INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–0346/2021/26–3–425–436
DOI: 10.17957/IJAB/15.1852
http://www.fspublishers.org

Short Communication

Selection and Molecular Identification of Lactic Acid Bacteria with Probiotic Potential from Girolando Calves Raised in a Semiarid Region

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Received 19 March 2021; Accepted 30 June 2021; Published 18 September 2021

Abstract

Diarrhea is one of the most important diseases in calves, resulting in high mortality along with the high costs of antimicrobial agents. For alternative control with probiotics, it is essential to isolate autochthonous bacteria that are effective against *Escherichia coli*. The aim of this study was to select lactic acid bacteria (LAB) showing probiotic potential from dairy calves raised in a semiarid tropical region. The feces of ten healthy weaned Girolando calves were sampled, and after dilution were incubated on solid de Man–Rogosa–Sharpe medium, in a microaerophilic atmosphere. A high population of LAB was observed in samples from each calf evaluated (average 2.8×10^9 colony-forming units g^{-}. Of the 31 LAB isolates, all of which were negative for catalase production and and 61.3% were Gram-positive rods were Gram-positive rods, compatible with the *Lactobacillus* genus. However, only seven of these isolates showed milk coagulation capacity after successive peak and were pre-selected. In the test for screening resistance to bile salts at 1% and to pH4, growth was observed in three (42.8%) of the seven LAB. After 16S rRNA gene sequencing and proteomic analysis, a selected LAB identified as *Lactobacillus plantarum* enabled higher inhibition of *E. coli* of bovine origin than other isolates, presenting probiotic potential for the control of colibacillosis. © 2021 Friends Science Publishers

Keywords: *Lactobacillus* spp.; *Escherichia coli*; Colibacillosis; Microbial antagonism; Semiarid

Introduction

Diarrhea in calves represents one of the most frequent diseases in young animals; in particular, colibacillosis caused by *Escherichia coli* can promote death and considerable financial loss in dairy production (Duse *et al.* 2015; Hang *et al.* 2019). Calves are also the major reservoir of Shiga-toxigenic *E. coli*, which is associated with hemolytic uremic syndrome and hemorrhagic colitis in humans (Ferens and Hovde 2011; Nguyen *et al.* 2015; Aref *et al.* 2018).

Antibacterials are frequently used in the treatment of colibacillosis; however, these facilitate the selection of bacteria showing antibacterial resistance and can promote toxicity and allergies (Enany *et al*. 2019; Hang *et al.* 2019; Algammal *et al.* 2020). Thus, there is a need for studies which evaluate the use of probiotics as alternatives to antibiotic therapy. Probiotic lactic acid bacteria (LAB) have shown beneficial effects with respect to the intestinal immune system and to the indigenous microbiota, resulting in the control of calf diarrhea and contributing to better performance of these animals (Luongo *et al.* 2013; Soto *et al.* 2014).

Sharma and Singh (2014) reported that strains of LAB produce antimicrobial compounds, such as organic acids, lactic acid, hydrogen peroxide and bacteriocins that can inhibit different species of pathogens. Their study analyzed the effect of a daily dose of two selected LAB isolates on new-born dairy calves. The fecal score was positively influenced by LAB, and diarrhea incidence was significantly lower in treated calves. However, the authors reported that these results could vary depending on the calving season, which may be associated with pathogen seasonality and environmental conditions (Fernández *et al.* 2020). Additionally, most studies on probiotics in calves have evaluated LAB strains not autogenous to the gastrointestinal tract of these ruminants, which could compromise the effectiveness of these products (Fernández *et al.* 2020).

The autochthonous LAB population in calf feces may be influenced by factors, such as age, type of feeding, and rearing system **(**Alimirzaei *et al.* 2020). Little is known about the population of LAB present in weaned dairy calves reared in semiarid tropical conditions. In our previous study carried out in beef Nellore calves raised in semiarid conditions, while considering the results of preliminary biochemical characterization that reported the presence of

To cite this paper: Duarte ER, VDA Veloso, ADCR Veloso, F Guimarães, HC Fonseca, DS Malveira, TGDS Braz, MS Pinto (2021). Selection and molecular identification of lactic acid bacteria with probiotic potential from Girolando calves raised in a semiarid region. *Intl J Agric Biol* 26:425–430

Lactobacillus lactis sspp. and *Lactobacillus brevis* in newborn calves; however, for calves at three months of age, *Lactobacillus pentosus* species predominated, accounting for 46.1% of the isolates (Malveira *et al.* 2016). Therefore, we aimed to isolate, quantify, and characterize LAB from dairy weaned calves reared in semiarid tropical conditions and to analyze their probiotic potential and growth inhibitory effect of these isolates with respect to *E. coli* strains responsible for calf colibacillosis.

Materials and Methods

Sampling and isolation

Ten weaned Girolando calves aged four to five months were sampled. Calves were from a dairy herd belonging to a farm in the north of Minas Gerais, Brazil. Approximately 30 g of feces was collected from each animal, as per the procedures approved by the Animal Experimentation Ethics Committee of UFMG (protocol No. 219/2011).

The samples were homogenized by vortexing for 2 min and subjected to decimal dilutions. A 100-microliter subsample was inoculated on solid de Man–Rogosa–Sharpe medium (MRS; Merck Darmastadt, Germany) and the plates were incubated in anaerobic jars using a microaerophilia generator at 39°C for 48 h (Bujnakova *et al.* 2014). Smears were prepared on slides stained using the Gram method for 31 LAB isolates (approximately three isolates per calf). The non-sporulated Gram-positive rod-shaped bacteria were selected.

Selection and characterization of isolates

Out of the 31 isolates, 19 strains were selected as having milk coagulation capacity. These LAB were analyzed for survival in three consecutive incubations at 39°C in skim milk reconstituted from powder. The catalase production capacity was considered negative owing to the lack of oxygen micro-bubble formation around the colonies (Bujnakova *et al.* 2014).

To test their ability to grow under aerobic conditions, the 19 LAB isolates were inoculated onto plates containing MRS medium and incubated for 48 h at 39°C. Bacteria growth, or the lack thereof, was observed visually. Seven isolates selected for the ability of milk to coagulate after successive cultures were evaluated for resistance to bile salts. LAB subsamples (100 μL) were inoculated onto MRS agar, containing bile salts at concentrations of 0.0, 0.3, 0.5, and 1.0%. After incubation at 37°C for 48 h in an anaerobic jar, the growth of the colonies in the presence of bile salts was visually observed. These procedures were adapted from Noh and Gilliland (1993).

In a second screening, the hydrochloric acid resistance of these seven isolates was evaluated in brain heart infusion (BHI) broth (Merck Kgaa, Darmastadt, Germany) with pH values adjusted to 3.0, 4.0, 5.0 and 7.0, inoculated at a proportion of 5%. After incubation at 37°C for 6 h, growth was evaluated. After this period, 50 μL of the broth containing LAB was inoculated onto MRS agar plates and incubated anaerobically at 37°C for 48 h. This time, growth was observed *via* visual comparison to the control at pH 7.

Six LAB isolates, pre-selected as resistant based on the results of the previous tests, were biochemically characterized according to the catabolic profiles of 49 substrates, using the API 50 CHL gallery kit (BioMérieux SA, Marcy-I'Etoile, France). The readings were taken 24 and 48 h after inoculation in the API kits. The results were analyzed using the probability software LAB PLUS version 4.0 database from BioMerieux, Marcy I'Etoile, France.

A selected LAB was identified through DNA and proteomic analyses. Its DNA was extracted using LiCl and lysozyme treatments, as reported by Acurcio *et al.* (2014). The DNA was amplified *via* polymerase chain reaction (PCR) using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), as described by Lane (1991). The 16S rRNA gene was sequenced as reported by Sanger and Coulson (1975) on an automatic DNA sequencer Megabace 1000 (GE Life Sciences, Chicago, USA). The 16S rRNA gene sequence was verified using sequence scanners v. 1.0 (Applied Biosystems, Foster City, USA) and compared with that hosted on NCBI using the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). The strain was identified by applying a 99% similarity threshold. Additionally, the bacterial strain yielded identification scores higher than 2.0 when analyzed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) along with MALDI Biotyper v. 2.0 software (Farfour *et al.* 2012).

Growth inhibition of *Escherichia coli* strains

Three LAB isolates showing different biochemical profiles were tested for antimicrobial activity against three *Escherichia coli* strains, one of them (E1) being of human origin, the ATCC 25922 strain. The second *E. coli* isolate was from the feces of a female calf at one month of age (E2) and the third came from the small intestine of a two-month-old male calf (E3), with both calves having diarrhea. The calf *E. coli* isolates were obtained from cultures on MacConkey agar (Acumedia Manufacturers, Lansing, Michigan, USA) and were presumptively characterized using a biochemical test proposed by McFaddin (2000) in modified Rugai medium (MBiolog Diagnósticos, Belo Horizonte, Brazil) and identified *via* sequencing of ribosomal DNA as described by Ribeiro *et al.* (2018).

In the first antagonistic test, zones of inhibition of *E. coli* strains were evaluated using the disc diffusion method as described by Malveira *et al.* (2016). The pure cultures of each *E. coli* strain were inoculated over the entire surface of a plate containing Mueller-Hinton agar (approximately 10^6

CFU·mL^{-1}). Subsequently, 6-millimeter diameter filter paper discs, impregnated with the MRS broth containing one of the three identified LAB isolates (approximately 10^8 CFU·mL^{-1}), incubated at 37°C for 48 h, were placed on the agar surface. After the 48-h incubation period, the presence or absence of inhibition halos was verified using a method reported by by Tagg and McGiven (1971). All procedures were performed in triplicate and three LAB isolates showing the highest antibacterial effects were selected.

These LAB isolates were tested using the spot-on-the-lawn method (Tagg *et al.* 1976) in which they were reactivated and inoculated on the surface of MRS agar, with the aid of a swab, providing colony growth in a circular shape with an average diameter of 4 mm. The plates were incubated anaerobically at 37°C for 48 h. After the growth period, the plates were exposed to chloroform vapor for 30 min to promote cell death. The plates were then opened for 40 min to let the residual chloroform evaporate. Subsequently, a 20 mL overlay of Nutrient Agar was applied at approximately 40°C, having been previously inoculated with the E1, E2 or E3 strains of *E. coli* (approximately 10^6 CFU·mL^{-1}), through the pour plate method, according to Sarkar and Banerjee (1996).

Subsequently, the plates were incubated anaerobically for 24 h at 37°C. The diameters of the inhibition halos formed around the colonies of *Lactobacillus* spp. were measured in millimeters. All these procedures were performed in triplicate.

Statistical analysis

For the antagonism test, the diameters of the inhibition zones were compared using the Kruskal-Wallis test using the statistical package SAEG, version 9.1, at $P < 0.05$

Results

The presence of LAB was detected for all the evaluated calves, with an average of $2.8 \times 10^9 \pm 3.2 \times 10^6$ colony-forming units (CFU) g^{-1}. Of the 31 LAB isolates, 19 were characterized as Gram-positive rods, all of which were negative for catalase enzyme production. Only seven samples of these 19 LAB showed milk coagulation capacity after successive peaks and these LAB strains were visibly compatible with the *Lactobacillus* genus. Aerobic growth capacity was observed for six of these seven isolates evaluated from the calves' feces (Table 1).

When evaluating the growth of the seven LAB isolates in acidic media, in the medium of pH 5.0, there was growth of LAB for all samples, five (71.42%) of which had good growth; however only three grew at pH 4 (Table 1). Among these seven isolates in the test for screening resistance to bile salts at concentrations of 0.3 and 0.5%, growth was observed in six (85.71%) of the strains (Be1a, Be1b, Be1c, Be2b, Be2c and Be3a). At the maximum concentration of 1% bile salts, four isolates showed growth. The Be2a isolate did not show growth in any of the concentrations evaluated so it was not selected (Table 1).

The analyses of biochemical profiles showed that the six selected LAB did not ferment L-xylose, methyl β-D-xylopyranoside, erythritol, D-arabinose, D-adonitol, L-sorbose, D-melezitose, xylitol, D-lyxose, D-fucose, L-arabitol and potassium 5-ketogluconate (Table 2).

In the antagonism test, three strains with probiotic potential were evaluated (Table 2). Of these three strains, the isolate Be3a showed the highest activity against the three *E. coli* strains, E3, E2 and E1 (Table 3). This strain was biochemically characterized as *L. pentosus*, but was identified as *Lactobacillus plantarum* after DNA sequencing (considering a 99% similarity threshold) and exhibited scores higher than 2.0 when analyzed *via* MALDI-TOF, confirming the species identification.

Discussion

The feces of all calves exhibited elevated populations of LAB and homogeneity compared to the means reported in the literature. These bacteria may be influenced by factors, such as age, type of feeding, and rearing system (Chaves *et al.* 1999; Alimirzaei *et al.* 2020). However, the calves evaluated in this study, reared under the same conditions as calves from semiarid regions, showed homogeneous LAB counts. Chaves *et al.* (1999) reported an average viable LAB cell count of 4.7×10^8 CFU·g^{-1} in feces from calves from one to three months of age, values close to those observed in this study.

In this study, 61.29% of the isolates were compatible with members of the genus *Lactobacillus*; however, only 22.5% were able to coagulate milk. Strains of this genus utilize lactose and produce lactic acid, responsible for reducing the pH, consequently for milk coagulation (Bujnakova *et al.* 2014). The Be2c strain was the only strain that did not exhibit aerobic growth capacity.

The growth of four LAB isolates at the maximum concentration of 1% bile salts, detected in this study, is a characteristic essential for the survival of probiotic microorganisms in the gastrointestinal tract (Lebeer *et al.* 2008). Bile contains various antimicrobial compounds that play an essential role in the digestive system. Bile salts cause disruption of the cell membrane and damage the structure of bacterial DNA (Merritt and Donaldson 2009).

In the medium with pH 5, 71.42% of the LAB strains exhibited good growth; at pH 4, good growth (++) was observed in 43% of these bacteria. However, at pH 3 only the Be1c and Be2c isolates exhibited satisfactory growth (+; Table 1). The acid pH resistance reveals important probiotic characteristics, as the bacteria were able to overcome the first physiological barrier of the digestive tract, *i.e.*, the low pH of the stomach (Gibson 2004).

In terms of the biochemical profile analyses, the Be3a strain exhibited the biochemical characteristics of *L.*

Table 1: Resistance to bile salts, pH, and aerobic growth of *Lactobacillu*s spp. isolates from the feces of dairy calves reared in the northern of Minas Gerais, Brazil

Isolates	pH				Bile salt concentrations				Aerobic growth
	3	4	5	7	0.0%	0.3%	0.5%	1.0%	
Be1a	-	++	+++	++++	+	+	+	+	+
Be1b	-	-	+++	++++	+	+	+	-	+
Be1c	+	++	++++	++++	+	+	+	+	+
Be2a	-	-	++	++++	+	-	-	-	+
Be2b	-	-	++	++++	+	+	+	-	+
Be2c	+	++	+++	++++	+	-	+	+	-
Be3a	-		+++	++++	+	+	+	+	+

The pH resistance result was expressed in terms of growth intensity. The concentration of bile salts and the aerobic growth were evaluated for growth (+) or non-growth (-); (Noh and Gilliland 1993; Malveira *et al.* 2016)

Table 2: Catabolic biochemical profile of *Lactobacillus* spp. isolates from the feces of Girolando dairy calves raised in northern Minas Gerais, Brazil

	Isolates of *Lactobacillus* spp. from calves					
Substrates	Be1a	Be1b	Be1c	Be2b	Be2c	Be3a
L- arabinose	+	+	-	+	+	+
D- ribose	+	+	-	+	+	+
D- xylose	+	+	-	+	+	+
D- galactose	+	+	+	+	+	+
D- glucose	+	+	+	+	+	+
D- fructose	+	+	+	+	+	+
D- mamnose	+	+	V	+	+	+
L- ramnose	-	-	-	-	-	-
Dulcitol	-	V	-	-	-	-
D- Mannitol	+	+	V	+	+	+
D- Sorbitol	+	V	-	V	V	+
Methyil- αD- Glucopyranoside	+	V	V	-	V	+
N- Acetyl Glucosamine	+	+	+	+	+	+
Amigdalin	+	+	+	+	+	+
Arbutin	+	+	+	+	+	+
Esculin iron citrate	+	+	+	+	+	+
Salicin	+	+	V	+	+	+
D- cellobiosis	+	+	+	+	+	+
Glycerol	V	-	V	-	-	-
D- lactose (bovine origen)	+	+	-	+	+	+
D- melibiosi	V	+	-	-	V	+
D- sucrose	+	+	+	+	+	+
D- trehalose	+	+	+	+	+	+
Inulin	V	V	-	-	V	V
D- Rafinose	V	V	-	-	V	V
Starch	V	V	+	V	V	V
Gentiobiosis	+	+	+	+	+	+
D- turanosis	V	V	V	V	-	+
D- tagatose	+	V	-	V	V	+
L- Fucose	-	-	-	-	-	-
D- Arabitol	-	-	-	-	-	-
Potassium gluconate	+	+	-	+	+	+
L- Sorbose	+	V	-	V	V	+
Glycogen	-	-	+	-	-	-
Methil- αD- manopiranoside	V	-	-	-	-	-
Biochemical profile	L1	L2	L3	L4	L2	L1

Note: + (growth), – (non-growth), e V (variable growth); Biochemical profile: L1 - *Lactobacillus pentosus;* L2 - *Lactobacillus brevis*; L3 - *Lactobacillus crispatus*; L4 - *Lactobacillus lactis*

pentosus; it exhibited growth in the presence of all concentrations of bile salts, was resistant to pH 5 and 7, and demonstrated positivity for aerobic growth. The Be1c strain corresponded closely to *Lactobacillus crispatus* (98.9% similarity); it showed growth in the presence of all concentrations of bile salts and was also positive for aerobic growth in all the pH values investigated. The Be2c isolate corresponded to *L. brevis* with 66.4% similarity and exhibited growth in all tested bile salt concentrations and pH values; however, it was not positive for aerobic growth. The Be2b isolate corresponded to *L. Lactis* sspp. with only 51.9% similarity and exhibited growth in the presence of bile salts (concentrations of 0.3 and 0.5%) and at pH values of 5 and 7. The two remaining LAB isolates presented unacceptable biochemical identification profiles.

Table 3: Mean values of diameter of the inhibition halo and standard deviation (in millimeters) that reflects the growth inhibitory effect of *Lactobacillus* spp. (from feces of Girolando calves, raised in the North of Minas Gerais, Brazil) with respect to the three pathogenic strains of *Escherichia coli*, namely E1, E2 and E3

Lactobacillus spp. isolates	E1	E2	E3
Be1c	8.3 ± 1.1a	7.3 ± 1.5b	5.0 ± 1.3b
Be2c	9.0 ± 1.3a	8.11 ± 1.1b	6.0 ± 1.6b
Be3a	6.0 ± 1.7a	10.0 ± 1.0a	12.0 ± 2.3a

E1= *Escherichia coli* strain ATTC 25922, E2 and E3 are *E. coli* isolates from calves with colibacillosis

Different letters in columns indicate significant difference between bacteria strains as determined by Kruskal-Wallis' test at 5% significance

These biochemical results corroborate those of a study performed in Nellore calves raised in semiarid conditions by Malveira *et al.* (2016). The researchers observed *L. lactis* sspp. and *L. brevis* in the newborn calves, corresponding to 53.8 and 38.5% of the identified isolates, respectively. However, for the LAB isolated from calves at three months of age, *L. pentosus* species predominated, accounting for 46.1% of the isolates. Using the same biochemical methodology, Chaves *et al.* (1999) analyzed LAB isolates from the feces of one to three days old claves and identified 12 strains (2.28%) of *Lactobacillus acidophilus.* However, these authors did not utilize molecular techniques for the identification of *Lactobacillus* spp.

The selected *Lactobacillus plantarum* isolate exhibited the highest antagonism toward the three strains of *E. coli* and was identified *via* DNA sequencing and MALDI-TOF MS. This isolate presented the biochemical profile of *L. pentosus,* but proteomic characterization was demonstrated as the more accurate and viable test for identification of LAB strains from calves.

In an additional examination of this selected *L. plantarum* isolate, its viability after 24 and 48 h of fermentation was evaluated. This calf LAB isolate exhibited satisfactory growth in milk-based formulations with high concentrations of viable cells (> 8 log $CFU \cdot mL^{-1}$). Transition milk on the third day after calving represented a useful substrate for the growth of this LAB, which may be stored for up to 50 d at 28ºC. Therefore, fermentation of transition milk by this *L. plantarum* strain was shown to be a viable method for the production of probiotics. Additionally, the transition milk may be preserved *via L. plantarum* fermentation as a functional food and used as a milk substitute in artificially fed calves (Fonseca *et al.* 2020).

A recent study evaluated the strain *L. plantarum* GB LP-1 (LP) produced through a proprietary fermentation process as a viable microbial direct feed for neonatal calves. The authors reported that the fecal scores improved linearly with increased bacterium inclusion and that feed efficiency was greatest at 4 $g \cdot d^{-1}$, improving gut health and growth performance of neonatal calves (Casper *et al.* 2021).

The use of native microorganisms with probiotic capacity represents a sustainable alternative for the control of several animal diseases, such as neonatal calf diarrhea. A study of four native LAB strains (TP1.1 and TP1.6 as *Lactobacillus johsonii* strains, *L. reuteri* as TP1.3B, and *L. amylovorus* as TP8.7) were isolated from dairy calves, raised in a temperate climate region, and evaluated their capacity to colonize and persist in the calf gastrointestinal tract. The strains TP1.3B and TP1.6 were able to persist in the treated animals for up to ten days after the completion of the administration period, showing promise as candidates for further development of calf probiotics under these conditions (Fernández *et al.* 2018).

In the present study, on considering the parameters described by Chaves *et al.* (1999), only the Be3a strain (*L. plantarum*) exhibited moderate inhibition of *E coli* strains E2 and E3 isolated from calves with colibacillosis but showed less inhibition for ATCC 25922 strain (E1) of human origin. In short, only the Be3a strain could efficiently inhibit the growth of *E. coli* strains of calf origin, a phenomenon that could be explained by the ability of this species to produce several varieties of bacteriocins (Salminen *et al.* 2004).

The *Lactobacillus* spp.-mediated growth inhibition of pathogenic bacteria has been attributed to the action of bacteriocins that can inhibit the growth of *Enterobacter aerogenes*, *E. coli*, *Klebsiella pneumoniae*, and *S. aureus,* indicating a broad spectrum of action (Viswanathan *et al.* 2015). In addition to the variation between species of LAB, bacteriocins are associated with other factors that may influence production and activity. A study carried out by Maldonado *et al.* (2012) on bacteria isolated from the feces of calves revealed that LAB isolates exhibited inhibitory activity toward *E. coli*, *Yersinia enterocolitica*, *Salmonella enterica* subspecies enterica serovar Dublin, and *Klebsiella* sspp. In most cases, growth inhibition was attributed to the production of lactic acid.

Conclusion

In this study, high counts of LAB were detected in the feces of weaned Girolando calves. Of the bacteria isolates evaluated, three were resistant to bile salts and acidic pH. One LAB isolate, molecularly identified as *L. plantarum,* inhibited the growth of *E. coli* strains that cause colibacillosis in calves. Thus, this strain can be evaluated for inclusion in the feed of dairy calves raised in tropical conditions to reduce mortality and to improve performance during the initial rearing phase.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES), Finance Code 001; by Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (CNPq); Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG); and Pro Reitoria de Pesquisa, Universidade Federal de Minas Gerais.

Author Contributions

ERD, MSP, and TGSB designed the experiments and edited the manuscript. VZV, ACRV, FG, HCF, and DSA prepared the samples, performed the experiments, and wrote the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

In this study all the procedures adopted with the sampled calves were approved by the Animal Experimentation Ethics Committee of UFMG (protocol No. 219/2011).

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