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Full Length Research Paper

Screening and identification of lactic acid bacteria isolated from sorghum silage processes in west Algeria

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The lactic acid bacteria (LAB) isolated from sorghum (*Sorghum bicolor.* L.) silage were identified during different periods of evolution of sorghum silage in west Algeria. Morphological, physiological, biochemical and technological techniques were used to characterize lactic acid bacteria isolates. A total number of 27 representatives of lactic acid bacterial strains were retained and among them four dominant genus were identified as *Lactobacillus* (44%), *Lactococcus* (14.81%), *Weissella* (29.62%) and *Leuconostoc* (11.11%). The representative species identified were *Lactobacillus brevis* (25%), *Lactobacillus pentosus* (3.7%), *Lactobacillus manihotivorans* (11.11%), and *Lactobacillus fermentum* (3.7%). *Lactococcus lactis* subsp*. lactis* biovar*. diacetylactis* (14.81%), *Weissella cibaria* (7.2%), *Weissella minor* (11.11%), *Weissella soli* (3.7%), *Weissella viridescense* (7.2%) and *Leuconostoc mesenteroides* subsp*. mesenteroides* (11.11%). Only two strains of lactic acid bacteria were amylolytic. These results will enable future research on the relationship between LAB species and silage fermentation quality.

Key words: Lactic acid bacteria, identification, silage, sorghum, evolution, amylolytic, technology, species.

INTRODUCTION

Lactic acid fermentation, an ancient preservation method, is nowadays specially preferred as a “natural” process to increase the shelf life of various products (vegetable, dairy and meat). Natural populations of LAB on plant material are responsible for conservation of a crop as silage by converting water-soluble carbohydrates (WSC) into organic acids, mainly lactic acid. As a result, the pH decreases and the forage is preserved (Chen and Weinberg, 2009). The biochemical and microbiological events that occur during ensiling process can be divided into four distinct stages (Ashbell et al., 1997). The bacterial spectrum includes homofermentative species that exclusively produce lactic acid and heterofementative species, which produce a mixture of lactic and acetic acids and/or other by-products like ethanol and carbon dioxide (Vlková et al., 2012). Habitually, heterofermentative LAB are low in number. Thus, the concept of using microbial inoculants for silage involves adding fast-growing homofermentative LAB in order to dominate the fermentation, but is less effective if insufficient fermentable substrate is available. Some of the commonly used homofermentative LAB in silage inoculants include *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Pediococcus acidilactici* and *Enterococcus faecium*. Commercially available microbial inoculant contains one or more of these bacteria that have been selected for their ability to realize fermentation. The quality of silage could be improved by the addition of inoculants, consequently lactic acid production occurs more quickly and loss of nutrients during ensilage can be reduced (Widyastuti, 2008). The presence of LAB such as *L. plantarum* and *Pediococcus* spp. were able to maintain the silage quality through increasing lactic acid production (Filya et al., 2006). Recently, in order to prevent the aerobic deterioration of silage, heterofermentative LAB species, such as *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus reuterin*, were developed as silage additives for antifungal effect. Moreover, homofermentative LAB inoculum can provide substantial benefit by reducing the risk of the

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growth of other harmful spoilage organisms such as butyric acid bacteria including clostridia by reducing the pH (Saarisalo et al., 2007). Several factors influence the fermentation processes that transform green forage into silage.

From a microbiological point of view, to our knowledge, no information is available on the microbial ecology of sorghum, especially with regard to the indigenous LAB and their effects during the fermentation process. Especially, the sorghum plant is drought adaptive supporting the arid climate and rich food. The aim of this study was to identify and characterize naturally present microbial populations from the sorghum silage, especially dominants lactic acid bacteria on the basis of their important technological properties in order to select potential autochthonous as grass silage inoculants.

MATERIALS AND METHODS

Preparation of silage

Silage sorghum (*Sorghum bicolor*) was obtained from different experimental sites in the north west of Algeria and it was prepared using the method described by Filya et al. (2004). Whereas, the forage of *S. bicolor* was collected in Oran region. This study was done at different periods of silage incubation. Sorghum was harvested and chopped by a precision forage harvester to 3 to 5 cm theoretical length. A serum bottle of 500 ml was used as microsilo and was incubated at two different temperatures, 30°C and ambient temperature. In all the experiments, the serum bottles were sampled on 2, 5, 8, 10, 18 and 90 days in triplicate (Ashbell et al., 1991; Filya et al., 2004).

Analytical methods

Biochemical test

An amount of 10 g of silage was homogenized for 5 min with 90 ml of distilled water and then the pH of the filtered water was measured by pH meter (Xing et al., 2009). Dry matter was determined by oven drying for 48 h at 60°C for fresh material and silages (Weinberg et al., 1995).

Microbiological examination

For microbiological analysis, the samples of silage were prepared as follows: 1 g of sample was homogenized with 9 ml of 0.85% (w/v) sterile physiological saline in a Stomacher lab-blender for 1 min and serially diluted (10^{-1} to 10^{-7}) in the same diluents. One milliliter of these dilutions was pour-plated in the respective media for LAB. LAB were isolated on MRS agar, after incubation under anaerobic conditions at 30°C for 48 to 72 h. Representative strains of LAB were obtained from MRS plates of the highest sample dilutions. Aerobic mesophilic counts were determined using plate count agar incubated at 30°C for 48 to 72 h.

Isolation and identification of dominant lactic acid bacteria

The selection of colonies was randomly isolated from the plate containing between 25 and 250 colonies. Purity of the isolates was checked by streaking in MRS agar plates, followed by microscopic observation.

For the investigation of the fermentation properties of these isolates, inoculation was done in 10% skimmed milk and to which 0.3% yeast extract, 1% glucose, 1% $CaCO_3$ were added and incubated for 48 h at 30 and 42°C. Subsequently, the coagulation of milk was checked which indicates the presence of LAB (Sengun et al., 2009).

Preliminary identification and grouping was done based on cell morphology, gram staining, catalase activity and other phenotypic properties by using CO_2 production from glucose, hydrolysis of arginine in M16BCP plates, growth at different temperatures of 15°C for 14 days and 37 and 45°C for 2 days, and at different pH: 4 and 9.6 for 7 days as well as the ability to grow in different concentrations of NaCl: 3, 4 and 6.5% for 2 days of incubation in MRS broth (Badis et al., 2004; Bendimerad et al., 2012).

The identification at species level was carried out by the fermentation of carbohydrates determined on MRS broth containing bromocresol purple (0.04 g/l) as a pH indicator. The carbon sources were added to the medium at 1% (w/v) as final concentration. The carbohydrates tested were: glucose, fructose, lactose, galactose, sorbitol, arabinose, xylose, trehalose, raffinose, ramnose, maltose, mannitol, sucrose, starch and esculin to ensure anaerobic conditions. Each tube was supplemented with two drops of sterile liquid paraffin before inoculation.

All strains of lactic acid bacteria were stored without appreciable loss of properties in skimmed milk containing 30% (wt/vol) glycerol at -20°C. Working cultures were also kept in MRS agar slant at 4°C and streaked for every 4 weeks (Badis et al., 2004; Guessas and Kihal, 2004).

Technological properties of the isolates

The physiological group of lactic acid bacteria involved in the degradation of macromolecules (protein, cellulose and starch) in the vegetative parts of the silage sorghum (*S. bicolor*) was investigated by the method of Dubos (1928).

Proteolytic activity

For screening of the proteolytic bacteria, strains were grown on Yeast Milk Agar (YMA) and gelatin medium. The plate counts were evaluated by the presence of the hydrolysis zone around the colonies, and by the change in the appearance of gelatin in liquid medium (Huggins and Sandine, 1984).

Amylolytic activity

The amylolytic activity of isolates of sorghum silage was observed in MRS starch medium where glucose in the medium was replaced by 20 g soluble starch (Brabet et al., 1996). The enumeration of amylolytic bacteria was evaluated by the presence of the clear zone around the colonies after using iodine solution (lugol) (Thapa et al., 2006).

Cellulolytic activity

From retained isolates of lactic acid bacteria strain, the pre-cultures were inoculated by spot in Dubois agar supplemented with 5% of cellulose. The plates were then incubated at 37°C for 72 h. The plates were checked for clear zones surrounding the colonies.

RESULTS AND DISCUSSION

The study of the diversity and the dynamics of microbial

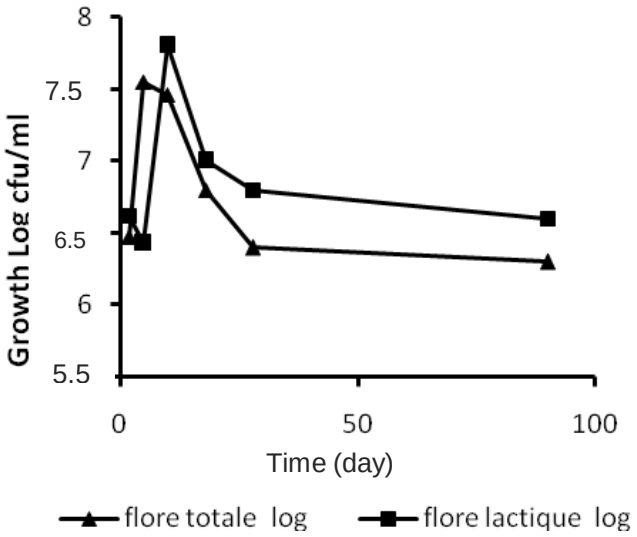

Figure 1. Growth of total flora and lactic flora during the fermentation process of sorghum silage.

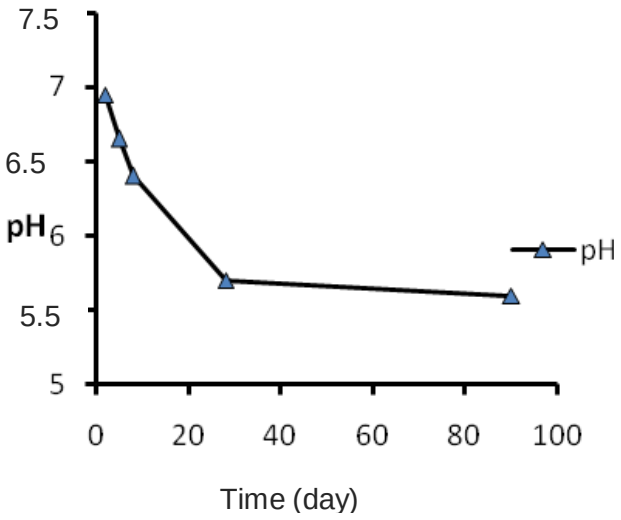

Figure 2. pH variations during the fermentation process of sorghum silage.

populations associated with sorghum silage were investigated for evaluation of the quality of silage which represent a major effect on the feed intake, nutrient utilization and milk production of dairy cows (Saarisalo et al., 2007).

Enumeration of total flora and lactic acid flora

The microbial composition of the sorghum silages is depicted in Figure 1. During the initial time of incubation, the number of total microflora is higher than lactic flora (7.5 and 6.5 log, respectively). But at day 8 of silage incubation, the number of lactic acid bacteria increased to 7.8 log, whereas, the total microflora remained stable at 7.5 log. The number of total microflora and lactic acid flora decreased gradually during the time of incubation. At 90 days, the number of total microflora is less than lactic acid microflora (6.3 and 6.7 log, respectively). The growth of the total flora during 90 days of silage incubation was found to be maximum on 2nd and 5th day, whereas it was least on the 8th day. The variation in the evolution of pH during sorghum silage was noted and shown in Figure 2. The initial pH was 7 which dropped gradually to 5.6 on the 28th day and after which no change in the pH was observed.

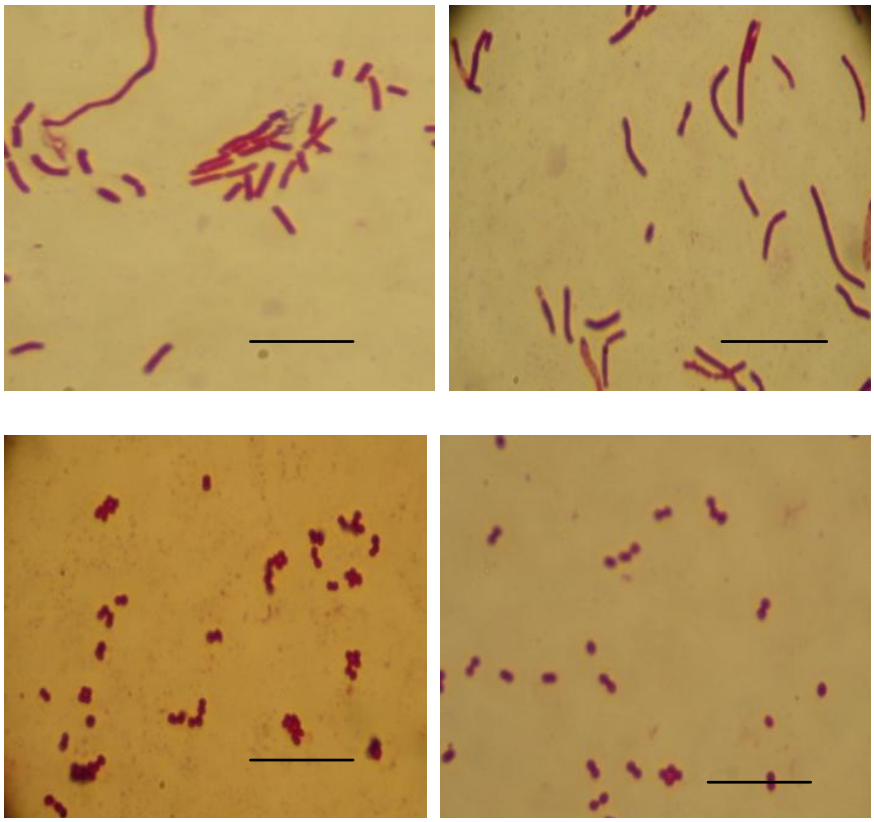

Figure 3. Morphological characters of lactic acid bacteria of silage sorghum observed with microscope (rod form and cocci form).

On the other hand, the growth of lactic flora which is a part of the natural heritage of flora associated with the plant material (Makimattila et al., 2011), acts differently in silage because as shown in Figure 1, the growth is minimal at 2nd and 5th day but in the 8th day it was maximum (ICMSF, 2005; Weinberg et al., 2010). During this period of silage incubation, many factors can present favorable or unfavorable effect on microorganism's growth. The total flora can be inhibited by the absence of O_2 such as yeast, molds and aerobic bacteria (Pahlow et al., 2003), pH sensibility by presence of the organic acid especially lactic acid produced during the growth of the lactic acid bacteria (Paragon, 2004) or acetic acid which has an antifungal effect (Lindsey and Kung, 2010; Keles and Demirci, 2011). The dominance of lactic acid bacteria at the end phase of silage is enhanced by environmental conditions especially gradual absence of oxygen and pH decrease.

Isolation and identification of dominant lactic acid bacteria

From 63 isolates screened, 27 were considered to be LAB by the properties like Gram staining, catalase activity, motility and production of spores or not, as per the method described by Sengun et al. (2009). This lead to the delineation of two principal groups of isolates, each one displaying a distinct carbohydrate fermentation pattern (Table 1 and Figure 3). The various groups presumably represented four different LAB and various percentages of the total genera were found to be *Lactobacillus* (44%), *Lactococcus* (14.81%), *Weissella* (29.62%) and *Leuconostoc* (11.11%).

The LAB isolates were mostly homofermentative and heterofermentative. Most of them showed growth in the pH 4 and at temperature of 45°C.

In the lactobacilli isolates, especially for homofermentative *Lactobacillus* group, the specie *of Lactobacillus manihotivorans* was detected and identified in our silage sample. The *Lactobacillus pentosus* is facultative heterofermentaire species which can produce CO_2 from gluconate and have the same characters of *Lactobacillus manihotivorans.* The later species was also isolated from sour cassava starch fermentation in Colombia (Morlon et al., 1998, 2001).

The eight species of heterofermentative *Lactobacillus* group were found to belong to two species of *L. brevis* (7 isolates) *and L. fermentum* (1 isolate). To differentiate between these two species, xylose and esculin were used. Our results for *Lactobacillus* identification were in agreement with those obtained by Abriouel et al. (2008).

Table 1. Physiological and biochemical characteristics of isolated strains from silage.

Characteristics		Rod group				Cocci group					
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
		14-13-A_5-12-D_{11}-19-A_9	**50**	**7*-22-D_1**	**D_{10}**	**11-A_8-A_6-20**	**4-32**	**7-43-A_4**	**25**	**A_1-42**	**E_1-E_2-E_3**
Number of isolates (27)		7	1	3	1	4	2	3	1	2	3
Form		rods	rods	rods	rods	cocci	cocci	cocci	cocci	cocci	cocci
Gram		+	+	+	+	+	+	+	+	+	+
Catalase		-	-	-	-	-	-	-	-	-	-
CO_2 from glucose		+	v	-	+	-	+	+	+	+	+
NH_3 from arginine		+	+	+	+	+	+	+	+	+	-
Growth at	15	+	+	+	+	+	+	+	+	+	+
temperature (°C)	45	v	+	+	+	+	+	+	+	+	+
Growth in a medium with	3	+	+	+	+	+	+	+	+	+	+
NaCl (%)	6.5	+	+	+	-	+	+	+	+	+	-
Growth at pH	4	+	+	+	+	+	+	+	+	+	-
	9.6	+	+	+	+	-	+	+	+	+	+
Citrate hydrolysis		+	+	+	+	+	+	+	+	+	+
Heat resistance 63.5°C for 30 min		v	+	+	-	+	+	+	+	v	+
Acid production from											
Trehalose		+	+	+	+	/	+	+	+	+	+
Raffinose		-	-	-	+	+	-	-	+	-	+
Xylose		+	+	+	-	/	+	+	+	+	+
Maltose		+	+	+	+	+	+	+	+	+	+
Galactose		+	+	+	+	+	+	+	+	+	+
Sorbitol		+	+	+	+	+	-	+	-	+	-
Arabinose		v	v	+	+	+	+	-	+	-	+
Mannitol		+	+	+	+	+	+	+	-	-	+
Ramnose		-	-	-	v	-	-	-	-	-	-
Sucrose		+	/	/	+	+	+	+	+	+	+
Fructose		+	+	+	+	+	+	+	+	+	+
Glucose		+	+	+	+	+	+	+	+	+	+
Esculin		+	+	+	-	+	+	+	+	+	+
Starch		-	-	-	-	-	-	-	-	-	-
Pre-identification		*Lactobacillus brevis*	*Lactobacillus pentosus*	*Lactobacillus manihotivorans*	*Lactobacillus* fermentum	*Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*	*Weissella cibaria*	*Weissella minor*	*Weissella soli*	*Weissella* viridescense	*Leuconostoc mesenteroides* subsp *mesenteroides*

+, Positive; -,negative; v, variable.

Seseña et al. (2005) always considered the predominant LAB, with increased growth of *L. brevis* at the end of fermentation in natural fermentation of green olives. The utilization of ribose, galactose, glucose, fructose, maltose, saccharose, raffinose and esculin was used for the identification of *L. fermentum* by Hammes et al. (1992 and Sawadogo-Lingani et al. (2010).

In cocci groups, three isolates were positively

identified as *Leuconostoc mesenteroides* subsp. *Mesenteroides* characterized by: Production of CO_2 from glucose, production of dextran from sucrose, inability to use arginine, but the use of arabinose is specific for this species. The properties of *Leuconostoc* species were reported in several works (Carr et al., 2002; Bendimerad et al., 2012). Generally, leuconostocs are found living in association with plant material and dairy products. Other studies have reported leuconostocs as the dominant microbial population on forage crops and silage (Cai et al., 1994). The lower numbers of *Leuconostoc* is probably due to their inability to compete with other LAB in mixed cultures (Teuber and Geis, 1981; Togo et al., 2002).

Eight strains in this group were identified as *Weissella* differing from the *Leuconostoc* by the arginine test and the sugar fermentation profile. The represented species include *Weissella cibaria* (7.2%), *Weissella minor* (11.11%), *Weissella soli* (3.7%) and *Weissella viridescense* (7.2%). The studies of Pang et al. (2011) indicate that perhaps several *Weissella* species could improve silage quality. Some species of *Weissella* have been isolated from a wide range of sources such as soil, fresh vegetables, meat, fish, fermented silage and foods (Björkroth et al., 2002; Sirirat et al., 2008; Valerio et al., 2009).

Four homofermentative strains can hydrolyze arginine and citrate and cannot grow at pH 9, 45°C and 6.5% NaCl. However, the three later physiological characters: pH, temperature and salinity cannot be used as reference for the identification of lactic acid bacteria because there are variations in these characters particularly in this group of bacteria Drici et al. (2010). They later had isolated from camel milk the species of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* which show growth at 50°C. The profile comparison revealed that homofermetaives isolates observed in the present study can be considered as *L. lactis* subsp. *lactis* biovar. *diacetylactis* (14.81%). Further, the research work of Sawadogo-Lingani et al. (2010) has observed the presence of *L. lactis* in sorghum grains fermentation.

Technological properties of the isolated lactic acid bacteria

Fermentation is one of the oldest methods of food preservation technology in the world. The process relies on the biological activity of microorganisms for production of a range of metabolites which can suppress the growth and survival of undesirable microflora in foodstuffs. As a result, fermented products generally have a longer shelf life than their original substrate and present very good safety records. In this study, the absence of proteolytic and cellulolytic activity in isolated lactic acid bacteria was noticed. In contrast, three species of *Lactobacillus* produced an enzyme amylase in culture medium, which was confirmed by the observation of a clear zone of starch hydrolysis when treated with iodine. Three amylolytic strains identified belonged to the species of *L. manihotivorans* which are considered as amylolytic lactic acid bacteria.

Conclusion

On the basis of phenotypic properties, the lactic acid bacterial population in silage sorghum prepared in laboratory consisted of: *Lactobacillus* sp., *Lactobacillus brevis, Lactobacillus pentosus, Lactobacillus manihotivorans, Lactobacillus fermentum, Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis Weissella cibaria, Weissella minor, Weissella soli, Weissella viridescense* and *Leuconostoc mesenteroides* subsp. *mesenteroides* which play an important role in fermentation to increase the shelf life and enrichment by degradation of macromolecules. The present investigation revealed the presence of different genus of lactic acid bacteria in sorghum silage. The study of their technological characters might help in the near future for the establishment of a local starter for silage process. To our knowledge, in Algeria, this is the first report on the isolation of amylolytic species of *Lactobacillus manihotivorans* in sorghum silage which can be exploited in the manufacture of fermented foods.

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