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Identification and characterization of *Lactobacillus* bacterial genera most prevalent used to improve silage digestibility of important forage species for livestock sector

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ABSTRACT

The objective of this study is to isolate and characterize lactic acid bacteria and their effect as a microbial inoculants in silage digestibility of *Lolium perenne –Trifolium pratense* (Rye grass - Red clover) (RG-RC), *Avena sativa – Vicia sativa* (Oat-Vetch) (O-V) and Corn Stover *Zea mays* (Corn) (C). The lactic acid bacteria (LAB) isolated from three mixtures were identified at 40 day of evolution of micro silages. Morphological, physiological, biochemical and molecular techniques were used to characterize the isolates lactic acid bacteria. The following species were identified from the mixture namely *Lactobacillus buchneri, Lactobacillus plantarum, Lactobacillus brevis* and *Pediococcus acidilactici*. 54 micro silages with each feed material were produced, 27 micro silos were inoculated with bacteria's and the rest was used as a control. The nutritional value of protein, ether extract (EE), ash, energy, neutral detergent fiber (NDF), acid detergent fiber (ADF) at 20, 30 and 40 days of ensilage was compared. The percentage of each in vitro digestibility of treatments performed on day 40 was obtained that corresponded best A-V and RG-TR inoculated with bacteria as they were 35% and 41% more digestible than the control treatments appropriate, concluding that the inoculation of lactic acid bacteria facilitated improved digestibility of silage obtaining good nutritional quality, with optimal values. These results will enable future research on the relationship between LAB species and silage fermentation quality. Use of lactic acid bacteria is recommended as an additive to improve the nutritional quality of food animals as alternative in times of scarcity of fodder or as a supplement to improve the nutritional status of livestock herd.

INTRODUCTION

Silage is a preservation technique, lactic-acidic fermentation based-on process, which can be carried out in a silo that meets the wet medium and anaerobic conditions to maintain the nutritional properties of the green fodder during the fermentation and the storage phases (Favre, 2012). Natural populations of Lactic Acid Bacteria (LAB) are responsible for conserving several cultures, such as silage. The silage process consists of the conversion of Water Soluble Carbohydrates (WSC) into organic acids (lactic acid mainly), which are capable of lowering the fodder pH, and therefore, preserve it (Guerrero, 2013). The ensiling system enables farmers to use their facilities in a more appropriate way. By suppressing the need to deforest or to purchase more land to build new cattle corrals or paddocks, the silage technique can be implemented whenever the farmers get to experience a fodder shortage period. Consequently, a nutrient-recycling process will begin, and cattle housing management process will improve (Vieyra 2006). Additionally, this legume-grass preserving process, which provides farmers with some cutting time flexibility (Lara 2011), might also work as a daily cattle supplement that enhances the balanced rations and mineral supplementation action. Farm animal's needs mineral supplements often are required to meet their nutritional needs continuously. Additionally, several weather conditions like floods, droughts, volcanic activity, and deforestation might also lead to food shortage. As a result, an economizing supplementation alternative, such as using digestibility bacterial boosters for fodder, is required. Silage quality depends on how much crude protein, ash, energy, crude fat, additives and ethereal fraction will be handled.

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Additional parameters, such as: Water Soluble Carbohydrate quality, temperature below 40°C, pH 4.2, Homofermentative (Lactic Acid Only producer) and Heterofermentative (Lactic acid, Acetic Acid producer) bacteria content and humidity level around 60-70% affect the final silage quality (Gutiérrez 2009, Contreras 2009).

Heterofermentative microbacterial inoculants purpose is to improve the aerobic stability of silage by decreasing the yeast levels. The most common bacterial inoculant in stock is the Homofermentative Lactic Acid Producers (Filya 2003). These bacteria, whose main purpose is preserving the silage properties as near to the original green condition as possible, is capable of fulfilling several tasks, such as: reducing ammonia formation and proteolysis, keeping dry matter losses under 2-3%, and enhancing the dry matter digestibility and production of lactic acid (Muck and Kung, 1997). Both Homofermentative and Heterofermentative Lactic Acid Producers work as pH level-lowers and latter aerobic stability controllers respectively. However, few lab studies involving both Lactic Acid Producers simultaneously have been conducted (Pahlow *et al*, 2003).

The main purposes of this current research project is to identify and typify the dominating bacterial genuses, which are bound to enhance the digestibility of livestock-relevant plants grown for fodder; and to determine the nitrogen percentage of in-vitro-microsilaged fodder.

MATERIALS AND METHODS

Preparation of silages

The whole crops were collected from a farm in Instituto Nacional de Investigaciones Agropecuarias (INIAP) in Pichincha Province. Silages were prepared using a small scale, a forage mixture divided into three treatments with: *Lolium perenne-Trifolium pratense* (RG-TR), *Avena sativa–Vicia sativa* (A-V) and *Zea mays* (RM), approximately 3 kg of mixture forage material (30% legume and 70% grass) chopped into about 1-3 cm length and packed into PVC tubes of 50 cm of large. The tubes were filled with a solution of water, molasses and urea in proportions of 10%, 5% and 0.1% respectively, according to the weight of micro silages. 9 micro silos was performed, three for each vegetable mix. The micro silos were stored in a room at ambient temperature (Fig. 1).

Fig. 1: Micro silos with air valves and hoses infusion.

Silage samples were collected after 45 days during the ensiling process. The samples (10g) were blended with 90ml of sterilized water and serially diluted from $10^{-1}$to 10^{-3} in sterilized water. The dilution was cultivated on Man Ragusa and Sharpe (MRS Neogen) agar (1.0% peptone, 0.8% egg extract, 0.4% yeast extract, 2.0% glucose, 0.5% sodium acetate trihydrate, 0.1% polysorbate 80 (also known as Tween 80), 0.2% dipotassium hydrogen phosphate, 0.2% triammonium citrate, 0.02% magnesium sulfate heptahydrate, 0.005% manganese sulfate tetrahydrate, 1.0% agar, pH adjusted to 6.2 at 25°C) and incubated at 37°C for 72 h under anaerobic conditions. The colonies were selected according to the morphology and each colony was isolated and purified twice by streaking on MRS agar plates. Pure cultures were grown on MRS agar at 30°C for 24 h, and then the purified strains were stored at -90°C in glycerol solution at 15%, for conservation.

Isolation and Identification of Dominating Bacteria Phase

The selection of colonies was randomly isolated from the plate containing between 35 and 300 colonies. Four dominant bacterial strains were isolated: R50, V74, M45, and T66. The identification of strains was performed after 24 h of incubation on MRS agar. The cultures were identified based on cell morphology, Gram stain, biochemical and physiological characteristics. Catalase and oxidase activity was determined according to Benson, 2001. Starch hydrolysis was done about describing Cheeptham, 2012. Carbohydrate fermentation tested were: Glucose, Fructose, Lactose, Galactose, Sorbitol, Maltose, Rumors, Mannitol, Sucrose and Raffinose. The tubes were added with 3 droops of sterile liquid paraffin before inoculation in order to ensure anaerobic conditions.

The growth at different temperatures was observed in MRS broth after incubation at 15, 35 and 45^{o}C. Growth of cultures at pH 4.0, 6.0, 8.0 was determinate in MRS broth after incubation at 30°C for 5 days.

The 16S ribosomal RNA gene sequence was amplified by polymerase chain reaction from chromosomal DNA, performed in a PCR Thermal Cycler (Techne FTC41H2D, UK). The sequences of the PCR products were determined with the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGA-3′). An automatic sequencer (Applied Byosistem, model No 3730XL) was used to obtain the 16S rDNA sequence, 1650 pb. Alignment of the different 16S rDNA sequences from Gen Bank data library using the BLAST program was performed using CLUSTALW software program (Hitachi Software Engineering Co., Tokyo, Japan).

Bacterial propagation for experimental phase

In order to make up a microbial consortium, the R50, V74, M45 and T66 strains were selected as additive at 6x10^{8} CFU/ g of vegetal matter (Cabeza 2013). In that sense, after the identification and isolation phases were conducted, a bacterial mass propagation phase was carried out on a cultivation peptone broth (which consisted of 5 grams of dextrose, 5 grams of peptone,

and 1 gram of NaCl per liter of distilled water). Bacterial concentration was subsequently determined by utilizing a Thermo Genesis, USA spectrophotometer that employed the McFarland Standards to reach a $6x10^8$ CFU/ml concentration at 560 nm.

Experimental design

According to the net weight of each ensiled fodder, 54 polyvinyl-chloride micro silos were built to contain 6 kilograms of each vegetal mixture along with a molasses (5%), urea (0.1%) and water (10%) dilution. On the other hand, the micro silos containing the microbial consortium had no water in the dilution, but the $6x10^8$ CFU/ml cultivation peptone broths replaced the water concentration. The micro silos consisted of a diameter of 4 inches and a 60-centimeter length.

Experimental treatments included: control micro silage (without bacteria), RG-TR + microbial consortium, RG-TR without microbial consortium; A-V + microbial consortium, A-V without microbial consortium; RM + consortium, RM without consortium.

Temperature and pH levels of the micro silos were measured after the loading and sealing stages were conducted.

Compositional Analysis

20, 30 and 40 days after the fermentation process took place, the micro silos RG-TR with bacteria, RG-TR without bacteria; A-V with bacteria, A-V without bacteria; RM with bacteria, RM without bacteria were harvested. The harvesting phase consisted of isolating the vegetal mixture found in the middle of the micro silo, and discarding the dilution found in the highest and lowest parts of the container.

Subsequently, an organoleptic test was conducted in order to value odor, color, texture, and fungi presence parameters, along with ethereal portion, crude protein, ash, pH, temperature and energy levels using a 300-gram sample of ensiled matter.

Digestibility tests

In vitro digestibility tests were conducted for the dried samples of each treatment. By following the methods described by Galyean (1996). The dry matter disappearance was determined. Afterwards, vegetal substrates had to be weighed (250 ± 1 mg) and then placed on 60-milliliter syringes, with artificial saliva (25ml) and ruminal liquid, which was obtained from the annulated cows (3:1 relation).

Figures 2 and 3 to avoiding any gas leak, the syringes were sealed, and finally incubated at 39 °C for 24 hours. Following the method that Quinn *et al.*, (2010) described, the gas production levels were measured and calculated. However, ruminal liquid, along with artificial saliva, was employed for the control treatment. Digestibility testing was done in triplicates for each treatment on each evaluation date.

Once the incubation period ended, the resulting treatments were filtered, for which CFP41 Quantitative Cellulose Filter Paper (125-millimeter diameter) was used. Finally, the liquid portion of the vegetal samples was dry in oven at 60°C for 24-hours so that the g4gb net weight of the digested dry matter can be measured.

Fig. 2: Ruminal liquid obtained from cannulated cows.

Fig. 3: Syringes with artificial saliva (25ml) and ruminal liquid.

RESULTS AND DISCUSSION

The dominating bacterial strains: R50, V74, M45, and T66 were isolated from micro silos RG-TR, A-V, ZM. The isolates turned out to be mostly homofermentative and heterfermentative lactic acid producers. Most of them managed to grow at 4, 6 and 8 pH levels, at 15 and 35°C. The *Lactobacilli* isolates (R50, V74, and M45) showed rod-shaped, Gram-positive; catalase-and-oxidase-negative features, while the *Pediococcus* sp. isolate (T66) was a round-coccus-shaped, positive-gram bacteria. Additionally, apart from being a Homofermentative producer, the *Pediococcus* isolate was unable to grow at an 8 pH-level. The four identified groups presented different carbohydrate fermentation levels. Some other specified physiology and biochemical characteristics are displayed in Table 1.

By means of the polymerase chain reaction technology, the dominating-strain identification was carried out. The identified strains, which turned out to be *Lactobacillus*, presented a 1650 bases pairs (pb) amplicon, just as the typical Gram positive coccus bacterial strain. The phenotypical and genotipical characterization

results proved that the R50 strain turned to be *Lactobacillus buchneri*, the V74 strain matches to be *Lactobacillus plantarum*, the M45 strain was identified as *Lactobacillus brevis*, and the T36 strain was a *Prdiococcus acidilacticci* recognized match.

Lactic-acid-producing bacteria presence is necessary for the ensiling process to take place (Contreras *et al*, 2009), for they are able to low pH levels in shorter periods of time. That way, vegetal tissue transpiration can be decreased so that yeast, along with other microorganism, is inhibited and adequate anaerobical stability is achieved.

Table 1: Physiological and biochemical features of isolated strains from silages.

Feature	Strain R50	Strain V74	Strain M45	Strain T36
Shape	Rods	Rods	Rods	Cocci
Gram Stain	+	+	+	+
Catalase	-	-	-	-
Oxidase	-	-	-	-
CO_2 from glucose	+	+	+	-
Histamine	+	-	-	-
Tiramine	-	+	+	-
Phenylalanine	-	-	+	-
Lactic acid	+	+	+	+
Acetic acid	+	+	+	-
Growth at (°C):				
15	+	+	+	+
35	+	+	+	+
45	-	+	-	+
Growth at pH level:				
4.0	+	+	+	+
6.0	+	+	+	+
8.0	+	+	+	-
Starch	-	-	-	
Glucose	+	+	+	+
Fructose	+	+	+	+
Lactose	+	+	+	+
Galactose	+	+	+	+
Sorbitol	-	+	+	-
Maltose	+	+	+	+
Ramnose	-	-	-	+
Mannitol	-	+	+	-
Sucrose	+	+	+	-
Raffinose	-	+	-	-
Pre-identification	***Lactobacillus buchneri***	***Lactobacillus plantarum***	***Lactobacillus brevis***	***Pediacoccus sp.***

+, positive; -, negative

The three micro silos holding bacteria within showed similar pH levels to those described by Cobos (2012) and did not surpassed those described by Elferink, (2001). On the other hand, the non-bacterial treatments experienced more variability. Particularly, the microbial consortium was determined to have had a positive influence on drastically lowering the pH level. This is due to the action of Heterofermentative bacteria, which releases high quantities of lactic acid, acetic acid, lactic acid and butyric acid (vegetal acidifiers) (Contreras *et al.*, 2009).

Table 2 shows the temperature variations of each treatment. It can be acknowledged that bacterial treatments experienced slightly higher temperatures than non-bacterial treatments. This variation lies in the metabolic cycle of lactic acid bacteria, which implies that bacterial growth leads to increasing the micro silo temperature. The effect that Heterofermentative bacteria have can be perceived after the first twenty days (stable temperature period). After this period, the Homofermentative bacterial populations decrease, and let the Heterofermentative bacterial population develop, which causes a temperature drop. After 10 days for Heterofermentative stabilization, to take place, the temperature finally becomes stable as well.

According to Gutierrez (2009), the temperature within the micro silos must never go above 40°C. This research project also reaffirms that the sealing phase was successfully conducted within that temperature range.

Component analysis

Proteins Analysis

After 20, 30 and 40 days of ensiling, three tubes per treatment were opened to analyze the fermentation quality. The results are shown in Table 3. The analysis of the chemical composition showed that, in contrast to the non-bacterial treatments, the protein contents for RG-TR + consortium, A-V + consortium and RM + consortium micro silages exhibited were higher.

Nevertheless, a low quantity of proteins was shown by the RM treatment. This might have occurred due to the fact that this particular ensiled Stover had a low-temperature and a low-ensiling quality background for coming from a seed-purpose corn field. Fuentes (2001), who utilized corn for human-consumption, known to have a high ensiling quality background.

Table 2: Temperature and pH and levels measurements.

Treatment	pH				Temperature (°C)			
	0 day	20 day	30 day	40 day	0 day	20 day	30 day	40 day
RG-TR + consortium	6.1	4.2	3.2	3.8	18	15.2	14.9	15
RG-TR without consortium	6.2	4.9	4	3.7	17	14.5	14.8	14.3
A-V + consortium	6.1	4.1	3.5	4.1	19	15	15.3	15.5
A-V without consortium	6.1	4.7	3.9	4.5	18	14	14	14.3
RM + consortium	6	4.0	3.9	4.2	18	14	15	15.2
RM without consortium	6.1	4.9	4.6	4.8	17	13.6	15.3	15.5

Table 3: Component analysis of micro silos with consortium and without consortium at 20, 30 and 40 days for ensiling.

Treatment	Proteins (%)			Etherealportion (%)			Ash (%)			Energy (kcal/100g)		
	day 20	day 30	day 40	day 20	day 30	day 40	day 20	day 30	day 40	day 20	day 30	day 40
RG-TR + consortium	13.7	14.9	14.6	2.85	2.84	2.82	11.9	13.3	12.4	294.3	285.4	290.7
RG-TR withoutconsortium	13	14	13.9	2.80	2.70	2.60	12.2	13.3	12.2	291.3	290.7	288.3
A-V + consortium	10.4	10.7	11	2.18	2.13	2.3	8.7	9.9	11.9	281.2	273.5	271.6
A-V withoutconsortium	10.1	11	10.5	2.08	1.98	1.88	8.7	9.4	9	280.3	272.7	275.1
RM + consortium	1.2	1.8	1.7	0.69	0.84	0.77	6.2	6.5	6.2	249.8	248.3	244.1
RM withoutconsortium	1	1.4	1.1	0.67	0.84	0.71	6.4	7	6.6	244.6	242.8	241.9

Ethereal Extract (EE) Analysis

In RM micro silos no differences were found among the treatments inoculated with bacteria and those without bacteria. The lowest percentage of EE were presented by micro silos with the RM treatment at day 30th (0.84 %). In the A-V and RG-TR micro silos, which held the microbial consortium, higher percentages of EE were observed in contrast to the non-inoculated micro silos (Table 3). The measurements shown on this research project happen to be in the range described by Flores and Rodriguez (2010), for an adequate silage process.

Ash Analysis

The higher value in ash content was observed in AV treatment + consortium and RG-TR + consortium micro silages at 30th days of ensiling (Table 3). The values obtained in this study have a close relation with those established by Calsamiglia *et al.*, (2004) which determined 12.8% as top quality silage value. The lowest values were presented by RM micro silage at 20 and 40 days of fermentation.

Energy levels Analysis

Non-bacterial micro silos showed lower values unlike the treatments that held the bacteria consortium within (Table 3). This is due to the fact that a greater bacterial action on the cell wall of stubble was experienced in the bacterial treatments, leaving energy reserves exposed and available for fermentation. On the other hand, the results of energy in RM micro silos were lower than the micro silos inoculated with consortium.

Digestibility tests

In order to determine the digestibility levels of silage, the gas production had to be measured for this parameter is directly proportional to the digestibility. Table 4 displays gas production levels at a 24 and 48-hours period of *in vitro* digestibility. According to statistical analysis at 24h, there are significant differences between treatment AV + consortium (Gas production = 162.13 cm^3) and the A-V treatments without consortium (Gas production = 105.27 cm^3); RM + consortium (155.24 cm^3) and RM without Consortium (137.32 cm^3) which reaffirm that bacterial inoculation enhance the digestibility of silage. Additionally, a numerical difference can be observed in treatments (RG-TR + consortium, RG-TR without consortium). In addition, it was observed that the best treatment was AV + consortium, for it showed a higher gas production in contrast to the other treatments. Comparing gas production at 48 h in different forages with the presence and absence of consortium, it appeared that there are significant differences between treatments RG-TR + consortium (PG = 155.94 cm^3) and treatments RG-TR without consortium (PG = 91.7 cm^3). The bacterial consortium helps digestibility of silage material efficiently even at 48 h.

Table 4: *In vitro* digestibility of dry matter in the different treatments with and without bacterial consortium.

	Treatment	Gas production at a 24-hour period (cm^3)	Gas production at a 48- hour period (cm^3)
Forage* Consortium	RM + consortium	155.24	129.32
	RM without consortium	13732	124.62
	A-V + consortium	162.13	145.1
	A-V without consortium	105.27	152.83
	RG-TR + consortium	158.88	155.94
	RG-TR without consortium	158.32	91.7

Finally, by comparing the treatments inoculated with bacteria and treatment without bacteria, it was observed that the use of bacteria as microbial inoculants improve and enhance the silage, obtaining higher production of gas in the treatments inoculated with bacteria at both 24 hours treatment (PG = 152.75 cc) and 48 hours (143.59 cc), compared to treatments without bacteria (24 hours, PG = 139.63 cc) and (48 hours, 124.72cc) as shown in Table 5. Corroborating the results obtained in investigations of Nsereko *et al.*, 2008 and Contreras, 2009 who affirmed that the application of bacteria improve digestibility of silage.

Table 5: Average gas production levels.

Ensiling time	Gas production with consortium (cc)	Gas production without consortium (cc)
24H	152.75	139.63
48H	143.59	124.72

Neutral Detergent Fiber analysis and Acid Detergent Fiber (NDF, ADF)

In Figure 4a, comparing the observed treatment RG-TR with and without bacteria, NDF % being slightly higher in the treatment with bacteria (35.66%). In the A-V is the FDN% range between 41% and 41.99%, determining that silage has a better impact in the present investigation and his results coinciding with the work of Calsamiglia *et al.*, 2004, who mention that the excellent quality silage is in the range of 39.3% - 45.8% NDF.

In the RM treatment ranged between 59.49% with consortium and 59.95% without consortium, which are similar to the value observed by Fuentes 2001, (57.73% NDF). But in this case not there is no significant difference between the treatments.

The evaluation of forages for NDF digestibility is being conducted to aid prediction of total forage digestibility, and

researchers have demonstrated that the lactating dairy cows will consume more dry matter (DM) and produce more milk when fed forages that have higher NDF digestibility (Hoffman, 2001).

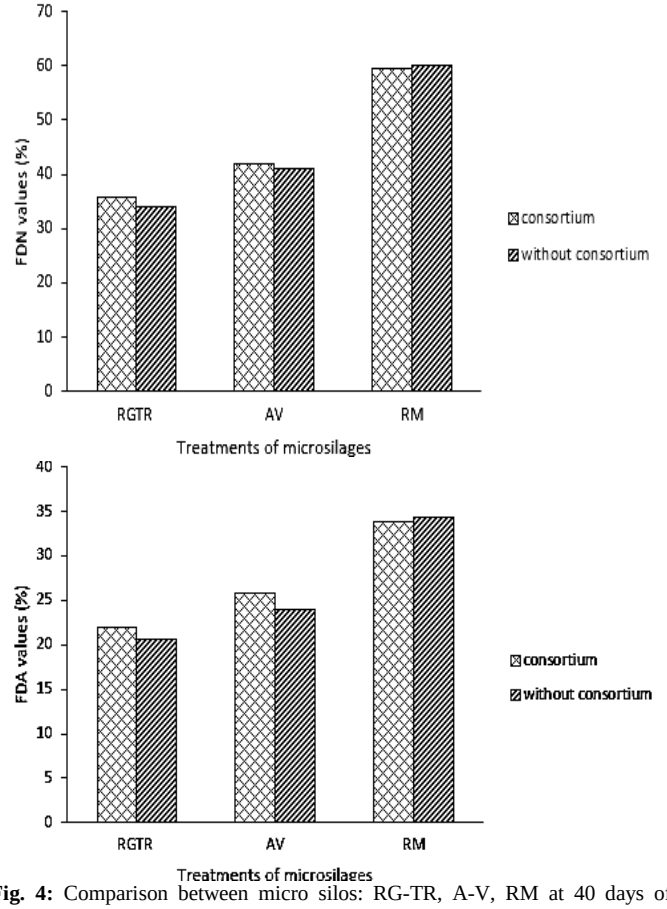

Fig. 4: Comparison between micro silos: RG-TR, A-V, RM at 40 days of ensiling. a) % NDF in the presence and absence of bacteria, b) % ADF in the presence and absence of bacteria.

The Figure 1b shows the results of ADF levels of bacterial and non-bacterial treatments. The RG-TR micro silo showed that the treatments with bacteria have a higher ADF level, with a value of 21.95%, while treatments without bacteria had a lower ADF level (20.59%). The AV micro silos treatments show that bacteria have a higher percentage of ADF (25.85%) in comparison to the non-bacterial treatment (24.02%). This value does not show any significant difference between treatments, but these values are lower than NDF values. ADF is used in many studies to define guidelines for high quality, as ADF increases, forage quality declines (Robinson *et al.*, 1998). The results of RM micro silage showed that the highest ADF values (43.39%) were displayed by the non-bacterial treatments, while treatments that held bacteria that reached to a 33.88% ADF value. These results are lower than those obtained by Fuentes (2001) 40.75% and 46.75%.

CONCLUSIONS

On the basis of phenotypic characterization, bacterial population in RG-TR, A-V and RM treatments consisted of: *Lactobacillus buchneri, Lactobacillus plantarum, Lactobacillus brevis* and *Pediococcus acidilactici*. These three treatments with bacterias played an important role in the fermentation process by increasing the shelf life and enriching by degradation of macromolecules.

The inoculation of suitable acid lactic bacteria facilitated obtaining good nutritional quality silage with optimum values: Neutral detergent fiber (NDF), acid detergent fiber (FDA), crude protein (CP), ether extracts (EE), ash and energy.

The present research project compared the nutritional parameters and the digestibility in order to pinpoint that the treatments holding bacteria within turned out to be more efficient than the non-bacterial ones.

Microbial consortium is considered to be the most suitable strains for improving the quality of silages and thus is potential inoculants for silage production.

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