

Review

Exogenous Enzymes as Zootechnical Additives in Animal Feed: A Review

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Abstract: Enzymes are widely used in the food industry. Their use as a supplement to the raw material for animal feed is a current research topic. Although there are several studies on the application of enzyme additives in the animal feed industry, it is necessary to search for new enzymes, as well as to utilize bioinformatics tools for the design of specific enzymes that work in certain environmental conditions and substrates. This will allow the improvement of the productive parameters in animals, reducing costs and making the processes more efficient. Technological needs have considered these catalysts as essential in many industrial sectors and research is constantly being carried out to optimize their use in those processes. This review describes the enzymes used in animal nutrition, their mode of action, their production and new sources of production as well as studies on different animal models to evaluate their effect on the productive performance intended for the production of animal feed.

Keywords: animal nutrition; enzyme; animal feed; zootechnical additive; digestibility; antinutritional factors

1. Introduction

Food security is a current challenge in most parts of the world, where the need to increase the intensive production of farm animals for the generation of meat, milk and eggs is a priority; however, to achieve this, it is necessary for the animals to consume nutritious and highly digestible feed, and their diet should not compete with humans' [1]. Traditionally, depending on the geographic location, animal feed has been based on grains, forages and silage, among others, but the need to improve the costs of feeding and animal production has led to the search for new ingredients for this purpose. The use of agro-industrial waste and agroforestry is a current trend; however, its use has disadvantages because the nutritional components are unbalanced or unavailable; therefore, they must be supplemented with grains of cereals, legumes or additives to meet the nutritional needs [2].

The use of exogenous enzymes has been shown to exert positive effects on the agro-industrial and agroforestry wastes used as animal feed by increasing the bioavailability of nutrients and digestibility as well as helping to eliminate some anti-nutritional factors. Although animals have endogenous enzymes involved in digestion, they do not have the ability to degrade them and to take advantage of all their nutritional components; therefore,

their treatment with exogenous enzymes is a trend with beneficial results on production and animal yield [3,4]. An example of this is the use of fibrolytic enzymes that, when added to fibrous substrates, produce small amounts of oligomers, and, therefore, will degrade both soluble and insoluble fiber. This causes breaks in the insoluble fiber, increasing the amorphous nature of the fiber and reducing the time for the attachment of fibrolytic bacteria, thus improving fiber digestibility and the ability of the microbiome to degrade fiber [5]. The addition of enzymes improves the availability of nutrients (starch, proteins, amino acids and minerals, etc.) [6]. Additionally, they offer a beneficial performance response most of the time, but their catalytic efficiency is associated with factors such as the types of food and environmental factors [7]. The enzymes with carbohydrase, protease, phytase and lipase activities are the most used in the improvement of animal feed. For its application, it must be ensured that the biocatalyst is capable of resisting feed processing extrusion and granulation as well as changes in the gastrointestinal tract [8].

Although there are several studies on the application of enzyme additives in the animal feed industry, it is necessary to search for new enzymes, as well as to make use of bioinformatics tools for the design of specific enzymes that work in certain environmental conditions and substrates, allowing for the improvement of the productive parameters in the animals, reducing the costs and making the processes more efficient. This review compiles the progress in obtaining enzymes and their use in the feeding of ruminants and monogastric animals to evaluate their effect on the productive performance.

2. Enzymes as Zootechnical Additives

Enzymes, considered biological catalysts, are proteins capable of accelerating the speed of chemical reactions, which are essential for the proper cellular functioning of all living beings. Due to their diversity, specificity and catalytic capacity, they have been widely accepted by the scientific and industrial community. Their use has shown benefits in various production processes, traditionally, in the food industry in the production of beer, bread, cheese, juice, etc. However, its use in the livestock feed industry was limited until a few years ago [2]; in the 1980s, the poultry industry was the first one to show interest in their use and over the years their use in animal feed grew remarkably, with an estimated commercial value of 1280 million dollars in 2019 [9].

The enzymes used in animal feed are considered zootechnical additives, which improve the consistency and nutritional value of the feed, increase digestibility, animal performance and reduce the effect of antinutrients. They also maintain intestinal health; in addition, the digestion process overcomes the growth of pathogenic microorganisms. They are added separately or as multienzyme preparations at all stages of ruminant and non-ruminant growth [1,4,9,10].

The effects of the action of enzymes used in animal feed processing have not been fully clarified; however, their success can be attributed to any of the following mechanisms [3,4,11].

1. Action on the bonds or components that cannot be hydrolyzed by endogenous enzymes.
2. Degradation of anti-nutritional factors that reduce digestibility and increase the viscosity of feed.
3. Cell wall rupture and the release of nutrients attached to the cell wall.
4. Digestion of nutrients.
5. Reduction in secretions and the loss of endogenous proteins in the intestine, reducing maintenance needs.
6. Increase in digestive enzymes, which are insufficient or non-existent in the animal, resulting in better digestion, especially in young animals with immature digestive systems.

The mode of action of each enzyme is different and interdependent, its use in combination with feed formulations must be carried out rationally and carefully to achieve maximum positive effects. Enzymes act directly or indirectly on nutrients, having main effects on the substrate to which it is directed as well as having side effects. For example, in the lignocellulosic complex, lignin degrading enzymes will attack their substrate as the

main effect and, consequently, they will access the nutrients linked to lignin (carbohydrates or proteins) as a side effect [12].

In addition, the catalytic activity of enzymes is influenced by temperature, pH, substrate specificity, among others; therefore, the enzymes used as additives in animal feed processing must be heat-resistant, stable and capable of preserving their activity through the digestive system of the animal, all of which are important factors in the catalytic response to pH, retention time, resistance to endogenous digestive proteases, microbial enzymes, water content and ionic strength [4]. One way to ensure that the exogenous enzymes are conserved until the place and moment where they will act is through their encapsulation, in order to stabilize them for the processing of the feed or their passage through the gastrointestinal tract, protecting them from adverse conditions and triggering their release at the action site [13,14]. The purpose of adding enzymes in animal feed is to improve food efficiency, production requirements and, consequently, reduce the cost of feeding [15].

Enzymes are generally classified according to the substrate on which they act; commercially, in animal nutrition, they are divided into three categories according to their purpose (Table 1), those directed to carbohydrates (fiber and starch), proteins and phytates [4,9]. Phytases act on phytate and release phosphorus from phytate, while beta-glucanase acts on non-starch polysaccharides and breaks down the fiber. Proteases act on protein and improve its digestibility. Cellulases act on cellulose polysaccharides and break down the fiber and alpha-amylase act on starch and improve its digestibility [10].

Table 1. Enzymes used in animal feed processing.

Enzymes	Substrates	Effect	Example	Ref.
Carbohydrases	Carbohydrates (fiber and/or starch)	Improves digestibility of plant biomass and increases energy. Beneficial effect on poultry and pig diets.	Xylanases and β -glucanases (degrade cell walls, used in poultry) β -mannanases Pectinases α -galactosidases α -amylase (improves digestibility of starch, body weight gain has been observed in poultry)	[16,17]
Proteases	Proteins	Some proteases increased apparent ileal nitrogen, digestibility and apparent nitrogen retention across the whole digestive tract in broiler chicks and broiler cockerels. Exogenous proteases can further improve protein digestibility of ingredients through solubilization and hydrolysis of dietary proteins. Antinutritional factor levels decrease. They can be of animal, vegetable or microbial origin.	Proteases isolated from microorganisms such as <i>Aspergillus niger</i> and <i>Bacillus spp.</i> Chymosin, pepsin A Bromelain, papain, ficine, aminopeptidase, bacillolysin 1, dipeptidyl peptidase III, chymotrypsin, subtilisin, trypsin.	[18–20]
Phytase	Phytates	Degrade phytate bonds releasing trapped nutrients. Improves cattle efficiency It increases the absorption of phosphorus, reducing the possibility of contamination of soil and water through excreta. Increase amino acid availability.	Acid phytases of histidine (pH 5.0) mainly applied to feed for poultry or pigs.	[4]

3. Use of Enzymes in Animal Diets

3.1. Enzymes in Poultry Feed

Poultry is a domestic species used as a source of high-quality meat and eggs. In its production, the feed represents between 70 and 75% of the total cost, and is mainly constituted by grains of cereals (corn, wheat, sorghum and proteins of vegetable flours) that provide energy to the animal; however, due to their high costs, producers have replaced them with cheaper ingredients such as barley, oats, rye, sunflower flour, etc., but these usually contain anti-nutritional factors (ANF) such as antigenic components, raffinose oligosaccharides, saponins, protease inhibitors, tannins, lectins and phytic acid, which are unable to be digested by monogastric animals. The presence of ANF can increase the digesta viscosity, decrease the absorption of nutrients and has even been associated with the incidence of pathogenic infections such as necrotic enteritis affecting the health of poultry and increasing production costs [21,22]. Poultry, when fed with cereals, cannot hydrolyze the starch-free polysaccharides present in the cell wall due to the lack of enzymes, which causes low feed efficiency; these effects can be counteracted by modifying the diet or adding exogenous enzymes. These have been a useful option to improve nutritional and economic aspects in poultry production, in addition to the decrease in the effects caused by ANF [23].

The poultry industry is the most experienced in the application of exogenous enzymes with more than 30 years of research and application [1]. In poultry supplementation, the most commonly used enzymes are xylanases, glucanases, pectinases, cellulases, proteases, amylases, phytases and galactosidases. Their use not only represents a nutritional improvement; it also allows the use of raw materials to be expanded [24]. In the feed industry, in order to neutralize the effects of the viscous, non-starch polysaccharides in cereals such as barley, wheat, rye and triticale have been mostly used. These antinutritive carbohydrates are undesirable, as they reduce digestion and the absorption of all nutrients in the diet, especially fat and protein [23].

The use of enzymes has been extended until the majority of intensively produced poultry diets contain carbohydrases to increase the bioavailability and assimilation of nutrients, as well as the reduction in digestive problems due to the decrease in viscosity [4,24]. In organoleptic matters, egg yolk color, specifically, has been better observed when xylanases and β -glucanases are added to the diet, and a greater energy generation and a better production of meat and eggs with diets supplemented with α -amylases [4], as well as a greater performance of poultry fed with a diet consisting of corn and soybeans added with α -amylase [25]. The phytases increase the utilization of phytate phosphorus. The ability of phytase to improve the digestion of phytate phosphorus and, subsequently, to reduce the output of organic phosphorus to the environment has attracted a great deal of scientific and commercial interest [23].

Café et al. [26] incorporated a multienzyme complex (Avizyme) composed of xylanases, proteases and amylases into the poultry diet, observing an increase in body weights, a decrease in mortality and a greater amount of net energy compared to the group without Avizyme. On the other hand, Babalola et al. [27] observed a better apparent absorption of nitrogen and fiber in poultry fed diets containing xylanases.

Furthermore, the addition of glucanases to whole barley (52.5 U/kg of barley) improves the nutritional value by decreasing the digesta viscosity in diets based on this grain, due to the depolymerization of glucans, in vivo results suggest that 1,4-glucanases act preferentially on cellulosic substrates and not on glucans mixed together [28]. The beneficial effects of the enzymes are reflected in the performance of broilers up to a 75% inclusion of treated barley [29]. Treatment with multiglucanases in wheat and barley (180 U/g) was shown to improve the growth rate and physicochemical properties of the broiler carcass; however, the feed conversion was decreased [30]. However, when combined with xylanases, they help reduce between 30 and 50% of viscosity in diets made from wheat and barley, respectively. In addition, they favor the increase in body weight and nutritional conversion [31]. Generally, xylanases are obtained from microorganisms

such as *Bacillus spp.* and *Trichoderma reesei* [32]. Xylanases, β -glucanases, pentosanases and phytases not only improve the nutritional quality and viscosity of the diets and productive parameters of the poultry, but also improve the intestinal health of the animal, since they restrict the proliferation of fermentative microorganisms in the small intestine [33].

Another exogenous enzyme of importance in the feeding of poultry are the phytases that, until the 1990s, were little used for their high cost. Currently, its application in Europe and countries such as the United States, Mexico and Brazil has been increasingly frequent in the main farms of broilers, not only for the economic benefits but also for the additional effects on the nutritional use of the diet, especially in the absorption of Ca, Zn, Mg and amino acids [34]. The importance of using these enzymes is due to the fact that phosphorus is a mineral associated with important metabolic functions and its excess or deficiency can cause problems in animal productivity. Poultry diets are made up of ingredients in which the phosphorus is mainly in the form of phytate, which, being poorly assimilated, is almost completely discarded through feces, which is why it is considered a source of environmental pollution [34]. To improve the use of phosphorus and reduce the environmental impact, the addition of microbial phytases is an option because it reduces waste and allows smaller amounts of inorganic phosphorus to be used in the diet [35].

Some studies have shown that the effects of phytases are not only limited to the improvement of the digestibility of phosphorus in monogastric animals, but that there has also been a greater retention of P, Ca and N in chicks fed with corn-soybeans and 600 U of phytase/kg compared to those with a diet to which the enzyme was not added [36]. Namkung and Leeson [37] showed that phytase supplementation (1149 IU/kg) created a positive effect of approximately 2% in the digestibility of protein and total amino acids in broilers.

3.2. Enzymes in Swine Feeding

The increase in the consumption of pork has led to a need to find alternatives that reduce the maturation and fattening time of piglets. Unfortunately, the increase in the number of piglets per litter results in animals with low birth weights, immune systems and immature digestive systems, in addition to the limited enzyme secretion reflected in the slow maturation of the animal [1,38]. Although the enzymes supplemented in the feeding of poultry and swine is similar, the results differ due to the digestive physiology of each species [39].

The addition of exogenous enzymes in pig feeding can improve the digestibility of feed, degrading their complex matrix. Carbohydrase supplementation (xylanase, β -glucanase, β -mannanase, α -galactosidase) increases substrate digestibility [38]. Furthermore, phytase is added to improve the digestibility of phytate with the consequent reduction to inorganic phosphorus, as well as improvements in the growth of piglets that are supplemented with this enzyme [38,40]. Although pigs have endogenous phytases present in the intestinal mucosa, they have almost zero activity, and although these enzymes are excreted by microorganisms in the large intestine, the released phosphorus is not absorbed and is almost completely excreted, which is why it is very important to supplement the diet with these type enzymes that can be obtained from fungi of the *Aspergillus* and *Peniophora* genera [41].

Tiwari et al. [42] showed that xylanase and mannanase can be used together or separately in diets rich in arabinoxylans and mannans (depending on the composition of the feed and the amount of the substrates) to improve their digestibility and, subsequently, the animal's intestinal health. Although the supplementation of the diets of weaned piglets with individual or combined mannanases, phytases, proteases and carbohydrases have shown positive effects on digestibility, growth and an improvement of the intestinal structure, the ability of enzymes to improve intestinal maturity and the health of weaned piglets is inconsistent [38,43].

3.3. Enzymes in Ruminant Feeds

The application of enzymes in the feeding of ruminants has developed slowly, the complexity of the digestive system (four compartments) of these animals and the existence of ruminal microorganisms that excrete enzymes and perform fermentation processes make it difficult to interpret the data obtained. Most of the research conducted in ruminants has been based on the use of fibrolytic enzymes, amylases and proteases; mainly multienzyme complexes composed of cellulases, xylanases, amylases and pectinases [1]. They are generally used to improve the digestibility of forage cell walls, increase the availability of the starch present in cereals and improve the performance of dairy cattle [44]. In the search to increase milk production and decrease its cost, the use of enzymes in ruminant feeding has been shown to have a positive effect on milk yield [45].

Enzymes such as xylanases have been used in the previous treatment of forages to improve their digestibility in ruminants [32] and to facilitate composting with glucanases, pectinases, cellulases, proteases, amylases, phytases, galactosidases and lipases, to break down feed components, reducing the viscosity of the raw material [46].

It is common to use agricultural waste in the feeding of ruminants; however, these wastes are not nutritious, have little protein, high amounts of fiber, low digestibility and contain anti-nutritional factors; therefore, the use of exogenous enzymes to improve the quality of these materials is a current trend. The use of cellulases in diets of farm animals has been shown to improve feed utilization and animal performance in vitro, in situ and in vivo through fiber degradation, as well as improved milk production in cows and small ruminants [47]. In dairy cows, the digestibility of dry matter and milk production has increased in diets with 34% silage of barley treated with fibrolytic enzymes (mixture of cellulase and xylanase) from *Trichoderma reesei* [45]. Recently, Golder et al. [48] characterized the response in the field of the application of fibrolytic enzymes in supplemented dairy cows before delivery and for 200 days from the start of lactation in three dairy farms in the United States; eight randomly assigned pens were controlled without enzyme administration and eight more pens received a dose of 750 mL/t of feed for five months. The results showed that milk production increased with the enzymatic treatment to 0.70–0.80 kg/day, this could be due to the higher digestibility of the feed. The body weight of the cows that were supplemented with enzymes did not show increases; however, a higher dry matter intake was observed (0.20 kg/head per day). Furthermore, in goats, it was possible to show an effect on daily weight gain, milk production and feed consumption with the inclusion of enzyme extract obtained from the spent substrate of *Pleurotus ostreatus* in the diet [49]. However, despite the benefits offered by fibrolytic enzymes, the use of these in the diet of livestock, specialized in meat production, does not show significant results [50].

In regard to amylolytic enzymes, they are potential additives to improve starch digestion in ruminant diets. Some amylases from *B. licheniformis* and *Aspergillus niger* may increase the digestibility of cereal starch such as sorghum and corn [44,51]; these enzymes have been able to act on the final non-reducing group of amylose and amylopectin, specifically on the α -1,4 or α -1,6 glycosidic bonds releasing glucose, maltotriose and maltose, which can be used as a substrate by ruminal microorganisms such as *Megasphaera elsdenii*, *Prevotella ruminicola* and *Selenomonas ruminantium*, which implies a greater degradation of dietary starch [52]. An isolated amylolytic enzyme of *B. licheniformis* recorded an activity of 4.19 mM/min, which was 69 times more active than the enzymes found in the rumen; their positive effects were demonstrated in studies 'in vivo' in sheep fed with a diet based on sorghum (70%) treated with these enzymes, achieving a decrease in the consumption of dry matter, organic matter and starch. However, the degree of use of starch is determined by the type or source, chemical and nutritional composition of the diet, the amount of food consumed per unit of time, mechanical alterations (degree of chewing) and physico-chemical properties (degree of hydration and gelatinization) and the adaptation of ruminal microorganisms to the substrate consumed in order to degrade it [44,52].

On the other hand, the use of phytase in ruminants improves the use of phosphorus and reduces the need to supplement the feed with inorganic phosphate, and it also

contributes to the decrease in phosphorus in feces, which represents an environmental benefit [13].

3.4. Enzymes in Fish Feeds

Another sector where exogenous enzymes have been applied is aquaculture. This industry uses fish meal with ingredients of vegetable origin in its processes; the addition of biocatalysts allows for a reduction in anti-nutritional factors, such as phytin, non-starch polysaccharides and inhibitors of proteases that affect nutrient utilization and interfere with fish performance and health [53]. In the larval stage, nutrition is a crucial factor that affects survival and performance, to achieve this it is necessary to have feed that meets nutritional requirements. An alternative can be the manipulation of the diet that allows for the optimization of the performance of the fish larvae. The addition of exogenous enzymes to the formulated diet can increase the assimilation by the larvae. On the other hand, it has been reported that the greater growth of fish larvae fed with live food is attributed to the activity of digestive enzymes present in live food [54,55].

The use of zootechnical additives in this industry is low; increasing research on this topic could be a useful tool to improve and sustain commercial aquaculture [56]. Phytases and carbohydrases are the most used in aquaculture; however, the latter have not been as common in aquatic species. Despite their promising effects to improve nutrient digestibility by hydrolyzing the non-starch polysaccharides present in plants food, their effects are not yet clear due to the difficulty of comparisons between studies [57,58]. The fish species that have been used as a study model to determine the effect of exogenous enzymes on larval growth are Dorada *Sparus auratus* [54], sea bass *Dicentrarchus labrax* [59], rainbow trout *Oncorhynchus mykiss* [60,61], Japanese sea bass *Lateolabrax japonicus* [62] and African catfish *Clarias gariepinus* [63].

Yigit and Olmes [64] mention that the cellulase supplementation obtained from *Aspergillus niger* in the diets for tilapia fingerlings (*Oreochromis niloticus*) did not show effects on growth, concluding that the addition of enzymes to the fish diet will depend on the enzymes, the species used and the source of the feed ingredients. In addition, an enzyme complex (hemicellulase, pectinase, cellulase) that allows the components of the cell wall, such as hemicellulose and pectin bound to cellulose, to degrade must be used.

In this sense, in another study, they evaluated the addition of multiple enzymes (Natuzyme[®] and Hemicell[®]) on the diet of Caspian salmon (*Salmo trutta caspius*), finding improvements in body weight gain and feeding efficiency. In addition to this, the authors mention that it is necessary to consider the effects of the enzymatic supplement on the intestinal microbial flora and the improvement of growth through the release of a growth-enhancing factor that cannot be ignored [65].

Adeoye et al. [66] found greater growth when using phytase, protease and carbohydrases in the diet of Tilapia (*Oreochromis niloticus*), in contrast to the fish fed with the control diet. Furthermore, they do not report changes in the hematological, intestinal morphological or intestinal microbiological parameters. However, carbohydrases showed a significant difference in the gut microbiota of the fish that were fed the tilapia diet compared to those fed the control diet. Although the species diversity parameters of the microbiota were not affected by dietary treatment, the analysis revealed differences in community profiles. Therefore, research is needed to confirm how exogenous enzymes (especially carbohydrase) modulate the gut microbiota and whether these modulations contribute to enhancing the host growth performance.

The aquaculture industry must face several challenges before applying exogenous enzymes in the fish diet, although phytase is the most used and has the best results. Research is needed on the effect of enzymes on amino acid availability, specific enzymatic modes of action, including interactions with endogenous enzymes during digestion, the effects of enzymes on target substrates, the consistency and predictability of the effects of enzyme doses and the effects of the quality of the ingredients on response predictability [67].

3.5. Enzymes in Dog's Feeds

The dog feed industry has also considered the use of exogenous enzymes in the diet, due to the exocrine pancreatic insufficiency (EPI) that these can present. The most widely used are amylase, protease and lipase from the porcine pancreas. Moreover, some vets recommend the use of plant and animal enzyme supplements for all pets, including those without EPI. The possible benefits that are expected range from an increase in the digestibility of nutrients to the support of the immune system; however, they have not been proven thus far [68].

The type of processing that dog feed has inactivates the enzymatic activity; therefore, it is necessary to superficially apply enzymes, finding freeze-dried enzymes on the market, which must be added to the feed. One of the parameters evaluated has been digestibility, where the results do not show significant changes when amylase has been added, which does not increase starch digestion with respect to the control diets [69–71]. The use of proteases has also not shown effects on protein digestibility, which may be due to the type of protease used, the low concentrations of enzymes and their low specificity [72].

The use of amylases during the extrusion process in the production of feed for dogs has been evaluated, showing that they do not interfere with the final texture of the feed, the gelatinization of the starch, the digestibility of the nutrients nor the palatability of the feed, but it does increase production in extrusion and a possible reduction in electrical energy use, but its use must be evaluated in various feed extrusion and formulation systems to determine the optimal balance between enzyme cost and increased feed productivity and energy costs savings during extrusion [71].

In the preparation of feed for dogs, it is common to use barley and wheat, but due to their composition, these have negative effects on digestibility and fecal consistency due to the high content of dietary fiber. To counteract this, the effect of glycanases in the formulations has been evaluated, finding that they digest the soluble fraction of the fiber, which increases the fermentation of these substrates when they reach the large intestine. Moreover, the addition of the enzyme improves the antinutritive effects, but it did not improve the problems associated with increased bacterial fermentation and an accumulation of lactic acid in the large intestine, yet the use of these enzymes is recommended due to the acceptable quality of the feces and good digestion in general [73].

On the other hand, Sá et al. [70] mention that adding wheat bran in the formulation increases the dietary fiber, promotes a reduction in nutrient and energy digestibility and there is an increase in fecal production when using a mixture of enzymes (b-glucanase, xylanase, cellulase, glucoamylase, phytase) before and after extrusion, concluding that enzyme supplementation, during feed processing or as exogenous enzyme supplementation for the animal, does not reduce the negative effects of wheat bran on digestibility. However, other combinations or doses of enzymes need to be tested to promote more extensive use of wheat bran in dog diets.

4. Production of Enzymes for Animal Feeding

Enzymes can be obtained from animals, plants or microorganisms. Initially their use was limited by the difficulties of their production and recovery as well as poor stability; however, current biotechnological advances allow for the production of biologically active enzymes in large volumes [74].

The development of recombinant DNA technology (DNAr) has allowed for the isolation and expression of genes of some microorganisms, such as *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Aspergillus oryzae*, *Aspergillus niger*, *Kluyveromyces lactis*, *Trichoderma reesei*, among others and the production of enzymes for industrial use, including animal feed processing [13,74,75]. Protein engineering is used to optimize enzyme performance characteristics for specific industrial applications such as changing the pH optimum, thermal stability and stability against chemical oxidation and altering the requirement for cofactors such as metal ions [13].

To ensure the safety, along with the chemical and microbiological purity, of the enzymes used in animal feed, these are produced under established standards by FAO/WHO and Codex Alimentarius, using safe documented microbial strains and GRAS (Generally Recognized as Safe) raw materials. Table 2 shows the enzymes commonly used in animal feed and the source from which they were obtained [13].

In response to environmental problems and considering the need to improve the nutritional quality of feed for animals, current research is committed to the use of ruminal fluid from slaughterhouses to obtain enzymes. Ruminal fluid is rich in ammonia and phosphorus, which represents an environmental problem; however, it is also rich in cellulase and xylanase enzymes with a potential use in the industry [89].

Sarteshnizi et al. [90] evaluated the use of ruminal fluid as a potential source of exogenous enzymes to improve the nutritional quality of ground corn, barley grain, soy flour and alfalfa hay to be used in ruminant feed. Cherdthong et al. [91] have proposed the use of dry ruminal fluid in animal feed. On the other hand, Sarteshnizi et al. [89] proposed spray drying of the ruminal fluid for subsequent encapsulation, using various hydrocolloids.

On the other hand, the limited stability and functional capacity of enzymes in difficult conditions, such as industrial processes, has long been recognized as a major problem. A current trend to obtain enzymes of industrial interest is made from organisms' extremities. Enzymes obtained from this type of organism have a potential use in the feed industry, since their ability to work under unfavorable conditions is well known, offering better enzymatic activities [92].

The fungal mannanases are attractive enzymes for the animal feed industry, which operate at a pH that ranges from 2.4 to 6. The conditions of their production from the fungi of the genera *Aspergillus* and *Trichoderma* are currently thoroughly investigated; however, they have successfully cloned and expressed mannanases in *Pichia pastoris*, which were evaluated in animal feed, managing to establish a significant source of β -mannanases [92].

The bacteria *Citrobacter braakii* and *Escherichia coli* as well as the fungi *Aspergillus niger*, *Aspergillus* sp. and *Peniophora* sp. have been reported to be producers of the phytase enzyme, which has been used in pig diets to facilitate the release of the P bound to phytate. [93–95]. Phytate-degrading enzymes, through stepwise dephosphorylation, can release P-phytate, thus improving the absorption of P and reducing its excretion; therefore, the use of these enzymes has nutritional and ecological benefits. In addition, it has been observed that minerals such as Ca, Zn, Fe, Cu and Mg are bound to phytate in mineral-phytate complexes, which reduces the degradation of phytate, thus reducing the digestibility of the minerals [93]. However, it has been reported that supplementation with phytase from *Escherichia coli* can increase the digestibility of Ca, Mg, Mn, Zn, Cu and Fe in pigs with doses of 500 to 1500 FTU of phytase/kg of feed [94]. It is worth mentioning that bacterial or fungal phytases have been shown to be effective when added to the feed of poultry and pigs, increasing the availability of P, the use of energy and reducing the excretion of P in feces [96].

Although many microorganisms have been molecularly characterized and the function and application of their enzymes have been described, work is being conducted to improve their biochemical properties that entail expanding their application. The development of molecular genetics, cell biology, genetic engineering, sequencing techniques and high-throughput omics have allowed modifications at the amino acid sequence level through rational design or molecular evolution [78,97]. With the above, the exploitation of numerous microbial enzymes with a greater pH and temperature stability is sought, as well as an increase in their yield with the use of promoters, which have been introduced as multiple copies in the gene that codes for the enzyme [98–100].

Table 2. Enzyme producing organisms used in animal feed.

Common Name	Classification	Function	Producing Organism	Ref.
α -Amylase	Carbohydrase	Starch Hydrolysis	<i>Bacillus licheniformis</i> , <i>Bacillus stearothermophilus</i> , <i>Bacillus amyloliquefaciens</i>	[76]
			<i>Aspergillus niger</i>	[77]
			<i>Aspergillus oryzae</i>	[76]
Maltogenic α -amylase	Carbohydrase	Starch hydrolysis with maltose production.	<i>Thermomyces lanuginosus</i>	[78]
			<i>Pseudomonas fluorescens</i>	[78]
			<i>Bacillus subtilis</i>	[78]
Cellulase	Carbohydrase	Breaks down cellulose	<i>Aspergillus niger</i>	[79,80]
			<i>Humicola insolens</i>	[81]
			<i>Trichoderma</i>	[80]
α -Galactosidase	Carbohydrase	Hydrolyzes oligosaccharides	<i>Pleurotus ostreatus</i>	[49]
			<i>Mortierella vinacea</i> var <i>raffinoseutilizer</i>	
			<i>Saccharomyces cerevisiae</i>	[82]
β -Glucanase Glucoamylase (amyloglucosidase)	Carbohydrase	Hydrolyzes β -glucans Hydrolyzes starch with glucose production.	<i>Saccharomyces carlsbergensis</i>	
			<i>Trichoderma reesei</i>	[83]
			<i>Aspergillus niger</i>	[79]
Hemicellulase	Carbohydrase	Breaks down the hemicellulose	<i>Humicola insolens</i>	[81]
			<i>Aspergillus niger</i>	[79]
			<i>Aspergillus niger</i>	[79]
Pectinase	Carbohydrase	Breaks down the pectin	<i>Bacillus licheniformis</i>	[78]
Pullulanase	Carbohydrase	Hydrolyzes starch	<i>Pleurotus ostreatus</i>	[49]
Xylanase	Carbohydrase	Hydrolyse xylan	<i>Bacillus circulans</i>	
			<i>Bacillus stearothermophilus</i>	
			<i>Bacillus polymyxa</i>	
Laccases	Oxidase	Oxidation of an organic or inorganic substrate and the reduction of molecular oxygen to water.	<i>Bacillus subtilis</i>	[84]
			<i>Bacillus amyloliquefaciens</i>	
			<i>Bacillus acidocaldarius</i>	
Lipase	Lipase	Hydrolyzes triglycerides, diglycerides and monoglycerides.	<i>Bacillus thermoalkalophilus</i>	
			<i>Fusarium venenatum</i>	[78]
			<i>Aspergillus oryzae</i>	[78]
Papain	Protease	Hydrolyzes proteins	<i>Pleurotus ostreatus</i>	[49]
			<i>Aspergillus niger</i>	[79]
			<i>Rhizopus oryzae</i>	[85]
			<i>Candida rugosa</i>	[86]
			<i>Carica papaya</i>	[87]

Table 2. Cont.

Common Name	Classification	Function	Producing Organism	Ref.
Pepsin	Protease	Hydrolyzes proteins	Animal stomach	[13]
Trypsin	Protease	Hydrolyzes proteins	Animal pancreas	[13]
Chymosin	Protease	Hydrolyzes proteins	<i>Aspergillus niger</i>	[78]
			<i>Escherichia coli</i> K-12	[78]
			<i>Kluyveromyces marxianus</i> var. <i>Lactis</i>	[78]
Catalase	Oxidoreductase	Hydrogen peroxide is needed for oxidation of compounds.	<i>Aspergillus niger</i>	[79]
Glucose oxidase	Oxidoreductase	It degrades glucose to hydrogen peroxide and gluconic acid.	<i>Aspergillus niger</i>	[79]
			<i>Aspergillus oryzae</i>	[78]
Phytase	Phosphatase	Hydrolyse phytate.	<i>Penicillium funiculosum</i>	[88]

The catalytic activity of enzymes at different temperatures and pH values can be improved using directed evolution [101], which is a tool that, together with mutagenesis and screening, helps to characterize new proteins from a parent protein under a particular evolutionary pressure, obtaining more efficient biocatalysts [102–104]. On the other hand, the use of protein engineering has contributed to generating biocatalysts with greater activity and stability at high temperatures and extreme pH values through directed mutagenesis. This has been achieved due to the advancement of bioinformatics, which allows the protein structure to be visualized in a three-dimensional way and, thus, achieve a rational design that allows the introduction of disulfide bridges, replacing the N terminal and increasing the number of hydrogen bonds [105]. Table 3 shows some enzymes designed from these techniques, taken from Victorino da Silva Amatto et al. [106].

Table 3. Engineered enzyme.

Enzymes	Source	Purpose of Modification	Method	Ref.
Alfa-amylase	<i>Bacillus</i> sp. TS-23	Improve thermostability, change glutamic acid 219, crucial for the thermostability	Site-directed mutagenesis	[107]
Xylanase (glycoside hydrolase-GH11)	<i>Neocallimastix patriciarum</i>	Improve thermostability. Single mutants Gln87Arg, Asn88Gly, Ser89His and Ser90Thr	Site-directed mutagenesis	[108]
Phytase	<i>Aspergillus niger</i>	Improve thermostability and catalytic efficiency. Change in Thr195Leu/Gln368Glu/Phe376Tyr; Gln172Arg/Lys432Arg/Gln368Glu; Gln172Arg/Lys432Arg/Gln368Glu/Phe376Tyr and Gln172Arg/Lys432Arg/Gln368Glu/Phr376Tyr/Thr195Leu; phyA: Gln172Arg; Gln172Arg/Lys432Arg; Gln368Glu/Lys432Arg	Error-prone PCR/Directed evolution	[109]
Endo-1,4 betaxylanase II	<i>Trichoderma reesei</i>	Improve thermostability, substituting Thr2 and Thr28 by cysteine	Error-prone PCR	[110]

The design of enzymes has also led to the choice of the expression system for the production of recombinant proteins, using mainly bacteria (*Escherichia coli*, *Bacillus* spp., *Lactobacillus lactis*), filamentous fungi (*Aspergillus* spp.) and yeasts (*Pichia pastoris*). Each of these microorganisms have characteristics that allow them to be used as hosts. In particular, *Escherichia coli* quickly and easily overexpress recombinant enzymes; however, they cannot express very large proteins that require post-translational modifications, in addition to producing toxins that reduce their use [111]. Despite this, bacterial expression systems remain attractive due to their rapid growth, use of inexpensive culture media, genetic characterization, number of cloning vectors and mutant host strains [112]. Recombinant enzyme production is still a promising field that will help meet the demand in the animal feed industry; but, to achieve this, it is necessary to use high-throughput expression technologies and to know the proteins at the proteome level to understand them at the systems level. Current advances in post-genomic technology make it possible to design improved cost-effective expression systems to meet the growing demand for enzymes [111].

Therefore, current processes in animal nutrition are using genetically modified microorganisms with the aim of increasing the productive capacity of the fermentation unit for the production of enzymes and avoiding undesirable activities [96]. In ruminant feeding, one approach to improve fiber digestion is by modifying the fiber inoculants, using genetically modified bacteria to produce enzymes that give new properties to the silage and/or pre-digest the plant material. Recombinant *Lactobacillus plantarum* is a strain used as a silage starter, which was constructed to express the alpha-amylase, cellulase or xylanase genes [113]. The possibility of genetically modifying rumen microorganisms to increase the degree of degradation of fiber components has also been proposed, and a possible strategy is through the establishment of recombinant organisms. One of the options has

been the manipulation of the predominant bacterial species in the rumen such as *Prevotella ruminicola*, *Butyrivibrio fibrisolvens* or *Streptococcus bovis*. In *Prevotella ruminicola*, one of the objectives has been to increase its fibrolytic activities, while in *Ruminococcus flavefaciens*, it has been chosen to induce the expression of endoglucanase/xylanase genes, but it should be noted that these studies are still in progress [113].

5. Future Developments of Enzymes for Animal Feeding

The industry dedicated to the production of food for animals should focus its efforts on investing in research and development and in the design of new enzymes that can play a key role in the improvement and nutritional quality of feed. The development of thermostable enzymes will simplify the application of the pre-granulation of dry product and will promote the use of the enzyme in granulated diets [114]. To achieve this, it is necessary to continue work on the rational redesign of existing biocatalysts and standardize combinatorial methods that seek the desired functionality in randomly generated libraries as well as employing robust computational methods combined with screening technologies and directed evolution to improve the properties of enzymes, the application of multistep reactions using multifunctional catalysts, the de novo design and the selection of specific catalytic proteins for a desired chemical reaction and, thus, meet process perspectives [115].

Suplatov et al. [116] mentioned that future computational advances will be the key to achieving success in the design of new enzymes. Currently, bioinformatics is used to predict structural changes that can be applied to wild proteins and produce more stable variants. The techniques used can be classified into stochastic approaches, empirical or systematic rational design strategies and chimeric protein design. Bioinformatic analysis can be used efficiently to study large protein superfamilies in a systematic way, as well as to predict particular structural changes that increase the stability of the enzyme. However, further development of systematic bioinformatics procedures is needed to organize and analyze protein sequences and structures within large superfamilies and link them to their function, as well as to provide knowledge-based predictions for experimental evaluation. Therefore, bioinformatics can become the cornerstone for the design of more stable and functionally diverse enzymes [116].

The next generation sequencing technologies and bioinformatics has facilitated the collection and analysis of a large amount of genomic, transcriptomic, proteomic and metabolomic data from different organisms that have allowed predictions to be made on the regulation of expression, transcription, translation, structure and the mechanisms of action of proteins as well as homology, mutations and evolutionary processes that generate structural and functional changes over time. Although the amount of information in the databases is greater every day, all the bioinformatics tools continue to be constantly modified to improve performance that leads to more accurate predictions regarding protein functionality [117].

The main databases used in computational biology are NCBI, GenBank, Protein Data Bank, Swiss-Prot, PIR, Flybase, TrEMBL, Enzyme, Prosite, InterPro, UniProt and PDB [117,118]. The high number of sequences that are stored in the different databases, have allowed the evolutionary relationships of different proteins to be inferred, which retain their function during long evolutionary times when presenting homology; however, homologous proteins can perform the same activity, but the substrates they use can come from different routes [117,119].

The study of ancestral enzymes has suggested that these presented a high thermostability, due to the Precambrian era that was thermophilic, in addition to the fact that most microorganisms and other organisms adapted to these environments with high temperatures. The ancestral protein alignments with the current ones show evidence of a slow evolution in structure, but not in amino acids [117,120]. Álvarez-Cervantes et al. [121] performed the phylogenetic analysis of β -xylanase SRXL1 from *Sporisorium reilianum* and its relationship with families (GH10 and GH11) from Ascomycetes and Basidiomycetes, demonstrating that groupings analysis of a higher-level in the Pfam database allowed the

proteins under study to be classified into families GH10 and GH11, based on the regions of highly conserved amino acids, 233–318 and 180–193, respectively, where glutamate residues are responsible for the catalysis. The phylogenetic relationship of xylanase SRXL1 of *S. reilianum* with the xylanases analyzed shows a monophyly and a relationship is observed with respect to their status as plant pathogens or saprophytic fungi, in this case the functionality of these enzymes is related to its adaptation to their ecological niche.

To analyze these changes in the sequences, bioinformatics programs use algorithms and mathematical models, based on empirical matrices of amino acid substitution, as well as those that incorporate structural properties of the native state, such as secondary structure and accessibility [117,122]. Protein phylogeny studies are currently necessary to know protein-protein interactions in biological systems. Molecular or structural analyzes on proteins will require more information to respond if a protein is present in one or several species, as well as to predict the common ancestor and evolution times [123].

The bioinformatics tools are TOPAL, Hennig86 and PAML; the computational packages that are allowed to occupy any of these are PHYLIP and PAUP, as well as MOLPHY, PASSML, PUZZLE and TAAR [117,124].

On the other hand, one of the challenges of protein engineering and biology is to improve industrial processes; to achieve this it is necessary to determine the tertiary structure of proteins from the amino acid sequence in order to design new proteins. Many of the protein structures that we know today have been obtained using X-ray crystallography, Nuclear magnetic resonance spectroscopy (NMR) or cryo-EM [117,125].

The use of algorithms and computer programs have achieved the design of new catalysts with the ability to use substrates, this considering their structural characteristics, structural dynamics and structural remodeling [126]. Computational design has obviously improved the possibility of finding active enzymes. However, it must be combined with optimized experimental protocols to obtain efficient biocatalysts [127].

The powerful and revolutionary techniques developed for protein engineering thus far provide excellent opportunities for the design of industrial enzymes with specific properties and the production of high-value products at lower production costs [127]. Table 4 shows the biochemical properties of some enzymes that the animal feed industry reported in the UniProt and PDB databases.

Table 4. Biochemical properties of some enzymes reported in the UniProt and PDB databases.

Protein	Gen	Organism	Catalytic Activity	pH	Temperature °C	Active Site	Glycosylation	Access Code UniProtKB	Access Code PDBe	Ref.
Endo-beta-1,4-glucanase B	eglb	<i>Aspergillus niger</i>	Endohydrolysis of (1,4)-beta-D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans. EC:3.2.1.4	6.0	70	160, 266	38, 100, 211, 288	O74706 (EGLB_ASPNG)	5i77, 5i78, 5i79	[128,129]
Xyloglucan-specific endo-beta-1,4-glucanase A	xgeA	<i>Aspergillus aculeatus</i>	Xyloglucan + H ₂ O = xyloglucan oligosaccharides. EC:3.2.1.151	3.4	30	-	-	O94218 (XGEA_ASPAC)	3VL8, 3VL9, 3VLB	[130]
Pancreatic alpha-amylase	AMY2	<i>Sus scrofa</i> (Pig)	Endohydrolysis of (1,4)-alpha-D-glucosidic linkages in polysaccharides containing three or more (1,4)-alpha-linked D-glucose units. EC:3.2.1.1	-	-	212, 248, 315	427	P00690 (AMYP_PIG)	1BVN	[131]
Alpha-amylase	amyS	<i>Bacillus licheniformis</i>	Hydrolysis of (1,4)-alpha-D-glucosidic linkages in polysaccharides to remove successive maltose units from the non-reducing ends of the chains. EC:3.2.1.2	11	100	260, 290	-	P06278 (AMY_BACLI)	1ob0	[132,133]
Beta-amylase	spoII	<i>Bacillus cereus</i>		-	-	202, 397	-	P36924 (AMYB_BACCE)	1J0Z	[134]
Endo-1,4-beta-xylanase	xylC	<i>Talaromyces cellulosus</i> CF-2612	Endohydrolysis of (1,4)-beta-D-xylosidic linkages in xylans	-	-	119, 210	-	W8VR85 (W8VR85_9EURO)	5HXV	[135]
Endo-1,4-beta-xylanase 2	Xyn2	<i>Trichoderma reesei</i>		4.5–5.5	40	119, 210	71, 94	P36217 (XYN2_HYPJR)	4HKW	[136]
Endopolygalacturonase I	pgaI	<i>Aspergillus niger</i>	(1,4-alpha-D-galacturonosyl)(n + m) + H ₂ O = (1,4-alpha-D-galacturonosyl)(n) + (1,4-alpha-D-galacturonosyl)(m). EC:3.2.1.15	-	-	207, 229	44, 46, 246	P26213 (PGLR1_ASPNG)	5ONK	[137]
3-phytase A	phyA	<i>Aspergillus niger</i>	Catalyzes the hydrolysis of inorganic orthophosphate from phytate.	-	-	82, 362	27, 59, 105, 120, 207, 230, 339, 352, 376, 388	P34752 (PHYA_ASPNG)	3K4P	[138]
3-phytase	phyC	<i>Bacillus subtilis</i>		7	55	-	-	O31097 (PHYC_BACIU)	3AMS	[139]
3-phytase	phy	<i>Bacillus</i> sp.		-	-	-	-	O66037 (PHYT_BACSD)	2POO	[140]

-: data not reported, EC: Enzyme commission.

6. Conclusions

The use of enzymes in animal feed is a dynamic research and development field; current research and its future application aim to assess its effect on the health and intestinal flora of animals, and to check whether or not there is a synergistic effect between the composition of the diet and the enzymatic action to improve the digestibility, to contribute to intestinal development and their effect on the productive performance, or if its application can diminish the use of antibiotics, by means of a decrease in the incidence of diseases or mortality. Furthermore, the search for new sources of obtaining enzymes for their use in animal feed is a current trend with a view to evolve in future work. The modification of commercial enzyme-producing strains that resist the conditions of the gastrointestinal tract or the search for new ways to encapsulate and protect enzymes are important aspects to be investigated and applied in the agro-industry for the production of animal feed.

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