MICROBIOLOGY

Biodiversity of wild *Lactococcus lactis* and their geo-spatial relationship with the environment

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Abstract: Lactic acid bacteria are distributed in nature, isolating themselves from diverse ecosystems and presenting a wide metabolic biodiversity. In Corrientes (Argentina), artisanal cheeses and their specific environment are an important source of autochthonous lactic acid bacteria. The objective of this work was to establish associations between the phenotypic characteristics of strains of *Lactococcus lactis* subsp. *lactis* native from Corrientes with climatological data of the Province and the characteristics of the soil and the landscapes. Physiological and biochemical characterization data of *Lactococcus lactis* subsp. *lactis* isolated from the dairy environment and from different localities of Corrientes will be used. The strains were space-located through Google Earth, flood and drought events were evaluated using Standardized Precipitation Evapotranspiration Index, and soil composition data (A and Bt horizons) in the study areas were obtained from the experimental station National Institute of Agricultural Technology - Corrientes. A statistical analysis was applied to these results (Infostat Software, Di Rienzo et al. 2008). The resulting consists in three conglomerates, differentiating strains from soils coming from "flooded landscapes" and those from "sandy hills landscape". The analysis by main components highlighted the preference of strains from flooded landscapes by a saline-alkaline environment, affecting during periods of drought, and strains from sandy hills landscape by a low medium in salts and acid soil, directly during period of high humidity resulting from previous floods.

Key words: autochthonous microorganisms, ecological niche, environment, climatic variability.

INTRODUCTION

Lactic acid bacteria (LAB) are a microbial group represented by several genera with common characteristics, which include an abundant lactic acid production from the original raw material.

A field of research of great interest over the years is the adaptive response of LABs to the environmental stress conditions. LABs are able to grow in different substrates and ecosystems according to growth factors such as salt concentration, pH, and presence of specific substances or temperature. *Lactococcus (Lc.) lactis* subsp. *lactis*, is particularly used as a component of starter cultures, mainly those destined for the manufacture of cheese and probiotic fermented milks, due to its rapid acidifying activity, as a food preservative, as a cellular manufacture of enzymes, metabolites of industrial interest, and recombinant proteins for their adaptation to hostile conditions (Kelly et al. 2010, Papadimitriou et al. 2016, Song et al. 2017, Silva et al. 2018, Tabla et al. 2019, Qi et al. 2020, Xiong et al. 2020).

In the province of Corrientes, Argentina, an important source of LABs are the artisan cheeses, whose production is made from raw

cow's milk and an artisanal rennet, obtained by immersing pieces of salted and dry abomasums in milk. The production of these cheeses is widely distributed throughout the Province, and their microbiota shows physiological and biochemical behavior influenced by the ecological characteristics of the area from which they were isolated (Vasek et al. 2008a, 2011, 2013, Vasek & Pimentel-Filho 2019).

Isolation of LABs strains from the dairy environment included, as starting material, to raw milk, artisanal rennet, curd at each stage of manufacturing and cheeses at different ripening times (Vasek 2003, 2005, Vasek et al. 2008b). The physiological, biochemical, and technological characterization, and some genetic studies of these strains, allowed to form the Institutional Collection of Autochthonous Microorganisms from Corrientes of the National University of the Northeast (UNNE), Argentina, according to Res. 701/2004 and 513/2015-Board of Directors-Faculty of Exact Science-UNNE), appoint a biological curator and be included in the National System of Biological Data of Argentina (Res. 030/2010-Scientific Technological Articulation Secretariat of the Ministry of Science, Technology and Productive Innovation of Argentina.

Among these characteristics above mentioned, soil properties and the present climatic variability stand out. Milk never comes into direct contact with the soil, but it does with the animals, utensils and operators involved in milking. So, through them and through cross-contamination, microorganisms can become part of the microbiota of milk.

The analysis of genomic sequence of isolated *Lc. lactis* strains from lactic starters and dairy product, and vegetable products, provides evidence that defines a common plant origin. It is believed that LAB species initially colonized the milk through contact of cattle with soil and plants used as fodder or bedding in barns. Thus,

these communities came to the surface of cow udders and from these to the milk during milking and from the feed, which may be the primary source of LABs' inoculation (Teuber 2009, Kelly et al. 2010, Vacheyrou et al. 2011, Cavanagh et al. 2015).

The formation of a beneficial biofilm by colonizing the internal surfaces of the udder and, therefore, building a barrier against pathogenic microorganisms is an important factor, its adhesion capacity explains the ability of bacterial strains to maintain their antimicrobial effects throughout of time, mediated by the secretion of inhibitory substances such as bacteriocins and other antimicrobial compounds (Hagi et al. 2013, Muruzovic et al. 2018a, b, Wallis et al. 2018, Zhang et al. 2019).

Types of soil and its characteristics vary from one geographic location to another, and these variations do not occur randomly (Mulla 2012, Monroy-Rodríguez et al. 2017). They are the result of climatic and biological factors acting over a considerable period of time on the relief, exerting a modifying influence. When this influence occurs in a regular and sustained natural way, the location of different types of soils, the location of their boundaries, and some of their properties can be predicted, facilitating scientifically based surveys (Infraestructura de Datos Espaciales de la Provincia de Córdoba 2019).

Unlike climate, the "Climate variability", depends on extreme weather conditions that exceed "normal". The phenomena that produce such contrasts include highly organized cold fronts, stationary dry cores, hurricanes, tropical disturbances and disproportionate humidity cores. Extreme weather events can include both excessive precipitation and prolonged drought (Organization of American States 2017).

Drought indices are calculated from time series of rainfall and evapotranspiration

reference, and they are generally suitable indicators for determining the impact of drought conditions on a variety of environmental conditions, hydrological and agricultural systems (Centro Regional del Clima para el Sur de América del Sur 2016).

Different studies (Fiorillo & Guadagno 2010, Pasho et al. 2011, Vicente-Serrano et al. 2012a) showed the relationship between the variability of drought indices and different environmental and hydrological variables, such as forest growth, ground water level, crop production, vegetation activity, among others. Among them, the most used at present, the Standardized Precipitation and Evapotranspiration Index (SPEI) stands out, which is (Vicente-Serrano et al. 2010) a meteorological indicator that determines climatic conditions that have been abnormally dry or abnormally humid. It is an index that was used in drought studies at various spatial and temporal scales. Particularly, in the Andean region of Argentina, Scordo et al. (2018), determined that the state of the vegetation and the extensions covered by the bodies of water, vary according to the periods of drought and humidity that were defined using the SPEI in basins of temperate climate.

The aim of this work was to correlate the phenotypic characteristics of *Lactococcus lactis* subsp. *lactis* strains autochthonous from Corrientes with the spatial, climatic and landscape characteristics of the Province.

MATERIALS AND METHODS
Microorganisms and their phenotypic characteristics

Physiological and biochemical identification data from 86 strains of *Lc. lactis* subsp. *lactis* (Vasek 2003), isolated from the dairy environment of different localities of Corrientes, were used (Institutional Collection of Wild Microorganisms

National University of the Northeast-Microbial Biotechnology for Food Innovation: UNNE-BiMIA).

Geo-referencing

Strains of *Lc. lactis* subsp. *lactis* under evaluation were spatially located using Google Earth, establishing the geographic coordinates of their isolation by means of Longitude-Latitude. These coordinates were registered in an additional column of an Excel spreadsheet containing the physiological and biochemical tests response of each bacterial strain. Geo-located strains were grouped according to the corresponding landscape in sandy hills or flooded areas (Contreras & Contreras 2017).

Landscapes in the study area

Sandy hill regions are seen as an undulating plain with an average height of 60 m above sea level and 15-20 m above the Paraná River. These undulations, with very gentle slopes, wide and flat backs, form long parallel cords with a general direction from Southeast to Northeast. Elevations are separated by shallow depressions and a flat bottom. Its soils are acidic, with maximum pH of 4.6 and 0.1 mEqNa/100g. The region is characterized by the presence of a large number of shallow lakes and the temporary permanence of its waters depends on rainfall. As they are shallow bodies of water, they have a rapid morphometric response during the wet and dry periods, which alternate every two years.

Regions of *flooded areas* are located around the sandy hills, with imperfect drainage, very slow runoff and low permeability, with frequent floods in times of large increases, and intense droughts in seasons of low rainfall. Its soils are saline-alkaline, with average minimum pH of 4.4 and maximum of 9.4 (4.1-13.4 mEqNa/100g).

Climatology

Corrientes province has a humid landscape that would depend on the same general subtropical climate, with abundant rainfall that decreases from northeast to southwest from 1,600 to 1,100 mm per year and which distribution is almost regular throughout the year (Scarpati et al. 2016). However, conditions in the landscape sub- units are strongly influenced by rainfall.

Flood and drought events

Standardized precipitation-evapotranspiration index (SPEI) was used to analyze data. This index consists of the conversion of rainfall data to probabilities based on long-term rainfall records. The probabilities are transformed into normalized series with an average of 0 and a standard deviation of 1. This index allows the analysis of drought impacts at different time scales, as well as the identification of different types of droughts, since dissimilar natural systems and economic sectors can respond to drought conditions in remarkably diverse time scales (Vicente-Serrano et al. 2012b). A scale of 1 month (SPEI 1M) and a spatial resolution of 0.5° was used for the period 1901-2018 of the SPEI Global Drought Monitor model (http://sac.csic.es/spei/home.html. This index uses monthly mean temperature data from the NOAA NCEP CPC GHCN_CAMS global model (ftp://ftp.cpc.ncep.noaa.gov/wd51yf/GHCN_CAMS/) and monthly cumulative precipitation data from the Global Precipitation Climatology Centre global model (ftp://ftpanon.dwd.de/pub/data/gpcc/first_guess/).This system estimates reference evapotranspiration using the methodology of Thornthwaite (1948). The data obtained by the SPEI, were represented against the months of the period under evaluation, adding the date of isolation of the strains under study.

A regional percentage analysis of SPEI data was conducted for the study area. Monthly values were averaged for each locality under study for each year.   Wet years were defined as those   in which the percentage of wet months was greater than the percentage of dry months. And as dry years were defined as those in which the percentage of dry months was greater than the percentage of wet months.

Soil composition in the study area

Records of the 1:50,000 soil maps provided by the National Institute of Agricultural Technology-Experimental Station (INTA-EEA) in the locality of El Sombrero were used. Characteristics cartographic units corresponding to the regions under study were selected to analyze the pH and salinity values (as Na^+ change cations), between the A and Bt horizons (0-20 cm and 20-91 cm respectively). This was done to correlate them with the phenotypic characteristics of the strains under evaluation.

Statistical analysis

Statistical software Infostat v.2008 (Di Rienzo et al. 2008) applied to the phenotypic characterization data was used, and a multivariate analysis by successive clusterings, with average linkage and Jaccard distance (d_j) was performed. The resulting dendogram was analyzed for a given cutting distance and the clusters obtained were compared with the areas of origin of the strains. The principal component analysis was applied to the strain characterization data and the resulting associations were compared with the soil characteristics of the environment of origin.

RESULTS AND DISCUSSION

Among the physiological-biochemical identification data of the 86 strains of *Lc. lactis* subsp. *lactis*, the characteristics that showed

the greatest variability corresponded to the results of growth in NaCl at 4.0 and 6.5%, growth at temperatures of 40 and 45 ºC and growth at initial pH 9.6. Only 2 and 4 strains among the 86 whose results were used in this study, showed negative reaction to produce NH_3 from arginine and hydrolysis of esculin to generate esculetin, respectively, which is why they were included to evaluate diversity. However, they did not show significant differences, when linked to the site of origin, whose environments have contrasting geographical differences.

Lc. lactis subsp. *lactis* strains under evaluation presented responses that differed from the expected behavior according to Teuber (2009), showing characteristics typical of LABs from the region. This implies the possibility of mutations induced by environmental conditions in the area. New genotypes capable of surviving in these wild environmental conditions have been generated.

Low-GC-content gram-positive bacteria often harbor more than one ArgR-type homologue, and separate studies recently demonstrated that two arginine regulators are necessary for the regulation of arginine metabolism in the lactic acid bacteria *Lactococcus lactis* and *Lactobacillus plantarum*. The deletion or mutation of any single arginine regulator, any of the three necessary enzymes or the antiporter that translocates ornithine and arginine across the cell membrane in these organisms, results in the complete disruption of arginine-mediated regulation, particularly in the wild-type strain. On the other hand, adaptive evolution has been extensively used in stress physiological studies of LABs, specially undergo a variety of environmental stresses, such as heat, salt and acid stress. Systems biology methods including omics analysis also have allowed us to understand how LAB respond to environmental changes, and other variables should be considered for the study of LABs communities

e.g., temporal and spatial change of community composition due to biotic/abiotic factors (Larsen et al. 2008, Wenzel et al. 2018, Jensen et al. 2019, Van der Meulen et al. 2019, Pols et al. 2020).

Based on Ponce De la Cruz & Hasang (2019), a saline soil is one in which there is a high concentration of combinations of different salts, with NaCl being the most frequent salt. Considering the saline composition of *flooded soils*, a period of intense rainfall would cause infiltration of salts and a drop in pH, and in periods of drought, water would evaporate from the soil by capillarity, dragging the salts from lower strata to the surface, accumulating there. In the *sandy hill* areas, with shallow lakes that are very reactive to climate change, dry periods would increase the typical acid pH, due to the increase in the concentration of sodium salts able to generating alkaline hydrolysis, of the carbonate and sodium bicarbonate type.

Records evaluation in the map of Corrientes, showed that these strains defined the region of study (Fig. 1), including the localities of Bella Vista, Empedrado, San Luis del Palmar, Ramada Paso and surrounding areas.

Table I. Shows the SPEI values and their corresponding definition, which were considered for this analysis.

Data analysis generated by the SPEI on a monthly scale and corresponding to the localities of Ramada Paso, San Luis del Palmar, El Sombrero and Bella Vista, revealed that dry periods (incipient dry + moderately dry + very dry + extremely dry) occurred more frequently in the years 1995 and 1997 with 58 and 66% respectively (Fig. 2). The years mentioned included data on which the isolations were carried out. Both years presented months with negative SPEI values, except for February in 1997, which showed an extremely humid month.

The humid period (incipient humidity + moderately humid + very humid + extremely
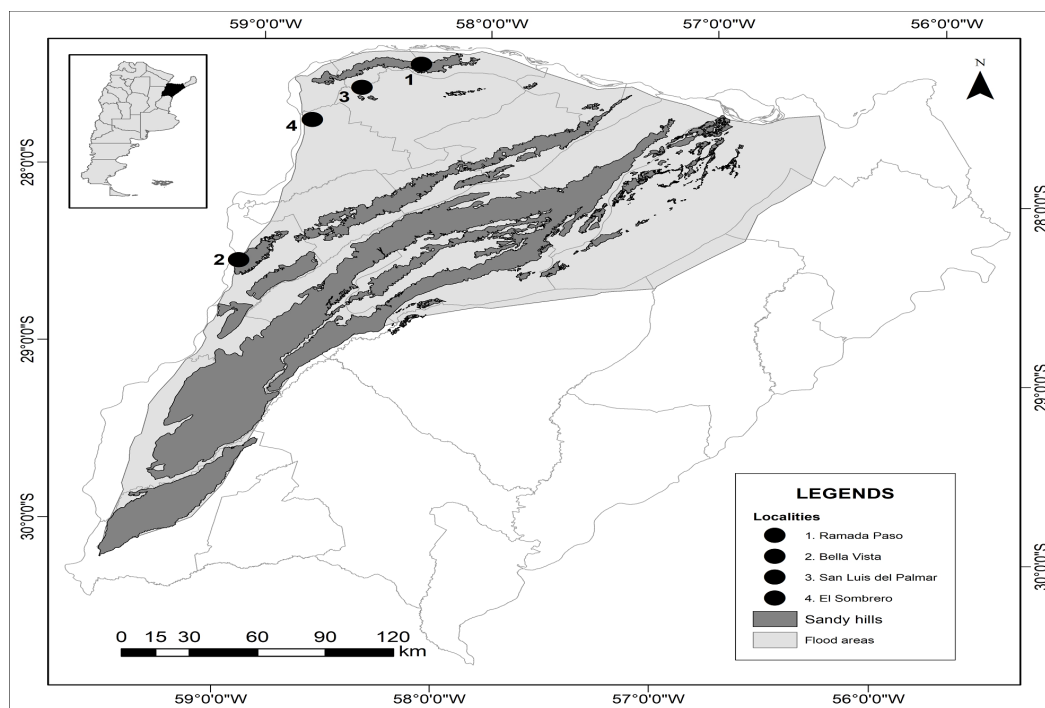
Figure 1. Study area and data collection points, Province of Corrientes – Argentina.

humid), was evident with a greater percentage in the year 1996 and in second place in the year 1994, exceeded only by the normal period in the same year. Both "extremely wet" and "extremely dry" events were more recurrent in the years 1995 and 1997, respectively. It should also be noted that in the period under evaluation, climatic variability presented the biannual cyclical behavior of drought-flooding (Fig. 3), characteristic for the area of study.

Statistical analysis by clustering, using strains as classification criteria and results of physiological and biochemical tests as variables, at a distance of Jaccard d_J = 0.53, separated five clusters (Fig. 4). The largest cluster grouped 61.63% of the total strains, and from which 92.00% belongs to Empedrado locality and the rest belongs to Ramada Paso locality. However, the sandy hills and the flooded areas could not be related. Both localities are located on the coast of the Paraná River, which gives them climatic and compositional peculiarities to the soil.

The second clustering brought together 25.58% of the total number of strains with 72.72

and 27.27% corresponding to the localities of Ramada Paso and Bella Vista, respectively and coming from sandy hills.

Conglomerate 3 was made up of 5.81% of the total isolated strains (Empedrado and San Luis del Palmar localities) coming from flooded areas.

In the Main Components analysis, the strains showed a considerable variability. The high positive weights achieved by the growth tests in NaCl (6.5%) and at initial pH = 9.6 as opposed to a growth medium in NaCl (4.0%) are emphasized. Based upon the foregoing, two groups were generated. One consists of typical strains of a saline-alkaline medium, and a smaller one that comprises strains characteristic of lower salinity habitats. The strains associated with a saline-alkaline medium were isolated from flooded regions corresponding to the Empedrado and San Luis del Palmar Departments. Those strains associated with a growth environment with lower salt concentration corresponded to isolations from sandy hills, where the sodium concentration is very low, as in the Ramada Paso locality.

Table I. SPEI Categorization (Scordo et al. 2018).

| Category	SPEI Values
Extremely wet	≥2
Very wet	(1.50, 2.0)
Moderately wet	(1.00, 1.50]
Incipient humidity	(0,50, 1.00]
normal	[0,50, -0,50]
Emerging Drought	(-0,50, -1.00]
Moderately dry	(-1.00, -1.50]
Very dry	(-1.50, -2.00]
Extremely dry	≤-2

Drought episodes occur whenever the SPI is continuously negative and reaches an intensity of -1.0 or less.
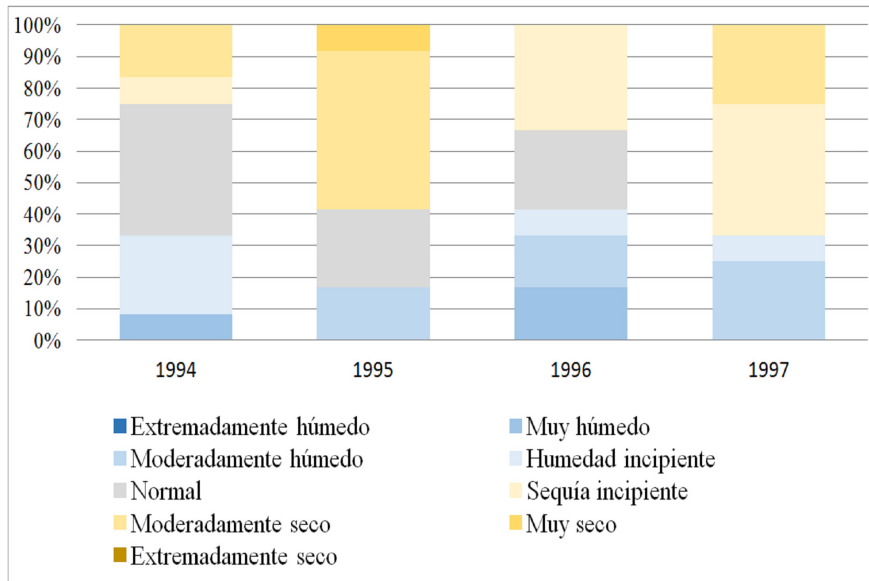
Figure 2. Monthly frequency for the years 1994 to 1997 according to the SPEI category during the period under assessment.

Figure 3. Evolution of the Standardized Precipitation-Evapotranspiration Index for the years 1994 to 1997 for the Corrientes localities under study.
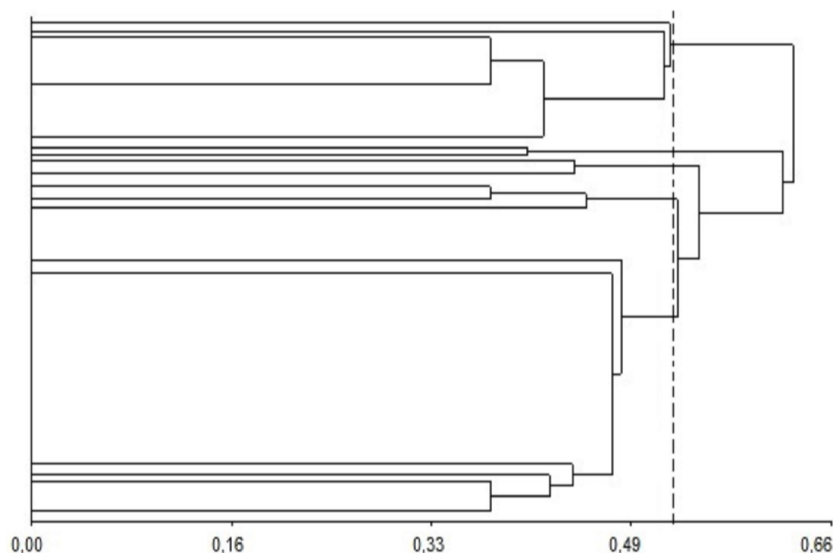
Figure 4. Dendogram showing the relationships between the 86 strains according to the metabolic characteristics considered, rC= 0.948.

CONCLUSIONS

The results obtained allowed the georeferencing of wild strains of LABs according to their place of isolation, and to characterize the regions of isolation from the point of view of some soil characteristics, climate and landscape. As for the area of study, two types of landscapes were defined: those that are *flooded* and those with *sandy hills*. The evaluation of the strains coming from both landscapes, allowed to distinguish them.

The analysis by Main Components highlighted the preference of strains from *flooded* landscapes to be in a saline/alkaline soil, isolated during drought periods. Likewise, it highlighted the preference of strains from *sandy hills* landscapes to be in a low in salt and acid soil, isolated during high humidity periods resulting from previous flooding. This proves the existence of associations between phenotypic characteristics of wild *Lactococcus lactis* subsp. *lactis* strains from Corrientes, soil characteristics and the relative strains abundance depending on the dry and humid periods that develop in the environment during isolation.

The adapted metabolic activity of native strains isolated from artisan cheeses is desirable compared to commercial starter cultures. This influences that the typical organoleptic properties of cheeses can be restituted and consumer preference is not altered (Cobo-Monterroza et al. 2019). The relevance of the findings of this work lies in detecting potential natural sources of lactic strains used for the production of cheeses that will allow characterizing cheeses from cheese-producing areas in Corrientes and designing starter cultures for each type of regional product.

Acknowledgments

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Mario M. Maidana was responsible for the collection and analysis of biochemical and edaphic data, as well as for the planning and revision of the manuscript. Félix I. Contreras contributed with the statistical analysis and interpretation of geographic and climatic data, planning and revision of the manuscript. Olga M. Vasek was responsible for sampling and phenotypic analysis of bacterial strains, planning and revision of the manuscript.