Review paper

Identification and degradation of mimosine, a toxic compound in *Leucaena leucocephala* (Lam.) de Wit

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Abstract

Leucaena leucocephala is known for being a shrub that generates nutritional benefits for animal husbandry in the world. Nevertheless, its consumption has been limited due to antinutritional factors such as mimosine, a toxic that exists in the plant. This review compiles studies by different authors who identified and characterized bacteria found in the rumen and in the rhizosphere of the plants, capable of degrading mimosine and its degradation products, 3,4- and 2,3-dihydroxypyridone (3,4 and 2,3 DHP). Mimosine (non-protein aminoacid which is found in *L. leucocephala* leaves and seeds) degradation by bacteria is a fundamental factor in the protection of animals when consuming leucaena. Mimosine and its degradation products have been used in the rhizosphere by the strain TAL1145 of *Rhizobium*, as carbon and nitrogen source; while at rumen level bacteria such as *Synergistes jonesii* can degrade it. Recent studies have proven that other rumen bacteria, like *Streptococcus lutetiensis* have the capacity to degrade this compound. Such advances will take the species *L. leucocephala* to another knowledge level, in order to search for higher efficiency and safety in its use for animal feeding.

Keywords: animal feeding, *Streptococcus lutetiensis, Synergistes jonesii.*

Introduction

In the tropics ruminant nutrition is based mainly on the consumption of pastures, harvest byproducts and, to a lower extent, preserved feedstuffs, such as silages and meals. Nevertheless, the grasses used in feeding have high fiber contents and low protein percentages, bringing about a considerable decrease in the general development of the animal, particularly in the dry season (Villanueva *et al*., 2013). A large number of forage trees, mainly legumes, are used as supplement in the diets of low nutritional quality; they show high protein content and good digestibility, compared with grasses (Pereyra *et al*., 2015).

Besides their favorable chemical composition, forage trees and shrubs have other positive properties, because they help to recover degraded soils (Rangel *et al*., 2016), increase their nutrient content due to the symbiosis they establish with bacteria of the *Rhizobium* genus (Higashide, 2014), protect against erosion, and can be also used as fuel and construction materials.

All the legume species have secondary metabolites, some of which are called antinutritional factors (ANF), that can cause a negative effect on the nutritional value of the feedstuff, as well as on animal health. ANFs can be defined as those substances generated by the natural metabolism of plant species, and which, by different mechanisms, exert adverse results on the optimum nutrition of the animals, because they decrease the digestive and/or metabolic effects (Rodríguez and Ledesma, 2014).

Legumes generate these substances through their secondary metabolism, as a defense mechanism against the attack of molds, bacteria, insects and birds; or, in some cases, as product of metabolism when they are subject to stress conditions. These plants, when contained in the diet ingredients, reduce intake and prevent nutrient digestion, absorption and utilization by the animal (Casso and Montero, 1995).

Among the most studied ANFs, present in most legume species, the following can be cited: lecithins, tannins, cyanogen glycosides, vicine and convicine, galactosyl sucrose oligosaccharide, galactomannan gums, saponins, non-protein aminoacids, neurolatherigens, arginine analogues, alkaloids, phytic acid, antigenic proteins and aromatic aminoacids; and within the last ones mimosine stands out [3-N-(3-hydroxy-4-pyridone) aminopropionic-ao-acid], present in *Leucaena leucocephala* (Lam.) de Wit. (Casso and Montero, 1995).

The objective of this review was to compile studies conducted by different authors who identified and characterized bacteria capable of degrading mimosine and its degradation products, which were found in the rumen and in the rhizosphere of the plants.

Characteristics of mimosine

Mimosine is a non-protein aminoacid, present in the tropical forage legume *L. leucocephala*, which has been responsible for toxicity symptoms in some species of domestic animals; among these symptoms the following stand out: alopecia, anorexia, weight loss, deep salivation, lesions through the esophagus, necrotic papillae in the rumen and reticulum, hyperplasia of the thyroid gland and low levels of circulating thyroxine hormone (Xuan *et al*., 2013).

Mimosine has an aromatic ring of 3-hydroxy-4-(1H)-pyridone (3,4 DHP) (Nguyen and Tawata, 2016). This compound plays an important role in the plant resistance to a large variety of phytopathogen agents; besides, as it is structurally analogue to thyroxine, it allows an inhibitor or antagonist behavior in many processes in which it is intermediary. In animals intoxicated by mimosine consumption an effect is shown on production, which includes low reproductive values due to precocious embryo mortality and perinatal death (Hammond, 1995).

The aromatic ring of 3,4 DHP is free in the rumen and in the circulation of intoxicated animals, which indicates that mimosine is easily hydrolyzed in the rumen and excreted at renal level (Barros-Rodríguez *et al*., 2012). Small quantities of mimosine have also been found in the nitrifying nodules of *L. leucocephala* and in the exudate adjacent to its roots, which is degraded through *Rhizobium* strains; on the other hand, when non-adapted animals are fed with leucaena over 30 % of dry matter in their diet, this can induce toxicity cases and cause death (Soedarjo and Borthakur, 1998).

Recently published studies show the efforts to decrease the mimosine contents of the *L. leucocephala* leaves using ethyl methanesulfonate (EMS), and thus improve the nutritional value of the plant. With this advance foresters intend to reduce significantly the price of animal feeding in the future (Zayed *et al*., 2014).

Molecular identification of mimosine

Although *L. leucocephala* is considered a promising alternative source of protein, and that it can also help to mitigate the emission of rumen methane (CH4) in the tropics (Soltan *et al*., 2013), the presence of mimosine in 2-10 % DM in the leaf and 2-5 % DM in the seed limits the quantity of foliage that can be supplied to cattle, because mimosine (depilatory agent) and its degradation product (3-hydroxy-4 (DHP)[3] (strong goitrogenic agent) are considered toxic for many species. The partial resistance to mimosine-caused toxicity in ruminants from certain geographical areas has been ascribed to the capacity of their rumen microorganisms, which, restrictively, metabolize mimosine and DHP. An example is the report in Hawaii, in which resistant goats, with efficient microorganisms that counteracted the effects of mimosine-caused toxicity, transferred the microorganisms to the rumen of Australian cattle, which was susceptible to such toxicity (Lalitha *et al*., 1993).

For the identification of mimosine and DHP, the most commonly used reaction method is the colorimetric one with $FeCl_3$ (Ilham *et al*., 2015). It has little sensitivity and specificity, because its poor solubility in aqueous and organic solvents limits its application, as it requires a high concentration to have accurate measurements. Liquid gas chromatography, liquid chromatography and ion-pair reversed-phase high performance liquid chromatography require sophisticated equipment and do not offer an improvement in sensitivity (Lalitha *et al*., 1993).

The spectrophotometric method for mimosine and/or DHP estimation is more sensitive than other reported ones, and can be most adequately coupled to an ion-exchange chromatography and paper chromatography for the specific essay of these compounds. It is essentially based on the formation of an intense azoic yellow coloring between mimosine and/or DHP and p-nitroaniline diazonium salt (Ilham *et al*., 2015).

Applying spectrophotometry it was estimated that the quantity of mimosine in the *L. leucocephala* leaves varies between 3,75 and 5,5 % DM, depending on the type of leaves, season, soil quality, etc. Due to the high sensitivity of the procedure, mimosine was detectable in the discolored extracts with activated carbon even after high dilutions, and the concentrations of other interfering compounds became negligible for these dilutions. A useful application of this technique was extended to the evaluation of mimosine toxicity in the experimental feeding material based on *L. leucocephala* leaves. The early observations indicated that, during the anaerobic degradation of these leaves using mixed specific inoculant, mimosine was actively metabolized, as shown by the method with $FeCl_3$ after the separation from paper chromatography. When applying the above-described method it was confirmed that only traces of mimosine and DHP remained, which indicated almost 99 % in 48 h, and the solid biomass made an adequate choice

of feeding material with high protein content. In the comparison of the method reported with other techniques of mimosine estimation it was proven that the colorimetric method with $FeCl_3$ requires a solution of 20-500 µl for an accurate measurement (Lalitha *et al*., 1993).

Mimosine degradation from *Rhizobium* sp. strains

Mimosine is a toxin found in large quantities in the seeds and leaves of legume trees and shrubs of the *Leucaena* genus. In its structure it is analogous to dihydroxyphenylalanine (L-Dopa) with a 3-hydroxy-4-pyridone ring instead of a 3,4 dihydroxyphenil ring (Nguyen and Tawata, 2016). This toxin is distributed throughout the plant, and is found from 4 to 5 % in the seeds (dry basis); in different parts of the plant, such as stems and leaves, it can vary from 1 to 12 %, and in the root from 1 to 1,5 % (Soedarjo *et al*., 1994). It has been proven that some *Rhizobium* strains can degrade the substance mimosine.

According to the work conducted in Hawaii by Soedarjo *et al*. (1994), a total of 32 strains have been collected and cultivated in optimum media, such as TY (Beringer, cited by Martínez *et al*., 2015); complete medium for rhizobia growth, YEM (Abrahamovich *et al*., 2014); yeast-mannitol extract (also used for rhizobia culture) and a *Rhizobium*-mimosine medium (RM), in order to know which of the collected strains had the capacity to grow in a medium with the toxin. It was determined that the strains TAL1145 and TAL1566 had capacity to use mimosine as only source of carbon and nitrogen. The growth rates of those strains in RM medium with different mimosine concentrations were determined through the inoculation of 50 mL of this medium in 250-mL bottles with screw top, which contained 0,5 mL of *Rhizobium* culture. The cultures were subject to 28 ºC, with agitation, and the growth was determined every 6 h by measuring the cell density as the optical density in a colorimeter.

It was determined that only a limited number of *Rhizobium* strains could use mimosine as selective growth substance; in contrast, the strains which did not have this capacity formed nitrogen-fixing nodules, as in the case of MS13. The utilization of mimosine can be a specialized mechanism which has been developed by some rhizobia that live in the rhizosphere of leucaena to survive, which provides a competitive advantage to certain *Rhizobium* strains. A direct relation could not be established between the capacity to catabolize mimosine and the capacity to fix nitrogen, because two strains, TAL1145 and TAL1566, degrade mimosine and use it as nitrogen and carbon source, but only TAL1145 is good forming nitrifying nodules, unlike TAL1566 which has deficiency to form them (Soedarjo *et al*., 1994).

In a study conducted later than this one, in the island of Guam, the strain with the best results regarding nodulation and utilization of mimosine for itself, in terms of nitrogen and carbon (TAL1145), was used. For such purpose native *Rhizobium* were isolated from the *L. leucocephala* nodules, in order to verify whether they had the capacity to degrade mimosine and examine them for the midA gene. As it was mentioned above leucaena nodulates with several types of *Rhizobium*, such as *Rhizobium* strain TAL1145, *Rhizobium* strain NGR234, and with strains of *Rhizobium tropici*, such as CIAT899. Among these rhizobia, only the strain TAL1145 and some related strains can degrade mimosine and use it as carbon and nitrogen source, while strains like CIAT899 and NGR234 cannot degrade it (Soedarjo *et al*., 1994).

After isolating the rhizobia, culturing the found strains and subjecting them in a RM liquid medium, and isolating and amplifying the genomic DNA of the *Rhizobium* strains that utilized the mimosine, it was concluded that eight of the 11 isolated strains used it, because the RM medium changed from brownish yellow to colorless. This was corroborated by Soedarjo *et al*. (1994), who proved that mimosine was not detected in the medium by high performance liquid chromatography (HPLC), when the medium became colorless, with the presence of rhizobia that degrade it. As shown in table 1, from the eight mimosine-degrading strains only three generated the PCR fragment 1055-pb, which suggests that these strains contained the gen midA of the strain TAL1145 (Marutani *et al*., 1999).

According to the above described facts, the importance of knowing further the strains belonging to the *Rhizobium* genus, which have the capacity to utilize mimosine and its degradation product 3,4 and 2,3 hydroxypyridone, was proven.

Soedarjo *et al*. (1994) isolated in different parts of the world some *Rhizobium* strains from the leucaena nodules which fulfilled this condition and which, additionally, could use it as carbon and nitrogen source. Although the capacity to catabolize mimosine for nodulation and nitrogen fixation is not required, this provides a competitive advantage to mimosine-degrading *Rhizobium* (mid+) in the

Table 1. Ability to utilize mimosine and detection of the midA gene of TAL1145 in different strains.

Sampling site/ soil type	N° of strain	Ability to use mimosine as C and N source	Detection of the fragment 1055 pb
Barrigada/	B1	+	-
Pulantat	B2	+	-
	B4	+	-
	B5	+	-
	B7	ND (contaminated)	+ (weak)
	B9	+	+
	B10	+	+ (weak)
	B12	+	+
	B19	-	-
	B24	-	+/- (very weak)
	B26	+	-
Yigo/	Y1	-	ND
Guam cobbly clay	Y2	-	ND
	Y3	-	ND
	Y4	+	ND
	Y5	-	+
	Y7	-	ND
	Y9	-	ND
Control/	*Rhizobium* TAL1145	+	+
	Bradyrhizobium sp.	-	ND

Source: Marutani *et al*. (1999).
+: strain with capacity to utilize mimosine as nutrient, and indicates the positive detection of the fragment 1055 pb.
-: strain without capacity to catabolize mimosine, and does not indicate the detection of the fragment 1055 pb.
ND: strain that was not included in the bioessay or in the PCR analysis.

rhizosphere of leucaena, by supplying a selective source of nutrients and, at the same time, inhibiting the growth of other microorganisms and rhizobia (Soedarjo and Borthakur, 1998). The *Rhizobium* strain TAL1145 is one of the mid+ strains, known for being competitive for the occupation of the nodule in leucaena (Siddiqi and Athar, 2013), and has been described as a very efficacious nitrogen fixing nodules in *Leucaena* spp. The coincidence in its efficient N_2 fixation capacity and its competitive capacity make it an ideal choice to be used in inoculant preparations.

To identify and characterize the mid genes present in the fragment from 12 to 6 kb of TAL1145, which are required for mimosine degradation, the cosmid pUHR181 was isolated from a clone library of TAL1145 (Borthakur *et al*., 2003). When this cosmid was transferred to non-degrading (mid-) strains, such as TAL182 and CIAT899, the degradation product 3-hydroxy-4-pyridone (HP) was accumulated in the culture medium. This suggested that pUHR181 contained genes for the degradation of mimosine to HP. The plasmid pUHR191 is a derivative from pUHR181 which contains an insert of 12 to 6 kb, constructed by the elimination of a fragment of approximately 10 kb of pUHR181. The transconjugants of TAL182 and CIAT899 contain pUHR191 which turn mimosine into HP (Borthakur *et al*., 2003).

Initially 12 defective TAL1145 in mimosine degradation (mid-) were established; they were made through Tn3Hogus (transposon), TnphoA (bacteriophage) or insertion of kanamycin resistance cassette. A PstI fragment (a restriction enzyme) of 5-0 kb of TAL1145, subcloned from a cosmid clone that contains mid genes for mimosine degradation, complements most mid- mutants. The sequencing of this fragment and the PstI fragment of 0-9 kb which is adjacent identified five genes: midA, midB, midC, midD and midR, from which the first three codify ABC transporter proteins implied in the mimosine absorption; while midD

codifies an aminotransferase required to degrade mimosine into HP, and midR is a regulating gene which codifies a LysR-type transcriptional activator. Thus, mid genes are specific for the *Rhizobium* of leucaena and are absent in the *Rhizobium* strains and in *Bradyrhizobium* spp. (Borthakur *et al*., 2003).

Just as in the strain TAL1145 the presence of genes responsible for mimosine degradation and utilization could be determined, nodulation-efficient strains, which have potential to degrade other antinutritional factors, should be further studied (Xu *et al*., 2013).

Degradation of mimosine from rumen bacteria

The tree legume *L. leucocephala* is a high-quality feedstuff used in ruminants, which is extremely important for livestock production in the tropics, in spite of the presence of mimosine in its leaves. This non-protein toxic aminoacid limits productivity and adversely affects animal health (Halliday *et al*., 2013). Given the fact that *L. leucocephala* has a high potential in production, studies have been conducted in order to know more specifically which rumen microorganisms could be involved in the degradation of the products derived from mimosine and thus reduce toxicity in ruminants.

In Venezuela, Domínguez-Bello and Stewart (1991) isolated a bacteria belonging to the *Clostridium* genus in sheep fed leucaena that did not develop toxic symptoms, which had the capacity to degrade 3-4 DHP. For the isolation of the bacteria clarified rumen content was used, which was subject to the growth of bacteria in a culture medium; it had micro- and macrominerals, resazurin, cysteine, phytone (soybean peptone) and sugars, which as a whole promoted bacterial growth. Additionally, the medium was gassed using CO_2 and became oxygen-free. For the identification of the anaerobic bacteria two systems of commercial tests were used (API-20A and 32A ATB), consisting in strips of essay domes that contained dehydrated substrates and propitiated the growth of bacteria from the *Clostridium* genus; mimosine, 3,4 DHP and 2,3 DHP were added to the bacteria that grew in the API systems. As a result the strain that degraded the above-mentioned toxics corresponded to a Clostridium (called strain 162), given its low proportion of guanine and cytosine. The strain 162 degraded mimosine, 3,4 DHP, 2,3 DHP and dihydroxyphenylalanine (DOPA), but no 3-hydroxypyridine or catechol when these compounds were added to the RPF medium (Baird-Parker rabbit plasma and fibrinogen base agar). The highest degradation was to mimosine (50-60 %) with regards to 2,3 DHP or 3,4 DHP (35-45 %).

Later, Allison *et al.* (1992) identified and characterized a bacterium isolated from the rumen of a goat (in Hawaii), capable of degrading 3-4 DHP and its isomer 2-3 DHP, which was named *Synergistes jonesii*. This research was based on the toxicity generated by the consumption of the legume *L. leucocephala* in ruminants; nevertheless, in some parts of the world resistant animals to the toxicity of the plant components, due to their capacity to degrade3-4 DHP and its isomer 2-3 DHP, were found.

S. jonesii was originated from four strains (78-1, 100-6, 113-4, 147-1) isolated from a goat, in Hawaii (Jones and Megarrity, cited by Halliday *et al*., 2014); these strains were cultivated in anaerobic media similar to the above-described ones for *Clostridium*, which were subject to essay kits (API-ZIM, AN-IDENT, API-20A) for the identification of anaerobic Gram-negative bacteria and of the enzymes they produced. Afterwards, the identified bacteria were subject to an antibiotic-sensitivity test, based on the inhibition of strain growth exerted by 3,4 DHP and visualized by colorimetry. This study allowed to conclude that *S. jonesii* differed from any organism, after having compared it with 600 different bacteria, because the 16S sequence of its ribosomal RNA is very singular; besides using as main energy source such aminoacids as arginine and histidine, factor that also separates it from the other bacteria with which it was compared.

When comparing among *S. jonesii* strains, the microscopic exams indicated that all of them had similar morphology, and no differences were observed between the strains 78-1, 100-6 and 113-4; while only 78-1 was subject to the complete range of physiological tests. The most definite and unique tests were considered for these bacteria with the four strains and no physiological differences were detected among the latter. All degraded 2,3 and 3,4 DHP, and used arginine, histidine and DHP, besides other energy substrates for growth.

From this work, it was described that *S. jonesii* is an anaerobic, non-spore forming, non-motive, Gram-negative bacteria; likewise, it has the capacity to ferment pyridinediols, its only known habitat is the rumen and it was originally isolated for its function of helping the Hawaiian goats that grazed *L. leucocephala* (Holland-Moritz *et al*., 2014). The organism is not omnipresent in the rumen populations, but defined geographical limits for its distribution have been proven (Allison *et al*., 1992).

When this bacterium was inoculated in the rumen of animals which were not adapted to the consumption of leucaena, the toxic effects were reduced (Palmer *et al*., 2010). Nevertheless, in spite of the inoculation, the animals from different latitudes (for example, Australia, Indonesia and Ethiopia) can only tolerate up to30 % of leucaena in the diet (Dalzell *et al*., 2012). In such cases, the hypothesis that the populations of rumen bacteria capable of degrading mimosine to DHP are saturated by the high levels of leucaena (mimosine) ingestion, has been stated.

It is known that the bacteria *S. jonesii* has the capacity to degrade 3-4 and 2-3 DHP; however, its presence or absence has not been confirmed in the animals fed leucaena in other places where this plant is native. In addition, it is not known whether some bacteria have the capacity to degrade mimosine, because the above-mentioned ones only have the capacity to degrade 3-4 and 2-3 DHP, but not mimosine (Jetana *et al*., 2012). Also, Dalzell *et al.* (2012) suggested that other bacteria with degrading capacity for mimosine and its metabolites can exist.

Mimosine degradation is ascribed to a type of mimosinase, enzyme which is found in the leucaena leaf; although the activation mechanism is not known, there is the belief that at the moment of chewing the leaf this enzyme is activated, when mimosine is hydrolyzed and 3-4 DHP is produced (Pereira *et al*., 2013). Reports have also been made about the hydrolytic activity in the rumen of the animals fed with leucaena; thus, it is assumed that there are some bacteria which could degrade mimosine (Kudo *et al*., 1984). Along with the above explained facts, the intoxication by mimosine is believed to be scarce, because the animals are capable of regulating the intake of *L. leucocephala*, with which they avoid the possibility of intoxication (Bacab *et al*., 2013).

The statements by Kudo *et al*. (1984) and Dalzell *et al*. (2012) about the idea of finding more bacteria responsible for the degradation of the most widely known toxics of *L. leucocephala*, were recently concreted. In this sense, in 2015 a study revealed four bacterial isolates (*Streptococcus lutetiensis*, *Clostridium butyricum, Lactobacillus vitulinus* and *Butyrivibrio fibrisolvens*) which could completely degrade mimosine within the seven days of incubation. It was also observed that *C. butyricum* and *L. vitulinus* were capable of partially degrading 2,3 DHP within the 12 days of incubation, while *S. lutetiensis* was capable of completely degrading 3,4 as well as 2,3 DHP (Derakhshani *et al.,* 2016).

Conclusions

Mimosine which is found in the rhizosphere of *L. leucocephala* is beneficial as it is captured by certain *Rhizobium* strains, which favors mimosine degradation and nitrifying nodulation. Thus, a selective isolation of these strains could contribute to the elaboration of specific inoculants for intensive *L. leucocephala* crops.

The DHP degradation activity by the bacteria mentioned in this review confirms the importance of the catabolic processes that occur in the rumen, for the tolerance of ruminants to the secondary metabolites of the plants.

An exhaustive tracing of the rumen content of livestock grazing *L. leucocephala* is needed, in order to identify other mimosine-transforming bacteria and to contribute to the development of most effective inoculants which could be used by farmers against mimosine toxicity.

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