post-pellet preparation. As expected by its versatility feature, the counts of *Bacillus* spp. spores were at least in the expected range, regardless of the temperature used during pellet preparation. These data highlight, once again, that the *Bacillus* strains included into our probiotic, **BOVACILLUS™**, tolerate high temperatures and can survive for a long period of time following the feed preparation.

Potential additional benefits:
Microbiota modulation: the beneficial effects of probiotics may also lead to alterations in the entire bacterial composition located in the gastrointestinal tract of the ruminants. The entire bacterial community is also called microbiota and a recent article demonstrated the ability of probiotics in consistently and permanently altering such composition. In this trial from South Africa (University of Pretoria), **BOVACILLUS™** supplementation resulted in the maintenance of the normal balance of the good bacteria in the rumen and lower GIT of beef animals (eubiosis; Figure 2) when offered a high-concentrate diet that often promotes the growth of potentially undesirable bacteria (Linde *et al.*; submitted).

In another experiment with dairy cows fed a silage-based diet, the authors demonstrated the ability of *Bacillus* spp. in modulating the rumen microbiota by increasing the abundance of bacteria associated with carbohydrate fermentation (Lamontagne *et al.*, 2023). This profile may lead to the formation of the fuel required by the rumen microorganisms and, therefore, optimize energy production and nutrient utilization in the rumen.

Changes in the lipid profile of the milk: from a human health standpoint, polyunsaturated fatty acids (**PUFAs**) exert many health-promoting effects, including anticarcinogenic, anti-mutagenic, hypocholesterolemia, and antiatherosclerosis effects (Bentsen, 2017), highlighting the interest on how dairy cow nutrition may change the PUFA profile of milk consumed by the human population. Lipidomics is a new scientific discipline that evaluates the pathways and networks of cellular lipids in biological systems, such as the mammary gland of the dairy cow. Preliminary data from a trial conducted by University of Florida (U.S.) demonstrated the ability of *Bacillus* spp. in changing the lipidomic profile of lactating dairy cows, with a more favorable PUFA profile.

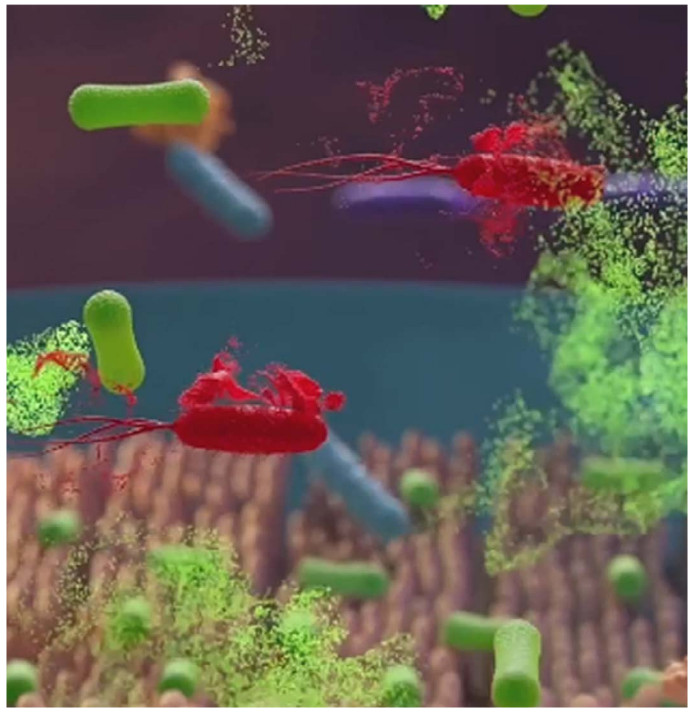
LIVE YEAST

Live yeast (mainly *Saccharomyces cerevisiae*) is the most common probiotic type included in the diet of ruminants, including lactating dairy cows and feedlot animals fed a high-concentrate diet. Some products in the portfolio of Chr. Hansen contain *S. cerevisiae* (PROBIOS® Precise, for example). Among the well-documented effects of live yeast products to cattle, we highlight:

Rumen health: The most pronounced benefit of live yeast is the support of normal health of ruminants fed a high-concentrate diet. In a comprehensive meta-analysis, Desnoyers and colleagues (2009) reported that live yeast increases rumen pH as the content of concentrate in the diet increased. On the other hand, the effects on lactic acid were also considered positive, with lactate levels decreasing as live yeast was fed, but its efficacy decreased as the amount of concentrate in the diet increased. This mediated control on rumen health

by live yeast might lead to the growth of cellulolytic bacteria (Chaucheyras-Durand & Fonty, 2002) and, therefore, improved fiber digestibility (Desnoyers *et al.*, 2009).

Figure 1. Direct inhibition of *Bacillus*-based probiotics against *Clostridium perfringens.*



Rumen bacteria function: Live yeast presents a high oxygen (O_2) scavenging ability. The live yeast included in PROBIOS® Precise was more effective than lactic acid bacteria in removing O_2 from an *in vitro* medium. This may benefit the growth and metabolism of ruminal anaerobic microorganisms, especially those that are more sensitive to O_2, such as cellulolytic and proteolytic bacteria needed for a well-functional and performant rumen.

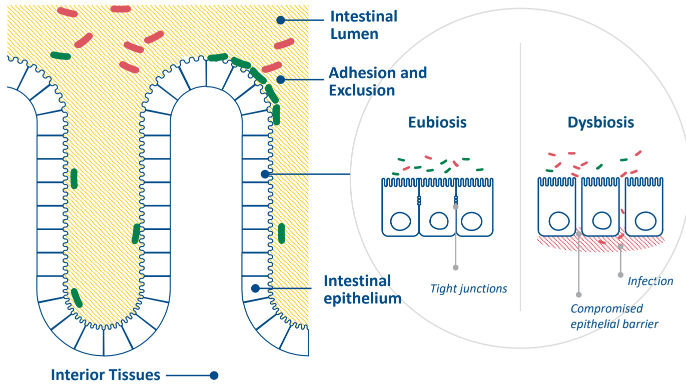
Enzyme production (nutrient utilization): Unlike *Bacillus spp.*, to the best of our knowledge, live yeasts do not produce enzymes that lead to greater nutrient digestibility. However, they help maintain an adequate rumen environment that favors the growth and activity of cellulolytic bacteria. This will, for example, increase organic matter, dry matter, and neutral detergent fiber digestibility (Desnoyers *et al.*, 2009; Chaucheyras-Durand *et al.*, 2015).

Pathogen inhibition: There is still some debate regarding the potential of live yeast supplementation to inhibit potentially harmful enteric microorganisms, but evidence suggests that such beneficial effects might be indirect (Chaucheyras-Durand *et al.*, 2008). In other words, live yeast might be able to promote the colonization of commensal bacteria in the GIT of the animals, which supports normal health of the animals with a reduction in the occurrence of loose stool associated with potentially harmful bacteria. Fibrolytic bacteria and protozoa are examples of commensal bacteria that are positively impacted by the feeding of live yeast (Chaucheyras-Durand *et al.*, 2008).

Competitive exclusion and enzyme production (defense): Some strains of *Saccharomyces spp.* may reduce pathogen load, or their effects, through competitive exclusion on intestinal cell binding, or degradation of the toxins produced by pathogens (Chaucheyras-Durand *et al.*, 2008). Recent trial conducted by Lee and colleagues (2019) demonstrated that supplementation of calves with *S. boulardii* reduced the counts of *Escherichia coli* and *Enterobacteriace*. Others have shown that the effects of *S. boulardii* on clostridiosis are by degrading the toxin produced by *Clostridium difficile* through the action of an defensive enzymes (Castagliuolo *et al.*, 1999).

Thermostability: Yeasts are not naturally thermostable, requiring additional protection technologies to be used in high temperature feed preparation, such as pellets. However, it is important to check and have the data that demonstrate the stability and recovery of these live yeast strains following a high-temperature process, such as blocks, pelleting, and milk replacer preparation.

Figure 2. Example of an integral gastrointestinal lining. Dysbiosis is characterized by a compromised intestinal barrier.



PREBIOTICS

The most common compounds used as prebiotics are β-glucans, mannooligosaccharides (**MOS**), fructo-oligo-saccharides (**FOS**), and galactooligosaccharides (**GOS**), as well as yeast fermentation by-products or culture products (Burdick-Sanchez *et al.*, 2021). Most of the benefits related to prebiotics have been observed when animals are exposed to challenging conditions, such as environmental stress (i.e., heat stress), management activities, or infections with pathogens (Broadway *et al.*, 2015; Burdick-Sanchez *et al.*, 2021).

Growth of commensal bacteria: The main benefit of prebiotics is to serve as a substrate for targeted native microbial species, being used to promote the growth of beneficial bacteria naturally present in the GIT or improving the pH balance and overall microbial health of the GIT. Therefore, a better rumen environment will lead to a better rumen function (Kido *et al.*, 2019) and, consequently, health, as previously described for live yeast.

BACTERIAL- AND YEAST-BASED PROBIOTICS: OTHER TYPES

Pathogen inhibition: Prebiotics, such as yeast cell wall that are rich in MOS and β-glucans, act to prevent the binding of pathogenic bacteria within the GIT (Broadway *et al.*, 2015). More specifically, prebiotics can act as decoys for binding the adhesive structures on the surface of pathogens, thus preventing these from binding to the intestinal mucosa. This allows for beneficial bacteria to promote the gut health of the host animals.

Modulation of the immune response: Several researchers have demonstrated that yeast-based prebiotics (i.e., yeast cell wall) can alter the numbers and responses of host immune cells following a pathogenic or stress challenge (Burdick-Sanchez *et al.*, 2021).

Heat stress: Ruminant trials have evaluated and reported the positive effects of prebiotics on reducing the body temperature of heat-stressed animals (Shwartz *et al.*, 2009; Colombo *et al.*, 2019; Broadway *et al.*, 2020), whereas the mechanism(s) by which it happens, are still unknown and are not broadly reported for all types of prebiotics.

Maintenance of the intestinal barrier integrity: Dead bacterial cells or yeast extracts also confer health benefits to the host by alleviating the occurrence of leaky gut, by strengthening the tight junctions of the gut (Lin *et al.*, 2020). However, additional studies are required to understand whether the same benefits also occur in ruminants challenged with pathogens or undergoing a stressful situation (Broadway *et al.*, 2015; Pisoni & Relling, 2020).

OTHER TYPES

There are plenty of other probiotic types and/or feed additives available to beef and dairy customers, including enzymes and fungi-based probiotics, such as *Aspergillus oryzae*. The fungi *A. oryzae* has been used in ruminant nutrition for a long period (Chiquette *et al.*, 1995) and the primary MoA include the production of fibrolytic enzymes (xylanase and cellulase) that, in turn, will lead to a greater fiber digestibility and rumen health (Williams *et al.*, 1991; Arriola *et al.*, 2017). Recent work has evaluated potential effects of *A. oryzae* on heat stress and, at this point, beneficial effects seem to be indirect rather than a direct effect on temperature control of the animals. In other words, modulation of the immune response might be one of the mechanisms by which *A. oryzae* might alleviate the negative effects of heat stress (Kaufman *et al.*, 2021).

Enzymes are proteins often fed to ruminants solely or in combination with other feed additives. The major goal of feeding enzymes is to increase rumen fiber (cellulases) and starch (amylases) digestibility in ruminants offered forage- and concentrate-based diets, respectively. The effects of feeding an exogenous fibrolytic enzyme have been evaluated by Arriola *et al.* (2017), with these authors reporting the cellulase-xylanase types more effective than other types. The MoA includes the breakdown of structural carbohydrates to facilitate the access and utilization of nutrient by rumen microorganisms, which, in turn, promote rumen health and the growth of beneficial bacteria.

On the other hand, increasing interest has been given on alpha-amylases, but its efficacy in terms of performance is limited by the amount of starch in the diet. Pech-Cervantes *et al.* (2022) reported that feeding an exogenous alpha-amylase increased dry matter (DM) digestibility and total-tract starch digestibility, suggesting that synergistic effects of alpha-amylases and rumen microbes could explain the aforementioned effects. Most, if not all, the commercially available alpha-amylase preparations are produced by solid-state or liquid-state fermentation to reduce the cost of production (Salim *et al.*, 2017) and, hence, the batch-to-batch variation could help to explain the differences in enzymatic activity among studies (Pech-Cervantes *et al.*, 2022).

CONCLUSION

In summary, probiotics and prebiotics have different modes of action to bring benefits to beef and dairy cattle. The differences among the types of probiotics (bacteria vs. yeast) are also something that should be considered when choosing a probiotic to be included in the milk replacer, supplement, or total diet of your herd. Overall, bacteria-based probiotics have more beneficial modes of action than live yeast and prebiotics. And when used together, the three types of additives may bring complementary benefits to beef and dairy producers.

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